Primary food resources for key waterbirds and benthic fish in the Coorong

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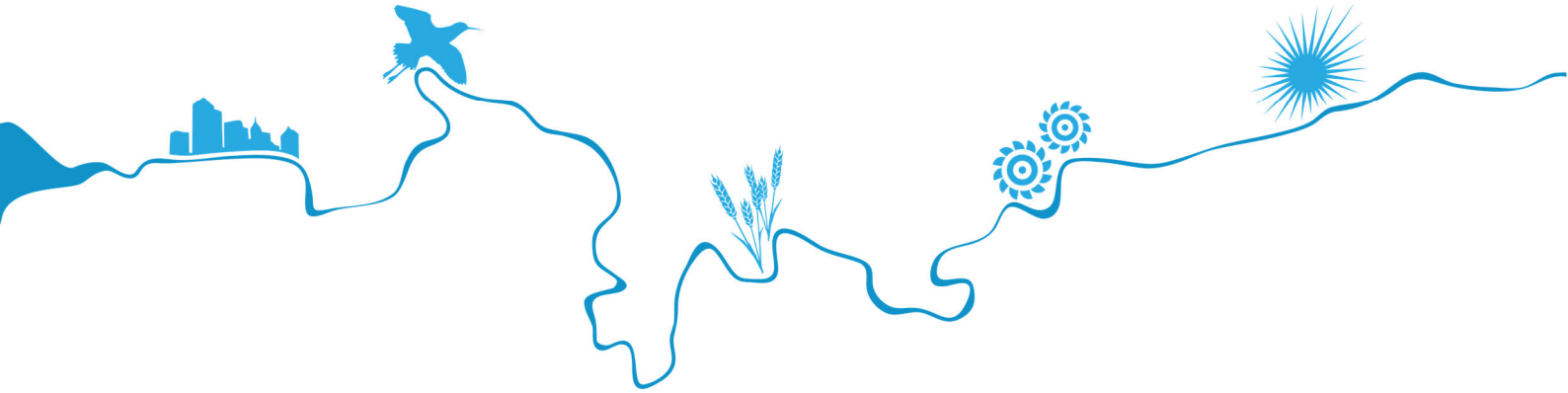

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This report has been prepared by the Department for Environment and Water, CSIRO, Flinders University, The University of Adelaide, the University of South Australia and the South Australian Research and Development Institute (the Goyder Institute for Water Research partner organisations) and contains independent scientific/technical advice to inform government decision-making. The independent findings and recommendations of this report are subject to separate and further consideration and decision-making processes and do not necessarily represent the views of the Australian Government or the South Australian Department for Environment and Water.

Contents

Figures

Tables

Respect and reconciliation

Aboriginal people are the First Peoples and Nations of South Australia. The Coorong, connected waters and surrounding lands have sustained unique First Nations cultures since time immemorial.

The Goyder Institute for Water Research acknowledges the range of First Nations' rights, interests and obligations for the Coorong and connected waterways and the cultural connections that exist between Ngarrindjeri Nations and First Nations of the South East peoples across the region and seeks to support their equitable engagement.

Aboriginal peoples' spiritual, social, cultural and economic practices come from their lands and waters, and they continue to maintain their cultural heritage, economies, languages and laws which are of ongoing importance.

Executive Summary

The Coorong and Lakes Alexandrina and Albert wetland in South Australia is internationally recognised under the Ramsar Convention as an important wetland for biodiversity. The wetland provides ecological services to a range of biota, including waterbirds and fish, which are supported at critical stages of their life-histories. Knowledge of the key food resources and diet compositions of waterbirds and less abundant fishes in the Coorong is limited, yet this information is critical for understanding trophic dynamics. The South Australian Government's Healthy Coorong, Healthy Basin (HCHB) program's Trials and Investigations (T&I) project includes a food web investigations component (*Component 3*). This component aims to develop an integrated quantitative food web model for the Coorong, using empirical investigations, that can predict trophic responses to various conditions including management actions.

In this study, we aimed to fill critical dietary knowledge gaps by identifying the major food resources and their relative contribution to the diets of a small-bodied, resident prey fish (lagoon goby *Tasmanogobius lasti*) and five important waterbird species in the Coorong. The five waterbird species were comprised of two migratory shorebirds (sharp-tailed sandpiper *Calidris acuminata* and red-necked stint *C. ruficollis*), one non-migratory shorebird (red-capped plover *Charadrius ruficapillus*) and two non-migratory waterfowl (chestnut teal *Anas castanea* and grey teal *Anas gracilis*). The diets of these species were explored using traditional gut content analysis (fish) and DNA metabarcoding of scats (waterbirds). For DNA metabarcoding, universal primers targeting the mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) and protein-coding chloroplast gene (*rbcL*) were used to amplify the DNA of animal (metazoan) and vegetation (plants and algae, 'plant' herein) food species, respectively, because the investigated waterbird species were considered to be omnivorous (i.e. feed on both animals and plants). Fresh scats were collected opportunistically from each waterbird species, with species-specific blocking primers designed and applied in the polymerase chain reaction (PCR) mix to reduce amplification of predator DNA for the *COI* gene assay. Two reference library databases of *COI* (animal) and *rbcL* (plant) sequences were also generated from potential prey samples collected from key foraging grounds in the Coorong to help resolve unknown taxonomic identifications.

Key findings

***Fish*:** In the brackish to slightly hypersaline regions of the Coorong (Murray Estuary, mean salinity 15–33 parts per thousand (ppt) and North Lagoon, 27–52 ppt), the diet of lagoon goby was almost exclusively amphipods.

Shorebirds: In the hypersaline (75–108 ppt) region of the Coorong (South Lagoon), the animal-based diet compositions of sharp-tailed sandpiper, red-necked stint and red-capped plover were similar to each other and dominated by chironomids (presumably benthic larvae). In the North Lagoon, the diet of sharp-tailed sandpiper was more diverse with corophiid and gammarid amphipods, chironomids and platyhelminths forming important dietary components and being supported by the polychaete *Simplisetia aequisetis*. At sites where chironomids and amphipods were both available, sharp-tailed sandpiper showed stronger prey selectivity for amphipods over chironomids. The submergent halophyte *Ruppia* spp. was the main plant species consumed in the South Lagoon by sharp-tailed sandpiper and red-capped plover, although the contribution of plants to the overall diet compositions of these species remains unknown.

Waterfowl: The plant-based diet of chestnut and grey teal consisted of a variety of submergent, emergent, amphibious and terrestrial vegetation from freshwater, estuarine/marine aquatic (including Coorong) and terrestrial environments. *Ruppia* spp., freshwater plants (*Ceratophyllum* spp. and *Myriophyllum* sp.), samphire (*Salicornia* sp. and *Suaeda* sp.), agricultural barley (*Hordeum vulgare*) and green algae (e.g. *Ulva* spp.) were the key food items consumed. Fish, chironomids, amphipods and millipedes were the major animal prey constituents in the diet of chestnut and grey teal.

The relative proportions of animal to vegetation food items in the diets of waterbirds could not be quantitatively determined by our methodology, as the *COI* gene specifically amplifies DNA from animals and the *rbcL* gene specifically amplifies DNA from plants. There is no overlap of animal and plant DNA both being amplified using only one of these gene markers, hence relative proportions could not be determined. Nevertheless, we consider the major constituents of the diet to be animals (benthic invertebrates) for shorebirds and vegetation for teal.

Management implications

The Coorong supports a significant proportion of the global populations of migratory sharp-tailed sandpiper and red-necked stint, and resident/nomadic chestnut teal. At the non-breeding, overwintering grounds of the Coorong, it is critical that migratory shorebirds obtain sufficient energy from food resources to survive their return flight to breeding grounds in the Northern Hemisphere and reproduce successfully. Under current conditions in the Coorong, diet diversity was higher in the North Lagoon than the South Lagoon. Amphipods and chironomids were the key food resources of short-billed shorebirds in the mudflats of the North and South lagoons of the Coorong, respectively. In the Coorong, amphipods are also the main prey of many small-bodied fishes, including lagoon goby, and are important in the juvenile diet of many large-bodied fishes. Management actions which promote conditions that favour: (1) an abundant and diverse benthic invertebrate prey assemblage that includes amphipods and chironomids; and (2) for shorebirds, an increased extent of suitable foraging habitat (e.g. bare mudflat) where benthic prey are accessible, will benefit shorebird and fish populations that use the Coorong.

Conclusions

This investigation documents the diet compositions of lagoon goby and sharp-tailed sandpiper in the Coorong for the first time and builds on previous waterbird dietary investigations conducted in the 1960s–80s. DNA metabarcoding proved to be a viable, non-destructive technique for assessing waterbird diet. The complementary use of a traditional approach (e.g. quantification of hard parts of prey in scats or regurgitates) in association with DNA metabarcoding, however, would help validate results and may resolve uncertainty about the relative proportions of animal to plant food items. These findings improve our understanding of food web interactions and the foraging ecology of waterbirds and fishes in the Coorong. Future research should be directed towards understudied aspects of waterbird foraging ecology. This includes the diets of long-billed shorebirds not studied here (e.g. curlew sandpiper, red-necked avocet and banded stilt), all shorebird diets in the northern part of the Coorong (i.e. Murray Estuary and North Lagoon regions) and nearby wetlands (e.g. Lower Lakes and Morella Basin), and the minimum prey requirements (e.g. diversity, abundance and distribution) needed to support healthy waterbird populations. Further, knowledge of diet and prey in each region of the Coorong and across seasons and years will complement this study and enable a more complete understanding of the relevance of prey diversity for waterbird diet and the food web in general. This study provides data vital for developing the quantitative food web models of the HCHB program, which ultimately aim to inform management decision-making in the Coorong that will optimise ecological outcomes.

Acknowledgments

This study (*Activity 2*) was part of the food webs investigations component (*Component 3*) of the Scientific Trials and Investigations (T&I) project. This project is part of the South Australian Government's Healthy Coorong, Healthy Basin (HCHB) program, which is jointly funded by the Australian and South Australian governments. Thank you to Alec Rolston and Daniel Pierce (Goyder Institute for Water Research) for project management, and to Amy Ide for project support (Department for Environment and Water, DEW).

Tom Prowse (The University of Adelaide, UoA), Dan Rogers (DEW) and Phill Cassey (UoA) provided expertise to guide the design at the beginning of the project. T&I *Component 4 Waterbirds* researchers from The University of Adelaide contributed their expertise in waterbird ecology and helped with the collection of scat samples during *Component 4* field sampling. UoA honours student Katelyn Markos collected red-capped plover scat samples for this project. Red-capped plover DNA metabarcoding data from March 2021 were analysed separately and presented in her thesis (Markos 2021). Waterbird capture associated with *in-hand* scat sampling was carried out under an approval from The University of Adelaide Animal Ethics Committee (approval number 34788) and DEW scientific research (Y27036-1) permits.

DNA extraction and amplification steps were performed at the South Australian Research and Development Institute (SARDI) Molecular Sciences Laboratory by Sarah Catalano and Adele Barca. A positive control algae culture sample was provided by the Algae and Biofuels Facility, SARDI (Sasi Nayar). Next generation sequencing (NGS) of two Illumina DNA metabarcoding libraries was performed by the Australian Genome Research Facility (AGRF), Victoria, with preliminary bioinformatics analysis performed by Martha Zakrzewaki (AGRF, Queensland). Predator (bird) and prey tissue samples to test the cytochrome c oxidase subunit 1 mitochondrial gene (*COI*) polymerase chain reaction (PCR) assay and newly designed blocking primers were provided by Sally South, Collection Manager at the South Australian Museum (SAM) Australian Biological Tissue Collection (ABTC).

To build a discreet DNA reference library, Flinders University field staff, including Anthony Newberry, Jordan Kent, Laura Schroder and Will Pyke, collected and live-sorted macroinvertebrates. Sabine Dittmann facilitated field collection. Jason Nicol helped with establishing a list of potential plant food items for herbivorous/omnivorous waterbirds in the Coorong region and provided knowledge on the habitat and ecology of these species. Fish samples for gut content analysis were provided through sampling under *Activity 3* of T&I *Component 3*. Ambient macroinvertebrate assemblage data from sampling under *Activity 3* of T&I *Component 3* were provided by Flinders University. Jess Henkens (Flinders University) conducted fish gut content analysis and helped with data entry.

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1 Introduction

Estuaries are highly productive and dynamic systems that are used by a range of biota, including fish and waterbirds. The services they provide are broad. Estuaries may support all life stages of resident species; for non-resident species, they may provide a refuge area from predation and adverse environmental conditions, breeding habitats, nursery areas for development of early-life stages, staging or stopover sites along migration routes, and foraging habitat (Elliot et al. 2007, Wołowicz et al. 2007, Tian et al. 2008, Potter et al. 2015). In the case of migratory shorebirds, their abundance in estuaries may reflect complex interactions between salinity changes and estuarine food webs including their prey species (Canham et al. 2021).

In southern Australia, the Coorong estuary and Lakes Alexandrina and Albert are internationally recognised under the Ramsar Convention as a wetland of high ecological and cultural significance (Phillips and Muller 2006). This site has a range of wetland types that provide ecological services to a diverse community of biota that includes nationally and internationally threatened, endemic and wetland-dependent species. Of particular importance at this site are the foraging habitats and food resources that support migratory shorebirds (e.g. sharp-tailed sandpiper *Calidris acuminata* and red-necked stint *Calidris ruficollis*) of the East Asian–Australasian flyway. At the non-breeding, 'overwintering' grounds of the Coorong, it is critical that individuals obtain sufficient energy from food resources to survive their return flight to breeding grounds in the Northern Hemisphere and breed successfully (Aharon-Rotman et al. 2016, Clemens et al. 2016).

The long-term decline in the ecological condition of the Coorong since the 1980s, due to reductions in freshwater inflows, is well documented (e.g. Phillips and Muller 2006, Paton et al. 2009, Kingsford et al. 2011, Mosley et al. 2018). During the Millennium Drought (2001–10), freshwater inflow ceased from 2007 to 2010 resulting in extreme hypersalinity of >150 ppt in the South Lagoon (Gibbs et al. 2018) and reduced abundances, diversity and distributions of key biota (Noell et al. 2009, Rogers and Paton 2009, Zampatti et al. 2010, Brookes et al. 2015, Dittmann et al. 2015). Despite some recovery of the ecosystem associated with increased inflows after the end of the Millennium Drought (Leterme et al. 2015, Ye et al. 2015, Dittmann et al. 2018, Paton et al. 2021), the Coorong South Lagoon – a region characterised by consistent hypersalinity (i.e. >40 ppt) – has not recovered as expected (Brookes et al. 2018). Over time, there has been a switch in ecosystem state from an aquatic macrophyte (e.g. *Ruppia* spp.) to algae dominated system associated with eutrophication (nutrient enrichment) (Paton et al. 2018a, Mosley et al. 2020, Waycott et al. 2020). The impacts of this change in ecosystem state on biota and food webs include: (1) reduced reproduction, density and condition of the *Ruppia*/*Althenia* community (Paton et al. 2018a); (2) increased sediment anoxia (Waycott et al. 2020); and (3) suppressing the emergence of insect larvae due to smothering by algal mats, impacting on shorebird foraging (Paton et al. 2018b). These changes in the ecosystem and the lack of recovery are probably caused by several interacting factors, which are not well understood (Brookes et al. 2018). The capacity to forecast ecological responses to restoration management is consequently limited. Knowledge of trophic interactions and the key food resources for important biota (e.g. fish and waterbirds) is vital to understand biotic and food web responses to management actions in the Coorong. While knowledge of the diets of abundant estuarine and marine fish species in the Coorong has advanced (reviewed by Ye et al. 2020a), limited data are available for freshwater and other less abundant species (e.g. lagoon goby *Tasmanogobius lasti*), which are prey for higher-level predators (Giatas et al. 2018). Also, understanding of waterbird diets in the Coorong is predominantly limited to unpublished feeding observations and studies conducted on waterfowl in the 1960s (Delroy 1974) and shorebirds in the 1980s (Paton 1982), during periods of extensive macrophyte (*Ruppia* spp.) distribution and cover (Geddes 1987), which were dissimilar to current conditions where filamentous algae is dominant.

The Phase One Scientific Trials and Investigations (T&I) project of the Healthy Coorong, Healthy Basin (HCHB) program consists of a series of integrated components that will collectively provide knowledge to inform the future management of the Coorong, including the South Lagoon. *Component 3* (*Restoring a functioning Coorong food web*) of the T&I project aims to understand food web dynamics in the Coorong, using empirical investigations on food resources for key biota (waterbirds and fish) to develop an integrated quantitative food web model that can assess food web responses to various conditions (e.g. through management actions and interventions). The food web component includes four main activities: (1) review, synthesis and conceptual food web models (Ye et al. 2020a); (2) diet and food consumption of key species (this study); (3) bioenergetics and key drivers for food resource availability; and (4) quantitative food web models. Identifying the major food resources and their relative contribution to the diet of key biota, such as fish and waterbirds

(*Activity 2*), is fundamental in understanding trophic interactions of food webs, and is critical input into the quantitative food web models (*Activity 4*).

1.1 Aims

To *determine the major food resources and their relative contribution to the diet for key waterbird and fish species* (aim of *Activity 2*) in the Coorong, we reviewed available dietary literature (Ye et al. 2020a) and quantified the diet compositions of lagoon goby, a less abundant prey species with dietary knowledge gaps, and five important waterbird species. The five waterbird species were comprised of two migratory shorebirds (sharp-tailed sandpiper and red-necked stint), one non-migratory shorebird (red-capped plover *Charadrius ruficapillus*) and two non-migratory waterfowl (chestnut teal *Anas castanea* and grey teal *A. gracilis*). The diets of these species were explored using traditional gut content analysis for fish, consistent with the collection of previous fish diet data in the Coorong, and DNA metabarcoding of scats for waterbirds, a modern, non-destructive method.

This study provides critical diet composition data for the diet matrix that will be used as input into the quantitative food web models of T&I *Component 3 Activity 4*, and intends to address the following key knowledge gaps identified during *Activity 1* (Ye et al. 2020a):

- *What is the diet composition of lagoon goby?*
- *What is the diet composition of waterbirds in the Coorong?*
- *What is the contribution of* Ruppia *seeds and turions to the diet composition of shorebirds?*
- *What is the contribution of filamentous algae and animal prey to the diet composition of waterfowl?*

1.2 Background

In the Coorong, fish diets have been assessed primarily by identification and quantification of gut contents (Geddes and Francis 2008, Deegan et al. 2010, Giatas and Ye 2015, Hossain et al. 2017) and secondarily through stable isotope modelling approaches (Lamontagne et al. 2016). Worldwide, waterbird diet composition data have traditionally been obtained through invasive or destructive methods such as stomach flushing and stomach or gizzard content analysis (reviewed by Marchant and Higgins 1990a, 1990b, 1993, Higgins and Davies 1996). More recently, non-invasive methods such as DNA metabarcoding of scats (faeces) have become a broadly applied technique for diet assessment for a range of biota, including waterbirds (e.g. Gerwing et al. 2016, Novcic et al. 2016, McClenaghan et al. 2019). DNA metabarcoding has advantages over the quantification of hard parts of prey in scats, which is often limited by poor taxonomic resolution and bias against small prey that are not easily seen or those that are completely digested (Braley et al. 2010, Bowser et al. 2013, Heller 2020).

DNA metabarcoding involves targeting and amplifying short, highly-variable DNA regions (McInnes et al. 2017a, Gerik et al. 2018). The unique genetic signature of targeted or universal taxonomic groups (representative of prey) within the sample, commonly known as 'barcodes', are simultaneously sequenced using fast and cost-effective high-throughput methods such as next-generation sequencing (NGS) (Gerik et al. 2018). For taxonomic resolution and identification, sequences are then matched to those in publicly available reference databases, such as the Barcode of Life (BOLD) and GenBank at National Centre of Biotechnology Information (NCBI) (Gerik et al. 2018), or to reference library databases generated in-house from collected and sequenced anticipated prey items. When designing DNA metabarcoding studies for dietary assessments it is important to consider the expected range of prey species present in the environment along with the taxonomic resolution of different gene markers and primers (Pompanon et al. 2012, McInnes et al. 2017c). For example, if the diet of a herbivore is to be investigated, barcoding primers that amplify DNA from a wide range of plants, particularly those known to occur in the ecosystem, should be used. Conversely, if a broad view of the metazoan (animal herein) community composition is to be evaluated, a universal primer set with relatively low taxonomic resolution may be applied. As general rules, primers should target short DNA fragments (e.g. <400 base pairs) to counter DNA degradation, be highly conserved among the target taxa and should amplify hypervariable regions for which publicly available reference databases exist to aid taxonomic discrimination (Leray et al. 2013b, Rees et al. 2014, Thomsen and Willerslev 2015).

For studies investigating animal prey, the cytochrome *c* oxidase subunit 1 mitochondrial gene *COI* is commonly used because it has a high copy number, strong capacity to discriminate taxa and one of the largest sequence reference databases (Leray et al. 2013b, Esnaola et al. 2018). Care must be taken, however, to avoid amplification of *COI* DNA from the predator itself, as it is often more abundant, less degraded and not fragmented compared to the prey DNA, and can prevent prey detection by predator DNA saturation (Deagle et al. 2009, Pompanon et al. 2012, Rytkönen et al. 2018). The design and inclusion of primers that specifically block predator DNA amplification (termed "blocking primers") can increase the number of prey sequences obtained significantly (Deagle et al. 2009, Bowser et al. 2013, Leray et al. 2013a). While there is no consensus for the application of a single universal barcode for aquatic or terrestrial plants, the plastid (chloroplast) coding genes *rbcL* and *matK* appear to be the optimal targets for species discrimination, sequence quality, recoverability and completeness of the reference database (CBOL Plant Working Group 2009, Coghlan et al. 2021). All waterbird target species in our study are omnivorous (i.e. feed on both animals and plants). In the Coorong, the main diet component for short-billed shorebirds are animals (invertebrates) (Paton 1982) and vegetation for teal (Delroy 1974). We therefore used universal primers targeting two gene markers, specifically the mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) and protein-coding chloroplast gene (*rbcL*), to amplify DNA of animal and vegetation (plants and algae, 'plant' herein) prey species, respectively.

2 Methods

2.1 Study site

The Coorong is a long (~140 km) and narrow (2–3 km wide) coastal lagoon that lies at the terminus of the Murray-Darling River system in southern Australia (Figure 1). The Coorong is separated from Lake Alexandrina by a series of tidal barrages, and freshwater inflow to the estuary and the Southern Ocean (through the Murray Mouth) is highly regulated through these barrages. The Coorong is commonly divided into three geographical regions (the Murray Estuary, North Lagoon and South Lagoon, Figure 1) based on distinct physical features and salinity properties, generally with increasing salinity from the Murray Estuary into the South Lagoon. The Murray Estuary receives freshwater inflow through barrages and tidal exchange through the Murray Mouth. The North and South lagoons run parallel to the coastline inland from the Younghusband Peninsula and are separated from each other by a narrow section (Hells Gate at Parnka Point) that restricts water exchange. The South Lagoon receives smaller volumes (relative to tidal barrages) of seasonal freshwater inflow from the Upper South-East Drainage System via Salt Creek, discharged from the Morella Basin. During the period of sampling from March 2020 to June 2021, mean salinities ranged from 15–33 parts per thousand (ppt) in the Murray Estuary, 27–52 ppt in the North Lagoon and 75–108 ppt in the South Lagoon.

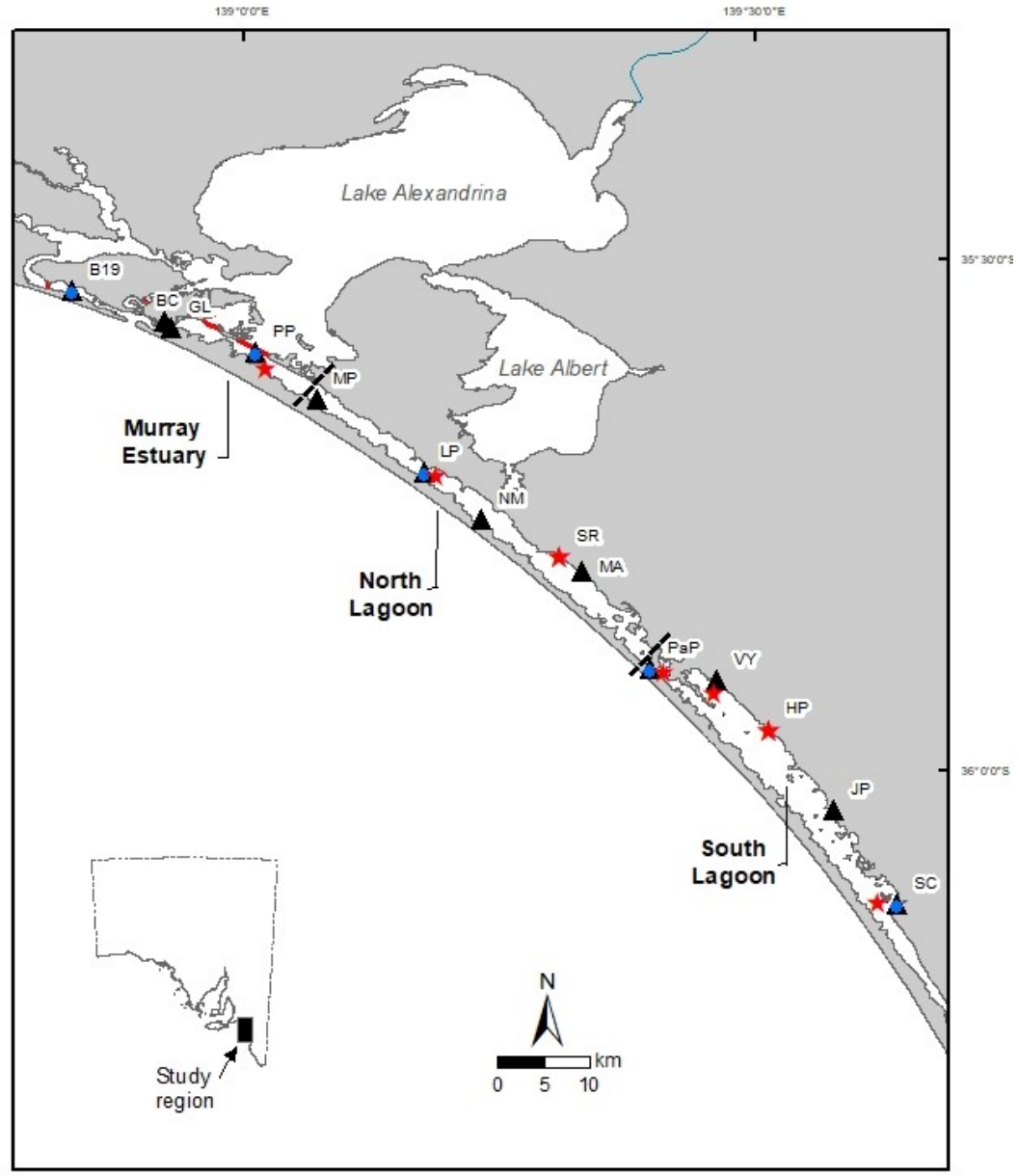

Figure 1. Macroinvertebrate (blue circle), fish (black triangle) and waterbird scat (red star) sampling sites in the Coorong during 2020–21. B19 = Beacon 19, BC = Boundary Creek, GL = Godfreys Landing, PP = Pelican Point, MP = Mark Point, LP = Long Point, NM = Noonameena, SR = Seven Mile Road, MA = Mt Anderson, PaP = Parnka Point (also called Hells Gate), VY = Villa de Yumpa, HP = Hack Point, JP = Jack Point, SC = Salt Creek.

2.2 Fish

2.2.1 Fish sampling

During seasonal (three monthly) fish sampling for *Activity 3* of T&I *Component 3*, from autumn (March) 2020 to winter (June) 2021, lagoon goby (*n* = 121) were collected using a standard seine net (61 m net length, 29 m wing length, 22 mm wing mesh, 3 m bund length, 8 mm bund mesh) (Table 1). Sampling occurred at 12 sites in the Coorong, with four sites in each region (Murray Estuary, North Lagoon and South Lagoon, Figure 1). After capture, all fish were frozen at -20 °C until processing. Most lagoon goby samples were collected from autumn–spring 2020. All lagoon gobies sampled were captured in the Murray Estuary and North Lagoon, with no individuals caught in the South Lagoon. A few (*n* = 4) samples of longsnout flounder (*Ammotretis rostratus*) were also collected during sampling and the diet of this species was opportunistically investigated to address a knowledge gap (*What is the diet composition of longsnout flounder in the Coorong?*, Ye et al. 2020a). No feeding selectivity or statistical analyses were considered for longsnout flounder due to low sample size and the diet composition results for longsnout flounder are presented in Appendix A.

Table 1. Number of lagoon goby retained for gut content analysis from seine net sampling in the Coorong from March 2020 to June 2021. Regions are ME = Murray Estuary, NL = North Lagoon. Empty stomach samples are included in the totals below and are different to non-empty gut sample numbers presented in Section 0. Seasons are autumn = March, winter = June, spring = September, summer = December.

REGION	SITE	2020				2021		TOTAL
		MARCH	JUNE	SEPTEMBER	DECEMBER	MARCH	JUNE	
ME	Beacon 19		16	4		1		21
	Boundary Creek				1			1
	Pelican Point			28				28
NL	Mark Point			13				13
	Long Point	35	13	1				49
	Noonameena	8					1	9

2.2.2 Gut content analysis

In the laboratory, fish were thawed and measured for total length (TL, to the nearest mm) and weight (to 0.01 g) before having their gut removed and preserved in 70% ethanol. Guts were dissected and the total gut content weight (to 0.01 g) was recorded. Items in stomachs were identified to the lowest taxonomic level possible under a dissecting microscope, enumerated and, for each prey category, volume was measured as a proxy for mass. Volume was estimated by squashing contents onto a laminated graph paper with 1 mm^2 squares to a consistent 1 mm height. Due to degradation from digestion, amphipods could not be consistently identified to a lower taxonomic level (e.g. to Family) and were recorded at the Order level. To quantify the contribution of prey to diet, the percentage by total number (%N) and total volume (%V) were calculated for different prey items (Hyslop 1980).

$$\%V = \frac{volume\ of\ food\ item}{total\ volume\ of\ all\ food\ items} \times 100\%$$

$$\%N = \frac{number\ of\ food\ item}{total\ number\ of\ all\ food\ items} \times 100\%$$

To investigate selective feeding behaviour and identify 'preferred' prey items of lagoon goby, diet composition was compared to prey availability in the ambient environment. Prey item selectivity was assessed by calculating the Strauss index of food selectivity (*L*, Strauss 1979), where *ri* is the relative abundance of a food item in the diet (proportion of total number in diet, %N) and *pi* is the relative abundance of the food item in the environment (proportion of total catch by number).

$$L = ri - pi$$

Benthic invertebrates were the key prey of lagoon goby, therefore ambient abundance data (individuals per m^2, 0.5 mm sieve size) of the benthic macroinvertebrate prey assemblage in the 'subtidal' zone (nearshore but always underwater) of selected sites were sourced from *Activity 3* of T&I *Component 3*. Proportions of prey in diet (*ri*) were recalculated after removing prey items that are not effectively sampled by macrobenthic monitoring methods (e.g. meiofauna including platyhelminths) and items that were not taxonomically resolved (e.g. unidentified annelids). The Strauss index was calculated for each individual prey item for site-season combinations where ambient data were compatible (i.e. same site and season of sampling). Calculations were restricted to site-season combinations with adequate ($n \geq 10$) stomach samples.

2.2.3 Statistical analysis

For lagoon goby, a data matrix comprising prey abundance volume (mm^3) data were entered into the software program PRIMER (v. 7.0.17) and PERMANOVA+ (Clarke and Gorley 2006; Anderson et al. 2008). Unidentifiable material (different to unidentified prey) was removed as a prey item prior to any analyses to reduce the influence on dissimilarity between factors. To investigate spatial and temporal variability in diet, a sample-similarity matrix was constructed using the Bray-Curtis algorithm (Bray and Curtis 1957) and samples were ordinated using non-metric Multi-Dimensional Scaling (nMDS) (Clarke et al. 2001). To test for spatial and temporal differences in diet composition, square-root transformed data were analysed using a two-factor (region, season) multivariate permutational analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity matrices (Anderson 2001). Analyses were confined to data from autumn to spring 2020 due to low sample sizes in other periods. Significance was set at $p = 0.05$ and p-values were obtained using 9,999 unrestricted permutations of raw data.

To assess the key dietary constituents of lagoon goby for the different site-season combinations, stacked bar plots of prey contributions to total diet by volume (%V) were generated in Sigmaplot (v. 14.0).

2.3 Waterbirds

2.3.1 Scat sampling

From January to June 2021, scats from sharp-tailed sandpiper (*n* = 39), red-necked stint (21), red-capped plover (69) and teal (grey and chestnut collectively, 143)[a] were collected from sites between Pelican Point (Murray Estuary) and Salt Creek (South Lagoon) (Figure 1, Table 2). Scat sampling was opportunistic and performed through or in conjunction with fieldwork undertaken for *Component 4* (*Maintaining viable waterbird populations*) of the HCHB T&I project. Scats were collected either from: (1) substrate surfaces ('ambient') or (2) directly from birds that were *in-hand* during the *Component 4* tagging study ('in-hand'). *Ambient* sampling occurred seasonally during January (summer), March (autumn) and June (winter), while *in-hand* sampling occurred broadly from February (summer) to June (winter). Scat sampling for migratory shorebird species (i.e. sharp-tailed sandpiper and red-necked stint) was prioritised during summer–autumn before their migration departure.

For *ambient* sampling, flocks of birds were observed foraging along mudflats or roosting in rocky habitats. When the birds had left the area, fresh scats were identified and collected. For quality control, a rating of 1 (poor) to 5 (high) was given to each scat sample for: (1) quality, as a measure of freshness and lack of contamination; and (2) species identification, as a measure of certainty of the species from which the scat originated. Scat samples with low (<3) quality and identification ratings were omitted from further processing. The identity of some low certainty bird samples (e.g. red-necked stint) were confirmed through DNA sequencing. Chestnut teal and grey teal often occurred as mixed species flocks and so scats from these species were combined (referred to herein as 'teal') due to uncertain species identity. Following other studies (e.g. Gerwing et al. 2016, McInnes et al. 2017a, Cavallo et al. 2018, Heller 2020), approximately 220 mg–1 g of fresh material was collected using a clean spatula, which was sterilised with 70% ethanol in between each sample to avoid cross-contamination. To avoid the sample containing known PCR inhibitors and contamination from the environment, only the dark, non-uric portion and top part of the scat were sampled

a The waterbirds assessed for diet were selected from a list of candidate species provided by DEW for the T&I *Component 4* (*Maintaining viable waterbird populations*) investigations (Prowse 2020). These species are abundant representatives of important life-history guilds in the Coorong. During the study period, insufficient scat samples were collected for a non-migratory wader target species (red-necked avocet *Recurvirostra novaehollandiae*) and so this species was not pursued further. The waterbird diet investigations focused on the South Lagoon as this was the key region for ecological restoration in the HCHB program. Lagoon goby was not present in the South Lagoon during sampling and so fish diet was assessed in other regions of the Coorong.

to avoid, respectively, inhibition by uric acid and contaminants from the substrate below. To screen scat samples for contamination with microbenthic animals from the environment, sediment scraping samples (*n* = 3) were collected from the mudflats at Parnka Point in the South Lagoon during scat sampling in January 2021. Scat material and sediment scrapings were placed in plastic vials with RNA*later*™ (ThermoFisher®) solution, at a ratio of 1 g scat:10 mLs RNA*later*. Vials were mixed by hand to ensure the sample was completely covered/submerged in the solution and stored on ice in the field before being transferred to -20 °C in the laboratory for downstream DNA extractions.

For *in-hand* sampling, sharp-tailed sandpipers were captured using walk-in traps (Lindström et al. 2005). Most captured birds defecated during handling and fresh scats were collected from surfaces using the same method described for *ambient* sampling.

Table 2. Summary of the number of bird scats opportunistically collected from the Murray Estuary (ME), North (NL) and South (SL) lagoons of the Coorong from January to June 2021. *indicates target species of the T&I *Component 4 Waterbirds* tagging study. Scats were sampled from mixed flocks of chestnut and grey teal.

SPECIES	REGION	MONTH				TOTAL
		JAN	FEB	MARCH	JUNE	
Sharp-tailed sandpiper*	NL		1	15		**16**
	SL	19	4			**23**
Red-necked stint	SL	21				**21**
Red-capped plover	SL			35	34	**69**
Teal (chestnut and grey)*	ME				22	**22**
	NL				41	**41**
	SL	60			20	**80**

2.3.2 Prey collection for DNA sequence reference library

DNA sequences of specific fauna from certain regions may be underrepresented in publicly available databases, meaning taxonomic classifications from next-generation sequencing data are at higher levels to ensure confidence in identification (Gerwing et al. 2016). Therefore, we collected invertebrates and plants that we identified as potential key prey for shorebirds and waterfowl from the Coorong and used them to establish Coorong-specific DNA sequence reference libraries for classifying food items in waterbird diet with greater resolution. From April to September 2021, individuals ($n < 10$) belonging to key animal prey taxa were collected during benthic invertebrate sampling for *Activity 3* of T&I *Component 3* (see methods in Ye et al. 2019) and *ambient* waterbird scat sampling (Table 3). Benthic invertebrates were collected using a PVC corer (83.32 cm^2 surface area) and retained through a sieve (500 µm mesh). During waterbird scat sampling, terrestrial insects were collected using 101 mm x 173 mm sticky traps sourced from Australian Entomological Supplies that were placed along the shoreline. Plant material was collected by hand during *ambient* scat sampling in June 2021 (Table 3). All invertebrates were preserved in plastic vials in ethanol (>70%) and stored at -20 °C. Plant material was rinsed with distilled water before being stored in zip-lock bags at -20 °C.

Table 3. Potential prey collected for establishing animal (*COI*) and plant (*rbcL*) Coorong-specific sequence reference libraries from April to September 2021. Some taxa (e.g. Chironomidae) were collected as multiple samples if thought to potentially be different species. Operational Taxonomic Units (OTUs) from scat samples that aligned with the reference sequences (<4 base pairs different) are indicated. Refer to Figure 1 for site locations. Single specimens per sample ID were often sequenced to generate the *COI* and *rbcL* sequence library.

SAMPLE ID/TAXA	TYPE	COLLECTION DATE	SITE	REF. NO.	OTU
COI reference library					
Australonereis ehlersi	Annelid	7/09/2021	Noonameena	1	
Capitellidae sp. a	Annelid	9/06/2021	Noonameena	2	#79
Ficopomatus enigmaticus	Annelid	14/09/2021	Beacon 19	3	
Aglaophamus australiensis	Annelid	28/04/2021	Pelican Point	4	
Phyllodoce novaehollandiae	Annelid	28/04/2021	Pelican Point	5	
Simplisetia aequisetis	Annelid	28/04/2021	Pelican Point	6	#51
Arthritica semen	Bivalve	28/04/2021	Pelican Point	7	#161
Amphipoda (Corophiidae sp. a)	Crustacean	9/06/2021	Noonameena	8	#5
Amphipoda (Gammaridea sp. a)*	Crustacean	28/04/2021	Pelican Point	9, 11	#63
Amphipoda (Gammaridea sp. a)	Crustacean	9/06/2021	Noonameena	10	#63
Ostracoda sp. a	Crustacean	7/09/2021	Parnka Point	12	#30
Ceratopogonidae sp. a	Dipteran	7/09/2021	Policemans Point	13	#662
Chironomidae sp. a	Dipteran	28/04/2021	Pelican Point	14	#1
Chironomidae sp. a	Dipteran	7/09/2021	Parnka Point	15	#1
Chironomidae sp. b	Dipteran	7/09/2021	Jack Point	16	#75
Chironomidae sp. a	Dipteran	9/06/2021	Noonameena	17	#1
Dolichopodidae sp. b	Dipteran	28/04/2021	Pelican Point	18	#516
Dolichopodidae sp. a	Dipteran	7/09/2021	Policemans Point	19	#40
Stratiomyidae sp. a	Dipteran	7/09/2021	Jack Point	20	#164
Diptera fly sp. a	Dipteran	11/06/2021	Salt Creek	21	#17
Diptera fly sp. b	Dipteran	13/06/2021	Villa de Yumpa	22	
***Hydrobia* sp. a**	Gastropod	28/04/2021	Pelican Point	23	
Salinator fragilis	Gastropod	28/04/2021	Pelican Point	24	#117
rbcL reference library					
***Ruppia* sp. a**	Plant	09/06/2021	Hack Point	1	#1
***Ulva* sp. a**	Plant	10/06/2021	Pelican Point	2	#12

*Amphipoda (*Melita* sp., Ref. No. 9) that was collected from Pelican Point on 28/04/2021 had a DNA sequence that aligned with the sequence from another species of Gammaridea. Therefore, Ref. No. 9 and 11 were amalgamated to Gammaridea sp. a.

2.3.3 Next-generation sequencing library preparation – DNA metabarcoding

The animal and plant dietary components of waterbirds were determined using DNA metabarcoding. Universal primers targeting two gene markers, specifically the mitochondrial cytochrome *c* oxidase subunit 1 (*COI*) and protein-coding chloroplast gene (*rbcL*), were used to amplify the DNA of animal and plant species, respectively, in waterbird scats. Species-specific blocking primers were designed and applied in the polymerase chain reaction (PCR) mix to reduce amplification of predator DNA for the *COI* gene assay. Sediment scrapings were analysed with the *COI* gene assay to determine the microbenthic animal community of the environment, which could be then used to screen for contamination in scat samples. Two reference library databases of *COI* and *rbcL* sequences (24 sequences for *COI* and 2 sequences for *rbcL,* Table 3) were generated from potential prey samples (e.g. annelids, bivalves, crustaceans, dipterans, gastropods and plants) collected from key foraging grounds in the Coorong to help resolve unknown taxonomic identifications of some Operational Taxonomic Units (OTUs). The remaining OTUs were classified by comparison with sequences in publicly available databases (see Section 2.3.4). A detailed description of the molecular techniques, including *COI* blocking primer design and optimisation, validation of *COI* and *rbcL* PCR assays, DNA extraction, amplification and sequencing, is provided in Appendix B.

To assess the animal dietary components of waterbirds, DNA extracts from 188 scat samples and three sediment scraping samples were PCR amplified using the universal *COI* primer set (Table 4). To assess the plant dietary component of teal (whose diet is predominantly vegetation, Delroy 1974) and explore the plant-based diet of sharp-tailed sandpiper[b] and red-capped plover (who predominantly feed on invertebrates but may consume plant seeds and turions, Paton 1982) at Parnka Point in the South Lagoon, 61 selected scat samples from these three bird species were PCR amplified with the *rbcL* modified primer set (Table 4).

Table 4. Summary of number of samples from each bird species and the ambient environment for the *COI* and *rbcL* gene markers that were PCR amplified, produced positive amplicons and were included in the sequencing library, and then in the final dataset post filtering following removal of samples with low total reads or high reads to predator OTUs. Refer to Table B4 in Appendix B for sampling site details. Teal = chestnut and grey teal.

BIRD SPECIES/ ENVIRONMENTAL SAMPLE	NO. SAMPLES PCR AMPLIFIED	NO. POSITIVE AMPLICONS & SEQUENCED	FINAL NO. SAMPLES IN DATASET POST FILTERING
COI			
Sharp-tailed sandpiper	39	32	28
Red-necked stint	21	17	17
Red-capped plover	69	69	59
Teal	59	41	38
Sediment scraping	3	3	3
COI TOTAL	**191**	**162**	**145**
rbcL			
Sharp-tailed sandpiper	10	8	8
Red-capped plover	10	5	5
Teal	41	34	34
rbcL TOTAL	**61**	**47**	**47**

[b] The plant dietary component was not assessed for red-necked stint but was considered to be similar to sharp-tailed sandpiper as they have similar feeding morphologies and strategies, i.e. have short bills adapted to probing, jabbing or pecking into sand and mud in shallow water (Higgins and Davies 1996, Dann 1999). Sample space in the next-generation sequencing libraries for the three shorebird species were prioritised for the assessment of the animal dietary component (*COI*) as this was considered the most important component in their diets (Thomas and Dartnall 1971, Poore et al. 1979).

2.3.4 Bioinformatics and taxonomic classifications

Sequence reads were paired using PEAR (version 0.9.5) (Zhang et al. 2014), where primers were identified and removed. The paired-end reads were then quality filtered, with removal of low-quality reads, full-length duplicate sequences (after being counted) and singleton sequences using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al. 2010), USEARCH (version 8.0.1623) (Edgar 2010) and UPARSE software (Edgar 2013). Reads were mapped to OTUs using a minimum identity of 97%. Rarefaction curves were used to inspect (retrospectively) sampling depth for each sample (Appendix C, Figure C1).

The datasets were then merged/split and analysed in two ways: 1) with all the bird species together ('all bird') in a single sheet for each gene marker, with total sample contribution to the whole dataset calculated and OTUs with a contribution of >0.01% to the whole dataset retained; and 2) with each bird species analysed separately in discreet sheets ('individual') for each gene marker, with sample contribution to the specific bird species dataset calculated and OTUs with a contribution of >0.01% to the specific bird species dataset retained.

Taxonomic identifications were assessed using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov) using the top hit with highest percent identity, e-value and query cover. Based on the percent identity value, OTUs were then assigned and collapsed to either Species (>99%), Genus (95–98.99%), Family (90–94.99%), Order (85–89.99%) or Class (<85%), with taxonomic classification checked using the Atlas of Living Australia (https://www.ala.org.au - *COI* dataset), Australian Plant Name Index – Australian Plant Census for Vascular Plants (https://biodiversity.org.au/nsl/services/search/taxonomy - *rbcL* plant dataset) or AlgaeBase (https://www.algaebase.org - *rbcL* algae dataset). The primers used in our study were universal, therefore they will amplify DNA from all animal or plant groups and respectively, they will also inevitably amplify unwanted DNA from non-food items such as parasites, contaminants from the environment or external organisms and the gut microbiota and fauna (McInnes et al. 2017a, 2017c). Using the taxonomic identifications, likely non-prey OTUs were hence removed from the *COI* dataset (e.g. vertebrates excluding fish, anthozoans, amoebae, bacteria, booklice, cephalopods, crinoids, diatoms, flagellates, fungi, gastrotrichs, hydrozoans, jellyfish, micro-algae, parasites, placozoans, rotifers, sea cucumber, sea urchins, single-celled eukaryotes, slime nets, sponges, stony corals, tunicates, ungulates and yeast). For the 'all bird' and teal individual *COI* datasets, unlikely fish taxa (e.g. open water pelagic and benthic marine species) were removed. All fish were removed from individual shorebird *COI* datasets because of uncertainty about their presence, although we note that a red-capped plover was observed consuming a small fish during this study (Markos 2021). Platyhelminths could include parasitic or free-living meiobenthic species and were left in the *COI* dataset due to non-definitive taxonomic classifications. For the *rbcL* dataset, only OTUs with taxonomic classification corresponding to likely-prey plant material were retained (e.g. angiosperms, conifers, filamentous green algae, flowering plants, stoneworts and seaweeds).

2.3.5 Statistical analysis

To assess the overall dietary patterns between bird species for each gene marker, the two datasets with all the waterbird species in a single sheet were entered into PRIMER (v.7.0.17) and PERMANOVA+ (Clarke and Gorley 2006; Anderson et al. 2008) as a data matrix comprising the percent standardised abundance of OTUs. To graphically represent similarities/differences in diet composition, a sample-similarity matrix was then constructed using the Bray-Curtis algorithm (Bray and Curtis 1957) and samples were ordinated using non-metric Multi-Dimensional Scaling (nMDS) (Clarke et al. 2001). The three shorebird species have similar feeding morphologies, such as bills adapted to foraging in sandy or muddy shallow water environments. However, they have different strategies for accessing prey; red-capped plovers primarily feed visually and take prey from sandy or muddy surfaces while sandpipers are more likely to probe, jab or peck into the mud in shallow water (Higgins and Davies 1996, Geering et al. 2007). To investigate inter-specific differences in the animal-based diet composition of the three species, we therefore tested for differences in their diet at Parnka Point – the only site where all three species were sampled during summer/autumn – using a one-factor (species) permutational multivariate analyses of variance (PERMANOVA) (Anderson 2001). Significance was set at $p = 0.05$ and p-values were obtained using 9,999 unrestricted permutations of raw data.

To explore intra-specific spatial variability in diet composition of waterbirds, we tested for differences in diet composition among sites (for each species separately using species-specific datasets) using a one-factor (site)

PERMANOVA. For red-capped plover, season was included as a factor in PERMANOVA (two-factor: site, season) tests because this species was consistently sampled at three sites during two seasons. To assess significant effects, PERMANOVA pair-wise comparisons were undertaken. To allow for multiple comparisons between sites and seasons, a false discovery rate (FDR) procedure (B–Y method correction) was adopted ($\alpha = \sum_{i=1}^{n}(1/i)$; e.g. for $n_{comparisons}$ = 6, B–Y method α = 0.05/ (1/1 + 1/2 + 1/3…….+1/6) = 0.020) (Benjamini and Yekutieli 2001, Narum 2006). For teal, data analyses were performed only on winter data (*COI* and *rbcL* datasets) because only one site was sampled in summer (Table 4). Where significant differences were detected for PERMANOVA pairwise comparisons between sites and seasons, the contribution of different prey items to the dissimilarity in diet composition was assessed using similarity percentage (SIMPER) analysis (Clarke 1993). A 50% cumulative contribution cut-off was applied. All tests were constrained to sample sizes ≥5.

To assess the key dietary constituents of each bird species for each gene marker, the single bird species datasets were used. Stacked bar plots were generated in Sigmaplot (v. 14.0) for those OTUs with a contribution of >0.01%.

To investigate selective feeding behaviour and identify 'preferred' invertebrate prey items of sharp-tailed sandpiper and red-capped plover, the Strauss index (*L*, Strauss 1979) was used, where *ri* is the relative abundance of a food item in the diet (proportion of total number of sequence reads for the *COI* dataset) and *pi* is the relative abundance of the food item in the environment (proportion of total catch by number).

$$L = ri - pi$$

Feeding selectivity was not assessed for teal because vegetation is considered their key food resource, for which there were inadequate ambient data. Ambient macroinvertebrate prey assemblage abundance data (individuals/m^2) from the foraging ('intertidal', regularly submerged and exposed by water level changes) zone of sites were sourced from *Activity 3* of *Component 3* of the HCHB T&I project. Ambient data from the 'subtidal' zone were used for Parnka Point in late March 2021 since no prey was found in the intertidal zone. Proportions of prey in diet (*ri*) were recalculated after removing prey items (OTUs) that: contributed to <0.01% of the total number of sequence reads in the *COI* dataset for a site-month combination; are not sampled by macrobenthic monitoring methods (e.g. meiofauna, including platyhelminths); are not taxonomically resolved (e.g. unclassified insects); or are considered to be from an external terrestrial, freshwater or marine habitat (e.g. coleopterans, hemipterans and freshwater decapods). The Strauss index was calculated for each individual prey item for site-month combinations where ambient data were compatible (i.e. same site and month of sampling). Calculations were restricted to site-month combinations with adequate ($n \geq 10$) scat samples. There were no compatible ambient data for adequate site-month samples of red-necked stint, and so this species was not assessed for feeding selectivity.

3 Results

3.1 Fish: Lagoon goby

3.1.1 General diet

Gut contents from 121 lagoon goby (22–53 mm TL) were identified, of which 25% (*n* = 30) were empty and excluded from further analysis. Amphipods were the major contributors to diet by number (91.0%) and volume (83.5%) (Table 5). The polychaete *Simplisetia aequisetis* and platyhelminths were the second and third most important prey items by volume (%V = 3.2% and 2.3%, respectively).

Table 5. Percentage contribution of prey items in the guts of lagoon goby (*n* = 91) by volume (%V) and number (%N). Gammaridae was observed to be the most dominant Family of amphipods. Unid. = unidentified.

PREY ITEM	%V	%N
Crustacea	**83.5**	**91.3**
Amphipoda	83.4	91.0
Ostracoda	0.1	0.3
Annelida	**3.6**	**0.8**
Polychaeta		
Simplisetia aequisetis	3.2	0.5
Annelida unid.	0.4	0.3
Mollusca	**0.4**	**0.8**
Bivalvia		
Arthritica semen		
Platyhelminthes	**2.3**	**7.2**
Unid. material	**10.2**	**N/A**

3.1.2 Intra-specific variability in diet composition

The diet composition of lagoon goby was similar across sites, and amphipods dominated their diet by volume (>65%) at all sites (Figure 2). Diet compositions at Long Point were similar during autumn and winter 2020, except for a greater contribution of the polychaete *S. aequisetis* during winter. The nMDS ordination also demonstrated a lack of grouping of samples by site and/or season (Figure 3a), and a large clustering of samples that were characterised by amphipods (Figure 3b). Due to limited comparable site-season comparisons (Figure 2), we grouped samples at the spatial scale by region (Table 1) to test for spatial and temporal variability in diet. Despite the polychaete *S. aequisetis* being exclusive to North Lagoon samples (Mark Point and Long Point, Figure 2), PERMANOVA indicated that there was no significant effect of region (PERMANOVA, *Pseudo-*$F_{1,85}$ = 0.64791, p = 0.602) and season (*Pseudo-*$F_{2,85}$ = 1.1461, p = 0.3219), nor the interaction of region and season (*Pseudo-*$F_{1,85}$ = 1.2679, p = 0.2701), on the diet composition of lagoon goby. This suggests that there were no spatial or temporal differences in diet composition, and that diet was predominantly characterised by amphipods in all regions and during all seasons.

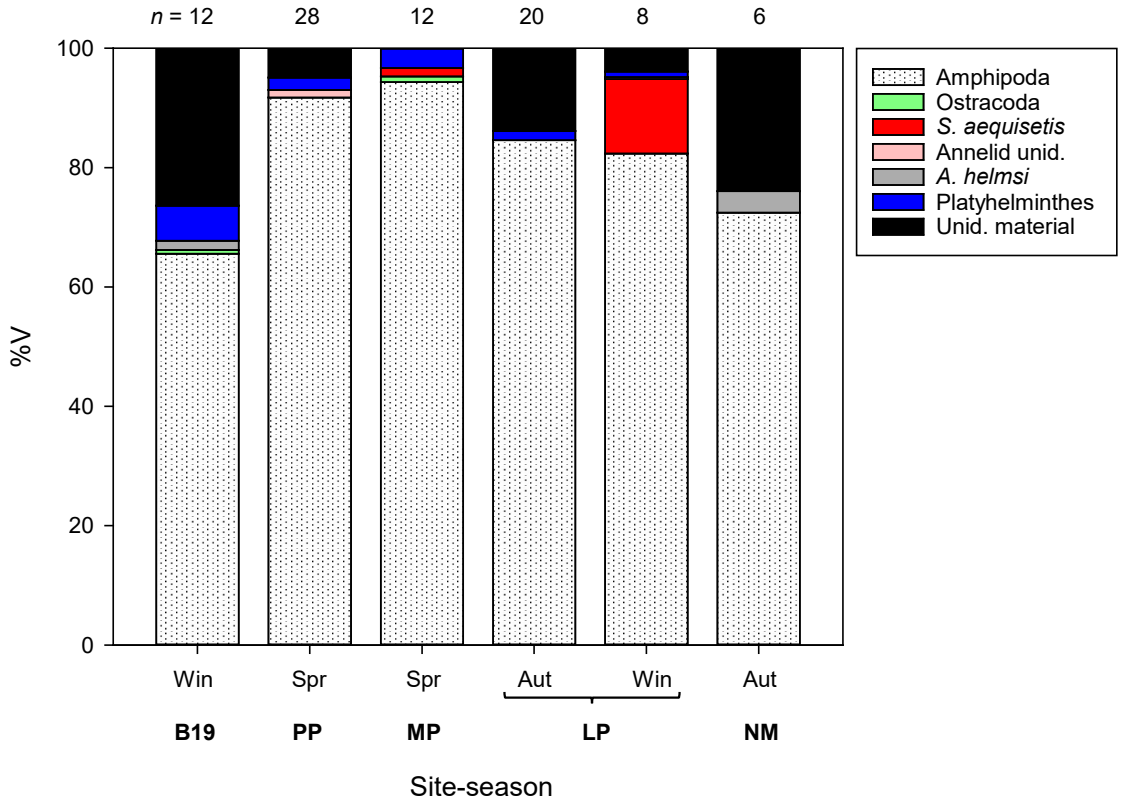

Figure 2. Diet composition (by volume, %V) of lagoon goby at five sites in the Coorong during 2020. Data are presented separately for seasons (aut = autumn, win = winter, spr = spring). Low (*n* < 5) sample numbers (provided at the top of each bar, as the number of non-empty stomachs) for site-season combinations are not presented. Sites are B19 = Beacon 19, PP = Pelican Point, MP = Mark Point, LP = Long Point and NM = Noonameena.

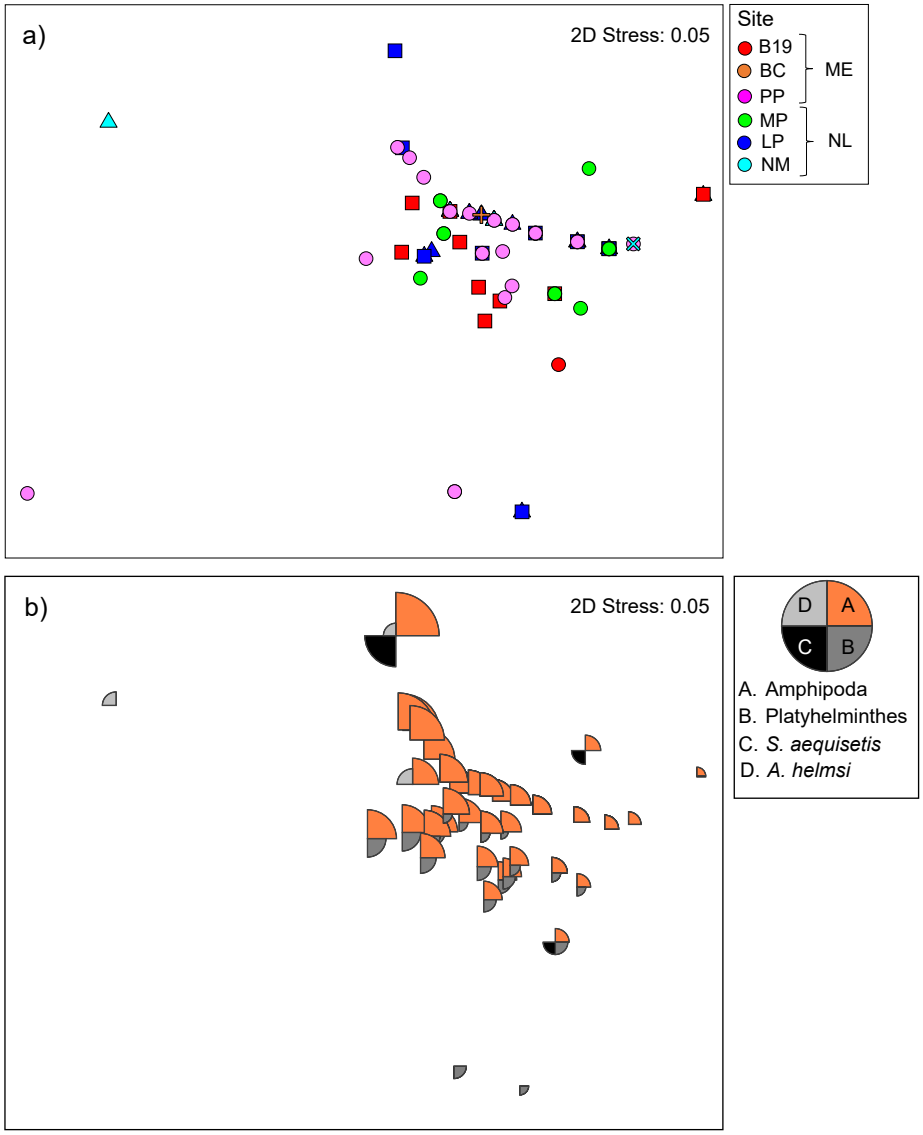

Figure 3. Two-dimensional non-metric Multi-Dimensional Scaling (nMDS) ordination of the diet compositions (volume, square root transformed) of lagoon goby at (a) different sites and seasons within the Coorong and (b) presented as a bubble plot showing the contribution of the four most dominant prey items to each sample by volume (maximum of 6 mm^3). Seasons are autumn 2020 = triangle, winter 2020 = square, spring 2020 = circle, summer 2020 = plus, winter 2021 = cross. Sites are B19 = Beacon 19, BC = Boundary Creek, PP = Pelican Point, MP = Mark Point, LP = Long Point, NM = Noonameena. Regions are ME = Murray Estuary, NL = North Lagoon.

3.1.3 Prey selection

High Strauss index values for amphipods at all three sites indicated that lagoon goby preferentially selected this prey item over others available (Figure 4). Lagoon goby showed the strongest selection (L = 0.84) for amphipods at Beacon 19, due to a lower proportional abundance of amphipods at this site, relative to other potential prey.

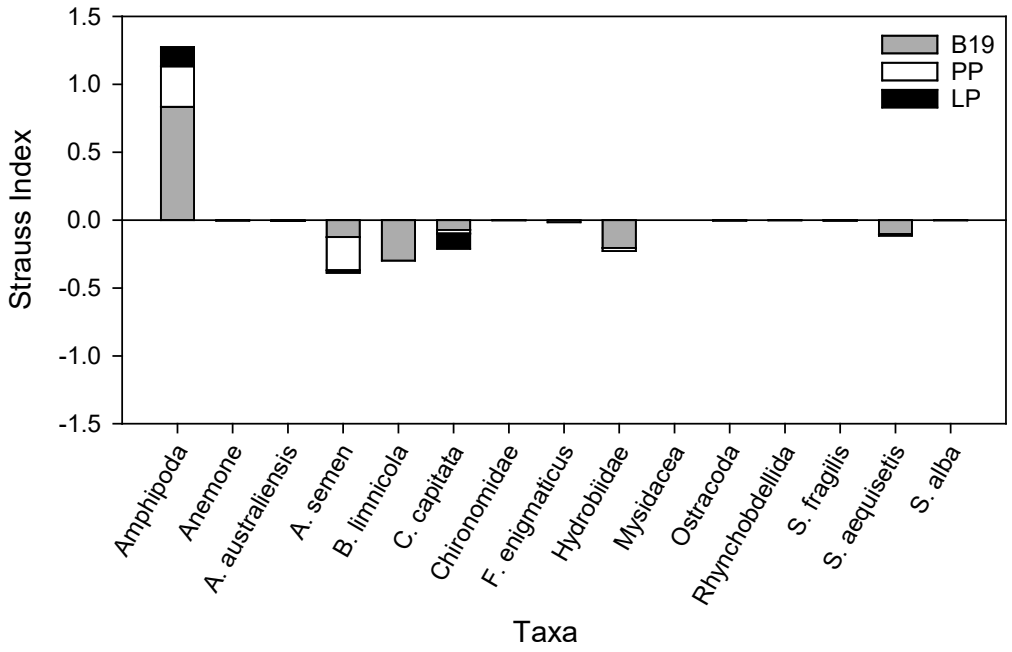

Figure 4. Prey selectivity by lagoon goby at Beacon 19 (B19, June), Pelican Point (PP, September) and Long Point (LP, March) during 2020, as determined by the Strauss index. Within each site, possible index values for prey taxa range from +1 (maximum selection for) to -1 (maximum selection against). Index values were calculated using compatible ambient subtidal benthic invertebrate data from *Activity 3* of T&I *Component 3 Food webs*. Species are: Bivalvia – *Arthritica semen*, *Soletellina alba*; Gastropoda – *Salinator fragilis;* Polychaeta – *Aglaophamus australiensis*, *Boccardiella limnicola*, *Capitella capitata*, *Ficopomatus enigmaticus*, *Simplisetia aequisetis*.

3.2 Waterbirds

For the animal prey (*COI*) dataset, 9,617,127 million sequence reads were derived from 162 samples, and for the plant prey (*rbcL*) dataset, 3,501,921 million sequence reads were derived from 47 samples (Table 4). Following further inspection of total read numbers per sample and reads mapped to predator (waterbird) OTUs, 17 samples in the *COI* dataset were removed. No samples were removed from the *rbcL* dataset. Hence, the final *COI* dataset comprised 145 samples and the final *rbcL* dataset comprised 47 samples (Table 4). The total number of sequence reads for an OTU (food item) across the bird samples was calculated, and the total sample contribution of that OTU (%) to the whole dataset or individual bird species was determined. Total reads in each bird sample were also calculated. After raw data processing and treatment, diet composition was calculated from the scats of sharp-tailed sandpiper (*COI*: 28, *rbcL*: 8), red-necked stint (*COI*: 17), red-capped plover (*COI*: 59, *rbcL*: 5) and teal (*COI*: 38, *rbcL*: 34) (Table 4). The percentage contribution of prey items (for OTUs >0.01% contribution) to each standardised dataset for each species is presented in Appendix D. The relative proportions of animal to plant material in diet could not be determined due to the non-comparable datasets. Therefore, the percentage of different items of animal and plants is relative in each case. The general trends in diet among species are discussed in Section 3.2.1 and species-specific diets are detailed in Section 3.2.2.

3.2.1 General diet

Animal items

The nMDS ordination of the *COI* waterbird diet composition data generally showed separation of Murray Estuary and North Lagoon samples from South Lagoon samples, which was consistent for the two species (sharp-tailed sandpiper and teal) that were sampled in multiple regions (Figure 5a). The distribution of samples appeared to be driven primarily by the contribution of the amphipod Corophiidae sp. a in North Lagoon samples, and dipteran insect larvae Chironomidae sp. a in South Lagoon samples (Figure 5b; Appendix D, Table D1). DNA sequences (OTU 5 and 1) from these two taxa matched with the Coorong-specific generated sequences (*COI* reference number 8 and reference numbers 14, 15 and 17, respectively, Table 3).

The ordination showed a general separation of teal samples from shorebird samples, which appeared to be driven primarily by the greater contribution of smallmouth hardyhead (*Atherinosoma microstoma*) in teal diet, and Chironomidae sp. a and Corophiidae sp. a in shorebird diet (Figure 5).

Variability in diet was observed for teal, particularly for the South Lagoon (Figure 5a). In contrast, there was a clustering of shorebird South Lagoon samples suggesting similarity and low variability in diet composition (Figure 5a). This was supported by PERMANOVA, which indicated that there were no interspecific differences (PERMANOVA, *Pseudo-*$F_{2,42}$ = 1.5059, p = 0.1554) in the diet composition of shorebirds (sharp-tailed sandpiper, red-necked stint, red-capped plover) at Parnka Point in the South Lagoon.

Sediment scraping samples from Parnka Point, which were collected to screen scat samples for contamination, were dominated by an unclassified hexanauplian crustacean and clearly distinguishable from other Parnka Point samples and all bird scat samples (Figure 5).

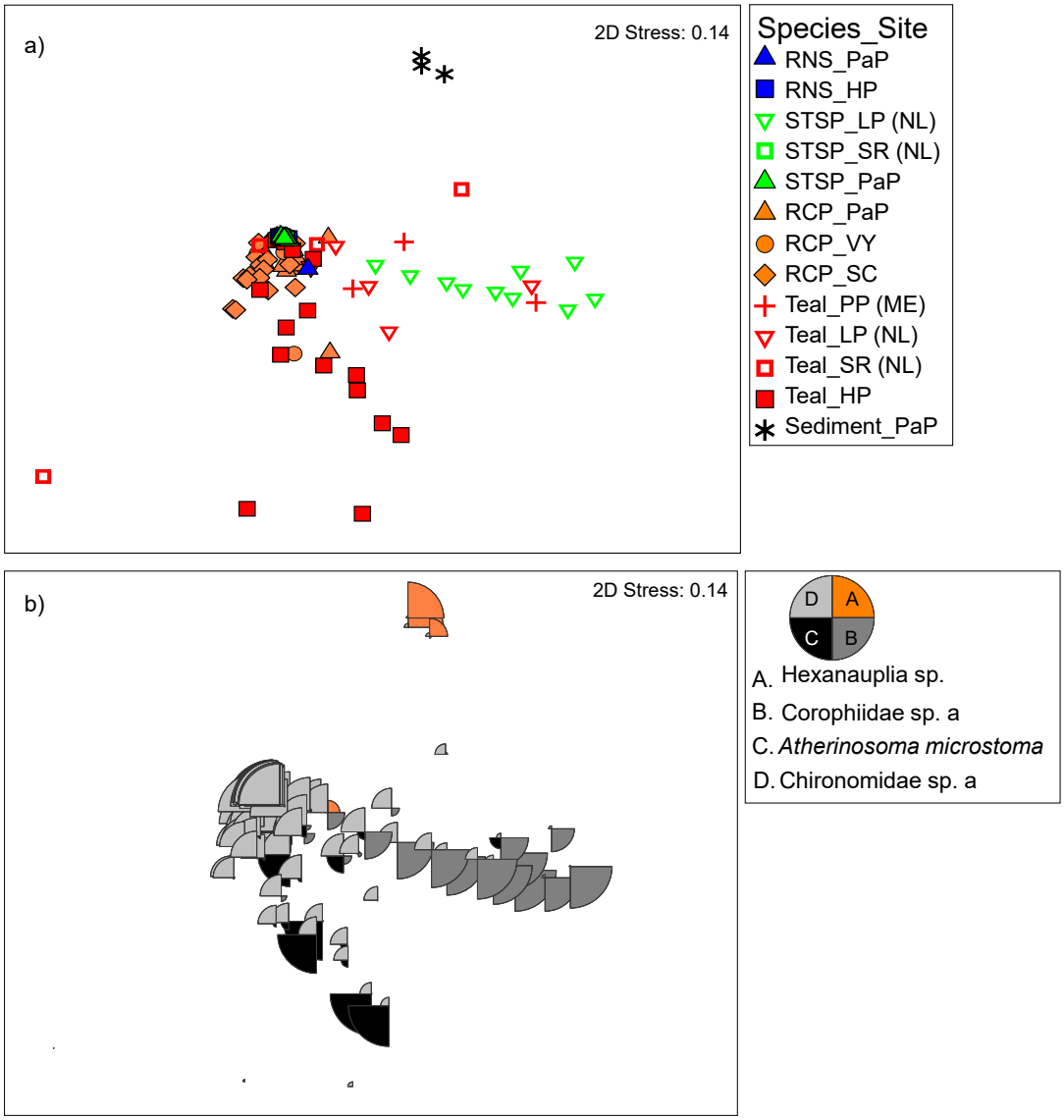

Figure 5. Two-dimensional non-metric Multi-Dimensional Scaling (nMDS) ordination of the *COI* (animal) dataset for red-necked stint (RNS), sharp-tailed sandpiper (STSP), red-capped plover (RCP), teal (chestnut and grey) and sediment scraping samples with OTU sample contribution >0.01% at (a) different sample collection sites within the Coorong and (b) presented as a bubble plot showing the contribution of four key prey/sample constituents (maximum of 100%) driving the spatial distribution of samples. Data are presented for the South Lagoon (closed symbols), North Lagoon (NL, open symbols) and Murray Estuary (ME, + symbol). Sites are HP = Hack Point, LP = Long Point, PP = Pelican Point, PaP = Parnka Point, SC = Salt Creek, SR = Seven Mile Road, VY = Villa del Yumpa.

Plant items

The nMDS ordination of the *rbcL* waterbird diet composition data showed some separation of samples by region of sampling (Figure 6a), although with some overlap of North Lagoon and South Lagoon samples. This separation of samples appeared to be driven primarily by the contribution of the submergent halophyte *Ruppia* sp. a (OTU 1) in South Lagoon and North Lagoon samples, which matched with the Coorong-specific generated sequence (*rbcL* reference number 1, Table 3) (Figure 6b). There was grouping of shorebird and teal samples from the South Lagoon, although greater variability in diet was observed for teal in the South Lagoon, compared to shorebirds (Figure 6a).

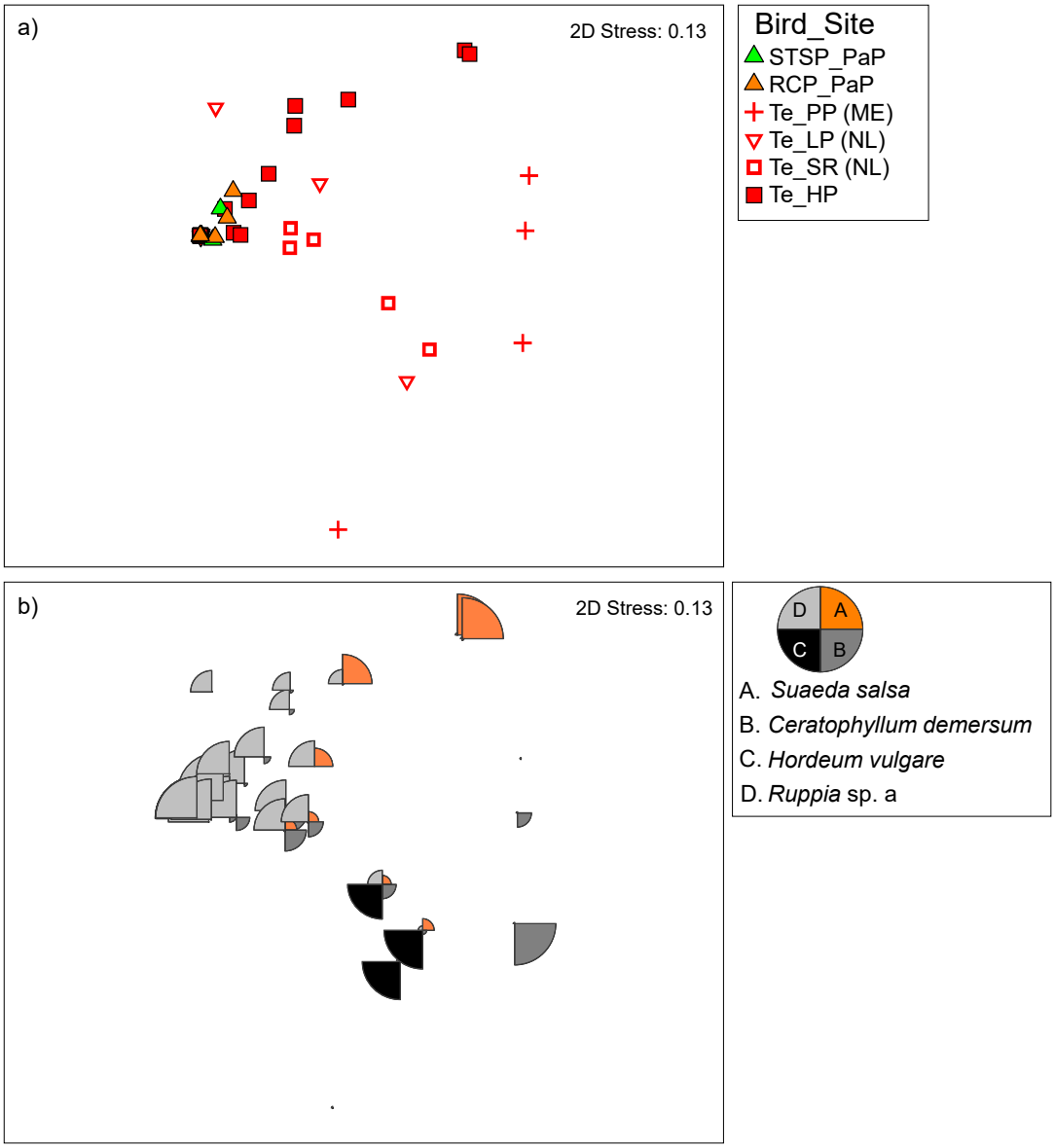

Figure 6. Two-dimensional non-metric Multi-Dimensional Scaling (nMDS) ordination of the *rbcL* (plant) dataset for sharp-tailed sandpiper (STSP), red-capped plover (RCP) and teal (chestnut and grey) samples with OTU sample contribution >0.01% at (a) different sample collection sites within the Coorong and (b) presented as a bubble plot showing the contribution of four key prey/sample constituents (maximum of 100%) driving the spatial distribution of samples. Data are presented for the South Lagoon (closed symbols), North Lagoon (NL, open symbols) and Murray Estuary (ME, + symbol). Sites are HP = Hack Point, LP = Long Point, PP = Pelican Point, PaP = Parnka Point, SR = Seven Mile Road.

3.2.2 Intra-specific variability in diet composition

Migratory shorebirds: Sharp-tailed sandpiper and red-necked stint

The animal-based diets of sharp-tailed sandpiper and red-necked stint in the South Lagoon were dominated (89 and 90%, respectively) by chironomids (Figure 7a). Unclassified insects largely formed the remainder (11 and 10%) of their diets. In the North Lagoon, corophiid and gammarid amphipods collectively contributed to 67% of the diet composition of sharp-tailed sandpiper. Chironomids (14%), platyhelminths (14%), the polychaete *Simplisetia australis* (2%) and unclassified insects (1%) contributed to the remainder of the diet composition in this region.

PERMANOVA indicated that sharp-tailed sandpiper diet composition was significantly different between Long Point in the North Lagoon and Parnka Point in the South Lagoon (PERMANOVA, *Pseudo-*$F_{1,26}$ = 139.7, p = 0.0001, Figure 5), with the dissimilarity in diet driven by a higher contribution of Chironomidae sp. a to diet composition at Parnka Point and the amphipod Corophiidae sp. a at Long Point (SIMPER, Appendix E). PERMANOVA indicated that red-necked stint diet composition was significantly different between Hack Point and Parnka Point in the South Lagoon (PERMANOVA, *Pseudo-*$F_{1,16}$ = 4.9967, p = 0.0083), influenced by one sample (Figure 5). Albeit low (14%), the dissimilarity in diet was driven by higher contributions of Chironomidae sp. a at Hack Point and unclassified insects at Parnka Point (SIMPER, Appendix E).

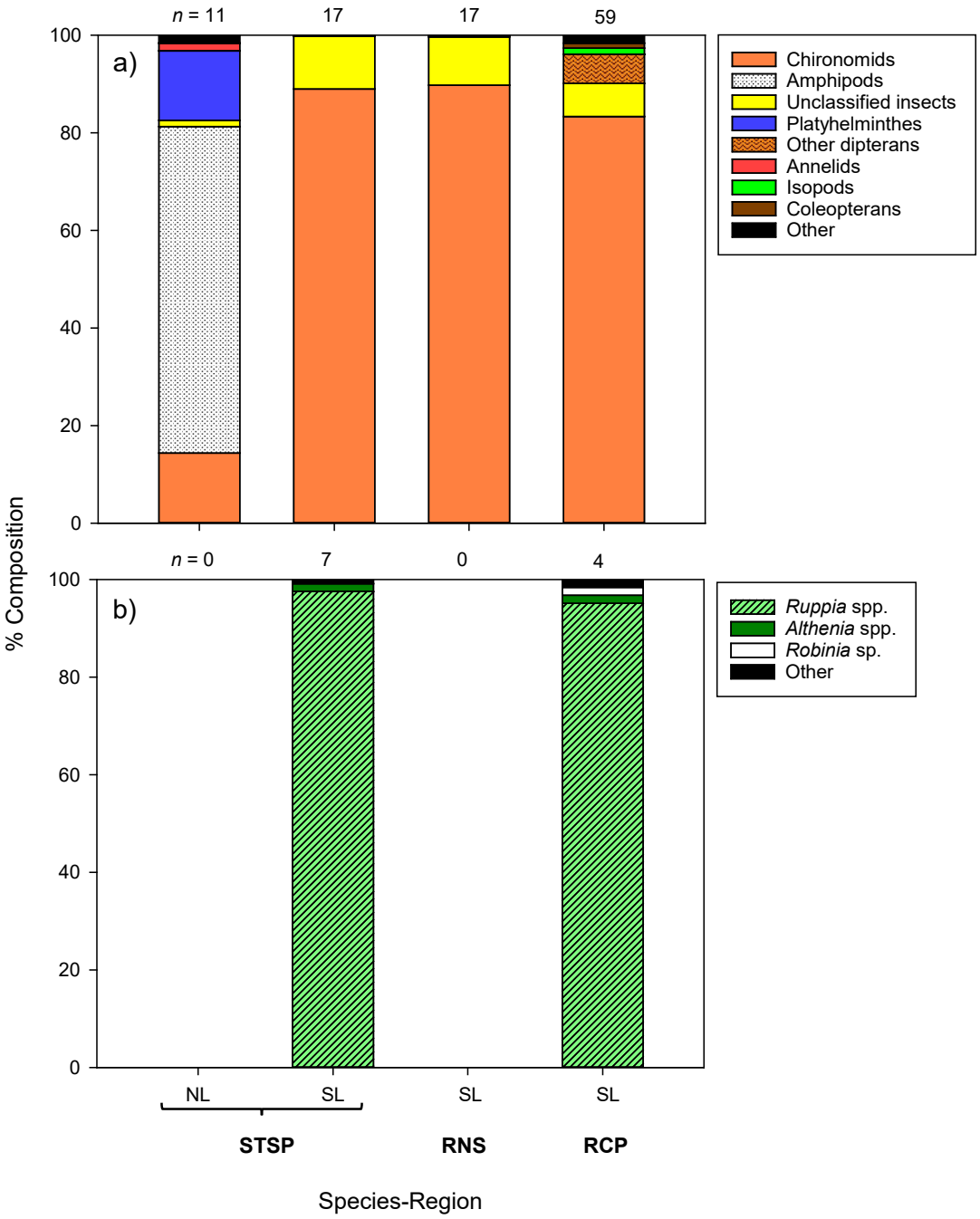

Figure 7. Contribution of (a) animal (*COI*) and (b) plant (*rbcL*) prey items to the diet composition (% of total reads in each dataset) of migratory (STSP = sharp-tailed sandpiper, RNS = red-necked stint) and non-migratory (RCP = red-capped plover) shorebirds in the Coorong during 2021 (sites and seasons pooled). Data are presented separately for regions (NL = North Lagoon, SL = South Lagoon). Sample numbers are provided at the top of each bar. The plant-based diet was not assessed for STSP samples from the NL or RNS samples from the SL. Other animals includes hemipterans, decapods, gastropods, millipedes, copepods, arachnids, hymenopterans, bivalves, cladocerans, ostracods, centipedes, collembolans, other insects and other crustaceans.

The plant-based diet composition of sharp-tailed sandpiper at Parnka Point in the South Lagoon was dominated (98%) by the submergent halophyte *Ruppia* spp. (Figure 7b). Another submergent halophyte *Althenia* sp. also contributed to a small (2%) proportion of the diet.

Non-migratory shorebird: Red-capped plover

The animal-based composition of red-capped plover diet in the South Lagoon was predominately chironomids (83%) and unclassified insects (7%) (Figure 7a). Other dipteran insects (e.g. true flies (Diptera fly sp. a (OTU 17 and COI reference number 21, Table 3), Sphaeroceridae sp., *Lispe* sp.) and frit flies (Oscinellinae sp.)), the isopod *Haloniscus searlei* and beetles (coleopterans) largely formed the remainder (8% collectively) of the diet (Appendix D, Table D1). PERMANOVA indicated that there was a significant interaction between site and season (*Pseudo-*$F_{2,58}$ = 8.603, p = 0.001) on the diet composition of red-capped plover (Appendix E, Table E1), suggesting that differences in diet composition between sites in the South Lagoon were not consistent among seasons and vice versa. Pairwise comparisons revealed that diet was different between Parnka Point and Salt Creek during both autumn and winter, while diet was different between Villa de Yumpa and Salt Creek during winter only (Appendix E). SIMPER indicated that these differences were driven, in autumn, by greater contributions of dipteran insects (Chironomidae sp. a and Oscinellinae sp.) at Salt Creek and unclassified insects and the isopod *H. searlei* at Parnka Point, and in winter by greater contributions of Chironomidae sp. a at Parnka Point and Villa de Yumpa and Diptera fly sp. a at Salt Creek (SIMPER, Appendix E).

DNA in red-capped plover scat samples collected from only March 2021 (n = 5) amplified successfully with the *rbcL* gene marker (Table 4). The plant-based diet composition of red-capped plover at Parnka Point in the South Lagoon was comprised primarily (95%) of *Ruppia* spp. (Figure 7b), with minor contributions (2%) of *Althenia* sp. and a deciduous terrestrial tree *Robinia* sp.

Non-migratory waterfowl: Teal

The plant-based diet of teal (chestnut and grey) consisted of a variety of submergent, emergent, amphibious and terrestrial vegetation from freshwater, estuarine/marine aquatic and terrestrial environments (Appendix D, Table D2). In the Murray Estuary, freshwater submergent (*Ceratophyllum* spp.) and amphibious (*Myriophyllum* sp.) plants collectively composed 52% of the diet composition of teal, while green algae (other Ulvales and *Ulva* spp., 29%) and samphire (*Salicornia* sp., 16%) were other major contributors (Figure 8a). In the North Lagoon, *Ruppia* spp. (40%) and agricultural barley (*Hordeum vulgare*, 25%) were the major diet components. Teal diet in the South Lagoon was dominated by *Ruppia* spp. (85%) but also included *Althenia* sp. and samphire (*Suaeda* sp.) (Figure 8a). PERMANOVA indicated that the plant-based diet composition of teal was not significantly different between sites (Long Point, Seven Mile Road and Hack Point) from the North and South lagoons (PERMANOVA, *Pseudo-*$F_{2,19}$ = 1.801, p = 0.072) (Figure 6).

The animal-based diet of teal in the Murray Estuary and North Lagoon was mainly comprised of amphipods, chironomids, fish (e.g. black bream *Acanthopagrus butcheri* and smallmouth hardyhead) and platyhelminths (collectively 86% and 66%, respectively) (Figure 8b). The gastropod *Ascorhis tasmanica* (6%) and millipedes (26%) were important contributors to diet in the Murray Estuary and North Lagoon, respectively. In the South Lagoon, chironomids (43%), the isopod *Haloniscus searlei* (13%) and fish (e.g. black bream and smallmouth hardyhead, collectively 25%) were the major contributors to teal diet (Figure 8b). Despite distinct separation of teal *COI* samples by site in the NMDS ordination (Figure 5a), due to high intra-site variability PERMANOVA indicated that the animal-based diet composition of teal was not significantly different between Long Point from the North Lagoon and Hack Point from the South Lagoon (PERMANOVA, *Pseudo-*$F_{1,14}$ = 1.7807, p = 0.0667).

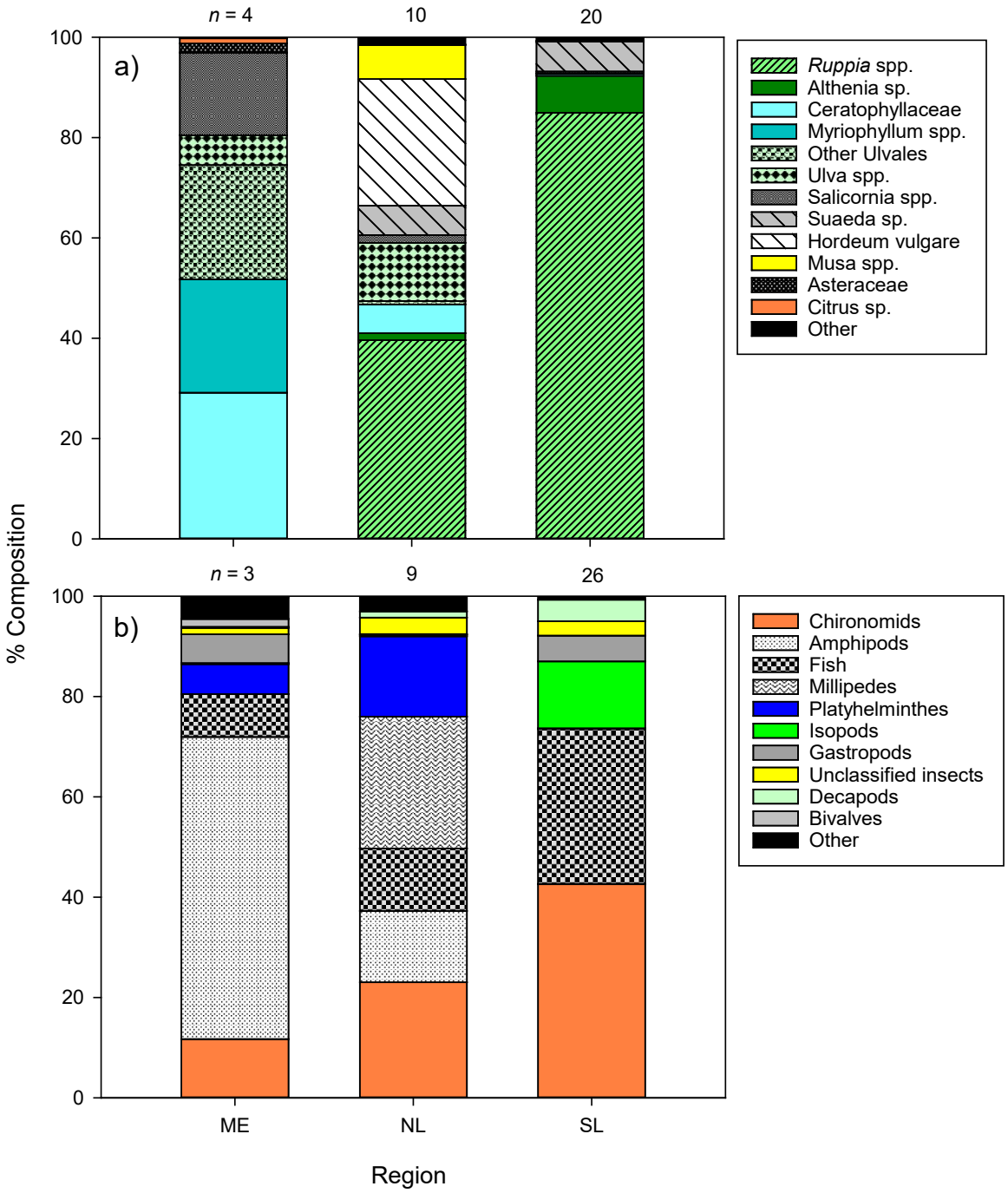

Figure 8. Contribution of (a) plant (*rbcL*) and (b) animal (*COI*) prey items to the diet composition (% of total reads in each dataset) of grey and chestnut teal (non-migratory waterfowl) in the Coorong during 2021 (sites and seasons pooled). Data are presented separately for regions (ME = Murray Estuary, NL = North Lagoon, SL = South Lagoon). Sample numbers are provided at the top of each bar. Teal may include chestnut and/or grey teal. Other animals include other dipterans, annelids, coleopterans, other crustaceans, hemipterans, other insects, copepods, arachnids, ostracods, centipedes, collembolans.

3.2.3 Prey selection by shorebirds

As determined by the Strauss index, sharp-tailed sandpiper showed strong selection for amphipods (*L* = 0.71) at Long Point in the North Lagoon and minor selection for chironomids at Long Point in the North Lagoon (0.12) and Parnka Point in the South Lagoon (0.06) (Figure 9a). With the exception of strong selection (*L* = 0.75) for chironomids by red-capped plover at Salt Creek in autumn, red-capped plover showed neutral selection (*L* = -0.07–0.04) for chironomids in the South Lagoon (Figure 9b). Red-capped plover showed a slight selection (0.00–0.07) for the isopod *H. searlei* as this species was not recorded in the intertidal zone of these sites during benthic monitoring in March and June 2021 (Figure 9b).

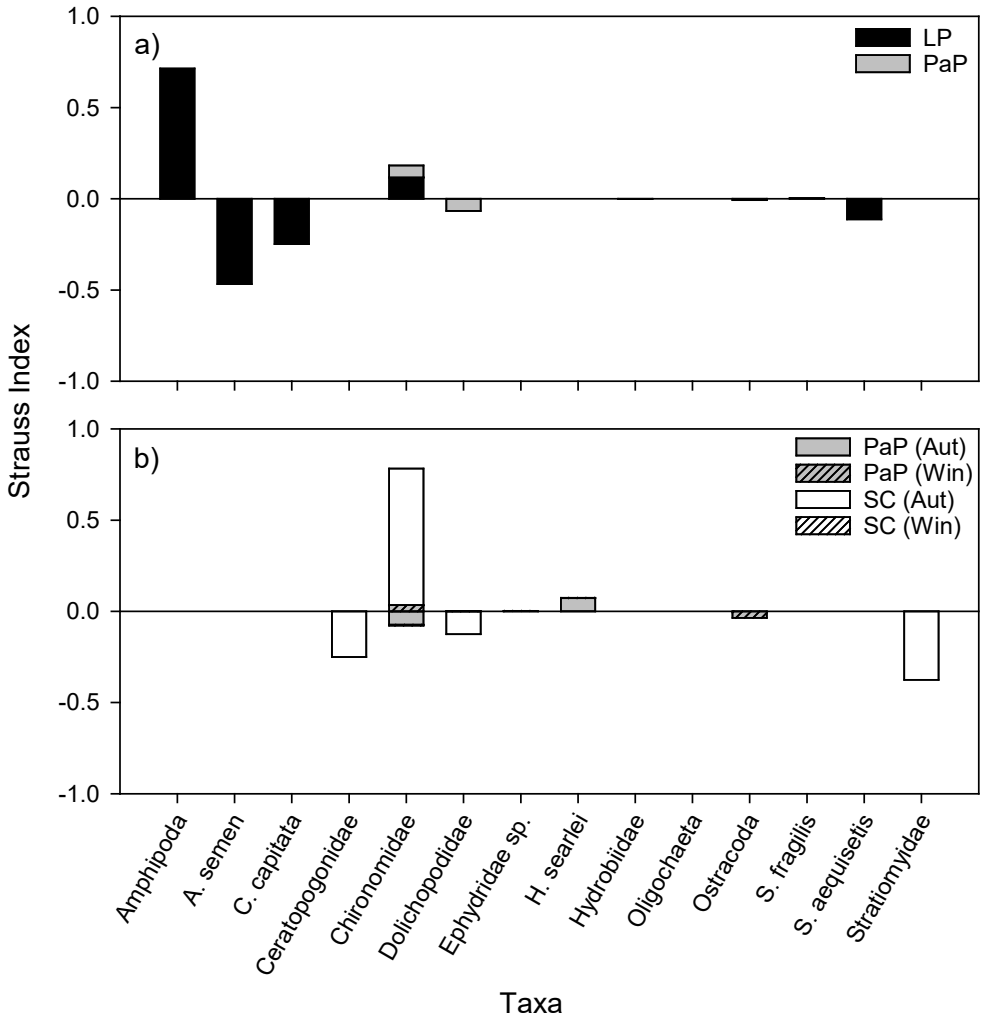

Figure 9. Prey selectivity by (a) sharp-tailed sandpiper at Long Point (January) and Parnka Point (March), and (b) red-capped plover at Parnka Point and Salt Creek during autumn (March, plain bars) and winter (June, dashed bars) during 2021, as determined by the Strauss Index. For each site, possible index values for prey taxa range from +1 (maximum selection for) to -1 (maximum selection against). Index values were calculated using compatible ambient intertidal benthic invertebrate data from *Activity 3* of T&I *Component 3 Food webs*. Species are: Bivalvia – *Arthritica semen*; Gastropoda – *Salinator fragilis;* Isopoda – *Haloniscus searlei*; Polychaeta – *Capitella capitata*, *Simplisetia aequisetis*.

4 Discussion

Through this work, we addressed key dietary knowledge gaps that were identified during a review and synthesis of the literature (Ye et al. 2020a). The knowledge gaps included classifying the diet composition of abundant waterbirds and less common fish species, and more specifically, determining the contribution of particular plant and animal prey items to the diets of shorebirds and waterfowl. To address these knowledge gaps, we determined the major food resources and their relative contribution to the diet for a small-bodied, benthic, prey species (lagoon goby) and five key waterbird species (shorebirds - sharp-tailed sandpiper, red-necked stint and red-capped plover; waterfowl - chestnut teal and grey teal, collectively referred to as 'teal') in the Coorong using traditional gut content analysis (fish) and DNA metabarcoding of scats (waterbirds).

4.1 Fish: Lagoon goby

What is the diet composition of lagoon goby?

The Lagoon goby is a small-bodied (<55 mm), benthic, estuarine resident species that is broadly distributed across south-eastern Australia. The diet of this species was undocumented. This study shows that the lagoon goby is a zoobenthivore that feeds on small benthic/epibenthic invertebrates, largely crustaceans, annelids

and molluscs. Amphipods were the key food resource for this species in the Coorong and were preferentially selected over other items available. In the Coorong, these small, epibenthic prey are abundant and broadly distributed from fresh (0 ppt) to hypersaline environments (~50 ppt) (Ye et al. 2020a). During 2020–21, amphipods were present at all sites where lagoon goby were sampled (*Activity 3* of T&I *Component 3*). Lagoon goby may be an important prey fish for other higher-order predators, such as piscivorous fish, birds and seals (Giatas et al. 2018), therefore our findings further highlight the underlying importance of amphipods in the system to maintain higher-level food web dynamics.

Other goby species occur in the Coorong; our findings have broader implications and knowledge gains in understanding coexistence and competition dynamics between Coorong gobiids, including those with overlapping or segregated habitat and diet preferences. For example, the larger-sized (<110 mm) Tamar goby (*Afurcagobius tamarensis*) also has a diet consisting predominately of amphipods (Hossain *et al.* 2017, Silvester 2011), and overlaps in habitat preference with the lagoon goby, forming burrows and showing preference for silty or muddy habitats (Lintermans 2007). Although there is overlap in diet and habitat preferences, these two gobiids coexist in the North Lagoon of the Coorong. Nonetheless, the Tamar goby is more abundant in the Coorong relative to lagoon goby (Ye et al. 2020a), suggesting that even though coexistence can occur, there is competition between these two species due to shared resource requirements. Other Australian studies have shown that when there is spatial segregation and dietary partitioning among co-occurring gobiids, both species can persist at high abundances (Gill and Potter 1993). Bridled goby (*Arenigobius bifrenatus*) and bluespot goby (*Pseudogobius olorum*) also regularly occur in the Coorong (Ye et al. 2020a). Unlike lagoon goby, these species are omnivores or detritivores (Robertson 1984, Gill and Potter 1993, Edgar and Shaw 1995, Becker and Laurenson 2007). All gobiids (lagoon, bluespot and bridled) co-occur at relatively equal, yet variable abundances in the Coorong (Ye et al. 2020a), highlighting the influence of spatial and dietary partitioning in the coexistence dynamics of these species.

4.2 Waterbirds

4.2.1 Shorebirds: Sharp-tailed sandpiper, red-necked stint and red-capped plover

What are the major food resources and their relative contribution to the diet for shorebirds in the Coorong?

Based on Parnka Point where data from all three species were obtained, the animal-based diets of sharp-tailed sandpiper, red-necked stint and red-capped plover did not differ from one another in the current study. Non-aquatic insects (e.g. coleopterans, true and frit flies), however, contributed more to red-capped plover diet. In the hypersaline South Lagoon (mean salinity 75–108 ppt), the diet compositions of these short-billed shorebirds were dominated by chironomids (presumably benthic larvae and pupae), which is consistent with long-term annual monitoring in the Coorong that discusses the importance of chironomids to shorebirds in the Coorong (e.g. Paton et al. 2021). In the predominately marine to slightly hypersaline (27–52 ppt) North Lagoon, the diet composition of sharp-tailed sandpiper was more diverse with corophiid and gammarid amphipods being the main constituents amongst other invertebrates, mostly chironomids and platyhelminths. The observed spatial difference in the diet of sharp-tailed sandpiper is reflective of the change in the benthic aquatic macroinvertebrate prey along the salinity gradient of the Coorong, which is the main driver of the macroinvertebrate assemblages in the Coorong (Ye et al. 2020a).

Prey items were consistent with other literature from temperate Australia and the foraging strategies used by these species. Sharp-tailed sandpiper and red-necked stint generally forage along the shoreline in wet sediment and display pecking, jabbing and probing feeding techniques (Higgins and Davies 1996, Dann 1999, Keuning 2011). In other southern Australian estuaries and tidal habitats, the diets of these species are predominantly aquatic larvae of dipteran insects (including chironomids), and amphipods and small gastropods (Thomas and Dartnall 1971, Poore et al. 1979). Red-capped plover may forage at higher elevations of the shoreline and exhibit a 'run-stop-peck' foraging technique (Thomas et al. 2006, Markos 2021), visually searching for prey and then running to the animal once detected (Pienkowski 1983). This strategy indicates that red-capped plover would primarily take surface-dwelling or shallow-burying prey (Barbosa and Moreno 1999; Martin and Piersma 2009), which is reflected by the prey items (e.g. coleopterans) detected in the current study and others (e.g. Poore et al. 1979). Our results contrast with findings by Paton (1982), where a coleopteran (*Clivina* sp.), a non-aquatic species, was the main prey item of red-necked stint and red-capped

plover in the South Lagoon of the Coorong. Low sample sizes in the Paton (1982) may have influenced this result.

Prey choice by shorebirds is influenced by multiple factors including prey availability (including accessibility), behaviour and profitability (Dann 2014, Finn et al. 2008, Spruzen et al. 2008, Estrella and Masero 2010). Competition among species is mitigated by differences in morphological specialisations and differences in bill size related to body size, both of which influence prey selection (Novcic 2016). The short bill penetration for these three species of shorebirds largely restricts them to feed on surface-dwelling (e.g. amphipods, gastropods) and shallow-burying prey (e.g. dipteran larvae) to 2 cm sediment depth (Keuning 2011), which is reflected in the findings from this study. The polychaetes *Simplisetia aequisetis* and *Capitella capitata*, which were relatively abundant in Long Point in the North Lagoon during scat sampling in 2021 (*Activity 3* of T&I *Component 3*), were not consumed in proportion to their ambient availability and contributed negligibly to the diet of sharp-tailed sandpiper. Such findings suggest that these burrowing prey species are less accessible or that too much energy is required by sharp-tailed sandpipers to find prey. In other comparative studies in south-eastern Australia, polychaetes (e.g. nereids) also contributed little to the diet of short-billed shorebirds (e.g. red-necked stint), but composed considerable proportions of the diet of longer-billed shorebirds including curlew sandpiper *Calidris ferruginea* (Thomas and Dartnall 1971, Dann 1999), which also occur in the Coorong.

The size of available shorebird foraging habitat (i.e. exposed mudflats) can greatly influence the presence and abundance of shorebirds within a region (Kraan et al. 2009). In the Coorong, mudflat exposure is influenced by water level, which is driven by tides and wind. The South Lagoon of the Coorong is shallow and not influenced by tide, and thus small changes in water level can translate to large changes in exposed foraging habitat. The benthic prey assemblage is depauperate in this region and comprised mostly of chironomid larvae and pupae (Ye et al. 2020a), which are tolerant to high salinities (e.g. >100 ppt, Dittmann et al. 2015). The consumption of chironomids by short-billed shorebirds in this region was non-selective (neutral Strauss index values) and suggests that birds are feeding in this region due to the available foraging habitat (e.g. exposure of wet mud), rather than selecting habitat because of high prey abundances or the occurring prey species. There is also suggestion of chironomid larvae being washed ashore by wind-induced waves in this region (Paton 2010), and so shorebirds may also feed on dislodged larvae along the shoreline. In the North Lagoon, where both chironomids and amphipods were available, sharp-tailed sandpiper showed strong selection for the latter. The importance of amphipods, in particular corophiids, in the diets of red-necked stint at other migration stopover sites in south-eastern Tasmania (Thomas and Dartnall 1971) and other *Calidris* species in Canada and USA (Hicklin and Smith 1984, Hicklin 1987, Novcic et al. 2016) has been documented. In the Bay of Fundy, the crawling behaviour of the corophiid amphipod *Corophium volutator* is considered to make them an easier target for predation by shorebirds (Boates and Smith 2011). Sharp-tailed sandpiper and other shorebirds are visual feeders and possess short bills, and may identify or feed on surface-dwelling amphipods in a more efficient manner than on burrowing benthic in-fauna (e.g. polychaetes) in the Coorong. Amphipods are therefore likely to be an important food resource for red-necked stint in the North Lagoon, as we found for sharp-tailed sandpiper in this study.

What is the contribution of* Ruppia *seeds and turions to the diet composition of shorebirds?

The relative proportions of animal to plant food items in the diet of shorebirds could not be determined by our methodology, because the gene markers used for DNA amplification do not overlap across animal and plant groups. Therefore, the proportion of plant seeds and turions in the diet by shorebirds could not be determined. Nevertheless, this study identified what food items were eaten and their relative proportions to each other within their respective animal and plant categories. In the Coorong, we considered short-billed shorebirds to be omnivorous, with animal items (benthic invertebrates) comprising most of their diets. Support for this is shown by the poor amplification of plant DNA in red-capped plover scat samples from June 2021, suggesting low amounts of plant DNA in scats, and, potentially, differences in foraging behaviour of this species between seasons. Based on a sample of sharp-tailed sandpiper and red-capped plover from Parnka Point in the South Lagoon, *Ruppia* spp. was the main vegetation species consumed, although the contribution to overall diet composition under current conditions is considered to be small. Contribution of *Ruppia* spp. seeds and turions in the diets of shorebirds has been documented for the Coorong (Paton 1982) and other parts of southern Australia (Poore et al. 1979). While the molecular technique we used did not identify which part of the plants were consumed, due to shorebird feeding ecology (Dann 1999, Thomas et al. 2006), it was most likely seeds or turions.

4.2.2 Waterfowl: Chestnut and grey teal

What are the major food resources and their relative contribution to the diet for waterfowl in the Coorong?

In the Coorong, we consider that grey and chestnut teal are omnivorous, with vegetation being the major component of their diets. We observed that grey and chestnut teal consumed a diverse range of vegetation from different environments, demonstrating foraging patterns outside of the Coorong. The halophyte *Ruppia* spp. ('wigeongrass'), freshwater plants (*Ceratophyllum* spp. and *Myriophyllum* sp.), samphire (*Salicornia* sp. and *Suaeda* sp.), barley (*Hordeum vulgare*) and green algae (e.g. *Ulva* spp.) were the key food items consumed. The prevalence of these items in diet are consistent with their distribution in the area; *Ruppia* spp. is the dominant submergent plant in the southern Coorong, hornworts (*Ceratophyllum*) and milfoil (*Myriophyllum*) are prevalent in Lakes Alexandrina and Albert and samphire and saltmarsh species are widespread in areas of the Coorong with moderate and high salinities (Nicol et al. 2018).

Delroy (1974) also recorded tubers and seeds of *Ruppia spiralis* (potentially mistaken for *R. tuberosa*) and other submergent halophytes *Lamprothamnium papulosum* and *Althenia cylindrocarpa* in the diet of grey and chestnut teal from the South Lagoon of the Coorong in the 1960s, during a period of extensive macrophyte distribution and cover (Geddes 1987). The consumption of seeds and vegetation matter from aquatic plants (e.g. *Ruppia* spp.) and seeds from terrestrial and amphibious plants (e.g. Cyperaceae and Amaranthaceae) by grey and chestnut teal and other *Anas* spp. ducks in Australia is well documented (Marchant and Higgins 1990a). More specifically, samphire seeds (Family Amaranthaceae) are recorded as food for other *Anas* spp. ducks (Figuerola et al. 2003) and critically endangered bird species (e.g. orange-bellied parrot *Neophema chrysogaster*, Mondon et al. 2009) that use the Coorong. The contribution of agricultural barley and fruit plants (e.g. citrus and banana) in the diet of teal in the current study suggests this species is an opportunistic feeder in the Coorong area and may forage in nearby agricultural land. The large within-site variability in animal and plant-based diets (i.e. there were no statistical differences between sites) reflects broad and variable foraging extents of these waterbird species in the Coorong. This is further supported by large movements over relatively short time scales undertaken by teal in inland regions of the Murray–Darling Basin (Roshier et al. 2006).

What is the contribution of filamentous algae to the diet composition of waterfowl?

In the Coorong, the green alga *Ulva paradoxa* occurs in filamentous form in hypersalinities and in non-filamentous (blade-like) form in estuarine and marine salinities (Collier et al. 2017). In our study, green algae (including *Ulva* spp.) was a relatively important food item for teal in regions of the Coorong with estuarine and marine salinities (Murray Estuary and North Lagoon). Other *Anas* spp. ducks have consumed *Ulva* spp., often by gleaning on plants in the drifting weed wrack, in brackish marsh habitats elsewhere (Lynch 1939, Weller 1975). Marine algae appear to be only partially digestible by ducks, however, based on the quality of algae in scats (e.g. Lynch 1939), and is less nutritious than *Ruppia* spp. in the Coorong (Moore et al. 2014). The absence of *Ulva* spp. (and other Ulvales) from the diet of teal in the hypersaline region (South Lagoon) of the Coorong suggests that filamentous green algae (e.g. *Ulva paradoxa*) contribute negligibly to the diet of teal in the Coorong. The increased cover of filamentous algae in the South Lagoon and associated negative effects on the growth of *Ruppia* spp. (Waycott et al. 2020), furthermore, is considered to reduce the availability of *Ruppia* spp., the primary food item for teal.

What is the contribution of animal prey to the diet composition of waterfowl?

Animal prey was not recorded in the dietary assessment of waterfowl in the Coorong by Delroy (1974), although the consumption of brine shrimp (*Parartemia zietziana*) by grey and chestnut teal was documented in the Coorong during the Millennium Drought (Paton 2010). We could not determine the relative ratio of animal to plant items from the molecular technique we used, however identification of the animal prey that were eaten and their relative proportions to each other were assessed based on the relative abundances of the DNA sequences of food item. Grey and chestnut teal are mostly herbivorous in the Coorong (Delroy 1974). Hence, it is unclear if animal prey in their diet is targeted or incidental consumed with vegetation. High proportions of animal prey in the diets of grey and chestnut teal (up to 98 and 90%, respectively) (Norman 1983) have been recorded from other locations in Australia, therefore we regard the animal prey as intentionally consumed, although plants may still be the main food resource. We found that fish, chironomids, amphipods and millipedes were the major animal prey of chestnut and grey teal in the Coorong. In inland freshwater lakes and rivers of southern Australia, where aquatic insects are abundant relative to

the Coorong, corixids, notonectids and dipteran larvae and pupae (including chironomids) were the key animal prey items of grey and chestnut teal (Norman and Mumford 1982, Briggs et al. 1985). In contrast to our study, in tidal, saline bays of southern Australia where animal prey predominate (i.e. 87–98%) in grey and chestnut teal diet, gastropods and nereid polychaetes were the key prey items (Norman 1983).

The bill morphology and 'dabbling' feeding behaviour suggests that teal are not specialised piscivores but opportunistic feeders. While fish have been recorded as prey of grey teal (e.g. Frith 1959, Lavery 1970, Briggs et al. 1985) in other parts of Australia, their contribution to diet was lower than we found (i.e. 25% of animal items). In the southern part of the Coorong, smallmouth hardyhead is the most abundant small-bodied fish (Ye et al. 2020a) and is the key prey resource for piscivorous birds (e.g. fairy tern *Sternula nereis*, Paton 2010) and other large waders (e.g. banded stilt and red-necked avocet, Paton 1982) in that area. Smallmouth hardyhead is a schooling, bentho-pelagic species that may be targeted in shallow water of the South Lagoon by teal. This prey fish has demersal, adhesive eggs that attach to submergent vegetation such as *Ruppia* (Molsher et al. 1994). Given that scat sampling occurred from January–June, outside the known September–December spawning period of smallmouth hardyhead in the Coorong (Molsher et al. 1994), it is assumed that juvenile or adult life stages, not adhesive eggs, were consumed by teal. The high contribution of black bream to the diet composition of teal was unexpected and is unlikely based on the average size and depleted biomass of this large-bodied species in the Coorong (Ye et al. 2020b). Instead, sample contamination may have caused this result. The presence of millipedes in the diet composition of waterbirds in the Coorong is unusual as they are not considered a common prey of waterfowl in Australia, but they have been recorded in the diets of other birds (McAuley et al. 2000, Gillings and Sutherland 2007). Platyhelminths were included in diet results of our study due to non-definitive taxonomic classifications, but it is likely that their contributions in scats are as parasites rather than prey. Parasitic platyhelminths have been documented in other *Anas* spp. ducks (Blair and Ottesen 1979, Green et al. 2010, Garvon et al. 2011).

4.2.3 Viability of molecular technique and general future research

DNA metabarcoding proved to be a viable technique for assessing waterbird diet in the current study. Other studies have also successfully used next-generation sequencing techniques to investigate and extend on the known diets of a range of bird species (e.g. Bowser et al. 2013, Gerwing et al. 2016, McInnes et al. 2017b, Cavallo et al. 2018, Rytkönen et al. 2018, Sullins et al. 2018). In our study, prey species identified via molecular methods were consistent with those identified in the literature using traditional stomach or gizzard analysis approaches, and matched with numerous reference library sequences which were generated in-house from prey collected direct from the Coorong region. Additionally, unlike traditional approaches where prey items (e.g. vegetation) may be poorly resolved due to digestion, we generally were able to resolve taxa to fine resolution using DNA metabarcoding. Collection of scat samples through *ambient* sampling, and to a lesser extent for *in-hand* sampling, also meant no physical destruction or harm to the sampled waterbirds. This is of high value for estimating the diet contributions of charismatic biota or those with conservational significance such as migratory shorebirds. Contamination of scats by microbenthic animals from the environment, particularly for the *ambient* scat sampling protocol, did not appear to be an issue, providing support for the sampling technique used in our study.

Next-generation sequence datasets have limitations in that absolute abundance cannot be measured. Nonetheless, we used the number of sequence reads as a measure of relative abundance of food items in the diet composition of waterbirds. Relative abundance was selected instead of frequency data to overcome environmental contamination or the contribution of low volumes of DNA from secondary consumption (i.e. DNA of food in the stomachs of prey which were consumed). The DNA of some food species may amplify preferentially to others, therefore the data may not be a true representation of the percentage contribution to the diet and assumptions need to be treated with caution. Biases also exist throughout the library preparation workflow, including DNA extraction, PCR, DNA pooling, sequencing and bioinformatic sorting, all of which can alter the quantitative nature of the data (Pompanon et al. 2012). Additionally, the DNA of food items may degrade differently after digestion, and so the relative amounts of DNA of food items in scats may not reflect proportions that were consumed (Gerik et al. 2018). These biases may have consequently affected prey selectivity analyses which assume the proportions of prey in diet to be accurate. Accompanying quantification of diet through other methods (e.g. feeding observations, gut content or hard part analysis of scats) would be useful to complement or validate molecular results. Using a combination of molecular and

traditional approaches allows for greater resolution of diet and trophic interactions compared to using one approach on its own (Braley et al. 2010). Including multiple loci (e.g. *16S*, *18S* and *COI*) in the molecular approach may also increase the coverage of prey groups and reduce PCR amplification biases (Bowser et al. 2013, DaSilva et al. 2019). Plant and animal food items were investigated separately, using gene markers/primers (*COI* and *rbcL*, respectively) that were optimal for amplifying DNA from the respective food categories. Consequently, it was not possible to identify the animal to plant fractions in diet and address the key knowledge gaps relating to the relative proportions of animal and plant prey in the diet of shorebirds and waterfowl. These questions remain unanswered. The complementary use of another traditional method would also help to identify these fractions, which could be applied to interpret and translate molecular results.

The reference library databases of *COI* and *rbcL* sequences from potential prey samples collected from key foraging grounds in the Coorong were useful in resolving unknown taxonomic identifications, including the most prevalent OTUs in the *COI* (Chironomidae sp. a and Corophiidae sp. a) and *rbcL* databases (*Ruppia* sp. a). Due to taxonomic uncertainty, many OTUs in the *COI* datasets belonging to insects were left unclassified because these sequences did not have matches in available databases. Since many abundant estuarine and marine aquatic invertebrates were sampled, it is likely that many of these OTUs belonged to insects from different aquatic or terrestrial habitats. Terrestrial insects are important in the diets of these species, particularly red-capped plover (Poore et al. 1979, Paton 1982). Collection of additional potential prey from beyond the Coorong and generating sequence reference library databases would benefit future research. Further taxonomic work to resolve reference collections would also be useful to determine the species identity of the DNA sequences for a range of prey taxa and show if these taxa are described and included in databases (e.g. BOLD, NCBI). Some species may be undescribed and these should be the focus of taxonomic description given their ecological importance.

Foraging beyond the Coorong by waterbirds, particularly teal, was evident from the prey taxa consumed. However, the foraging locations and time spent by teal in these locations remains unknown. It is unclear if freshwater food items were consumed from Lakes Alexandrina and Albert, the Morella Basin, or at another locality. Preliminary results from *Activity 4* of T&I *Component 4* show sharp-tailed sandpipers use various waterbodies and saltpans in the vicinity of Lake Albert. Movement results from this study may help to resolve some of these questions for teal. Energy (calorific) analyses will provide further information on the energy content (profitability) of prey items, which has implications for shorebird foraging. The energy (calorific) content of benthic prey are currently under investigation in *Activity 3* of T&I *Component 3*.

Waterbird scats were collected over a short time period in our study (January–June 2021), and conditions in the Coorong may not have been typical during this period. It would be optimal to complete similar analyses across all seasons and multiple years to solidify understanding of waterbird diet and its variability in the Coorong. Our study focused on assessing waterbird diet in the southern part of the Coorong, as the HCHB program's emphasis is on restoring the South Lagoon. This region has also been a focus in other waterbird dietary (e.g. Delroy 1974, Paton 1982) and foraging investigations (e.g. Paton et al. 2021). Future research should be directed towards understudied aspects of waterbird foraging ecology, particularly shorebird diets, across the broader spatial scale including the northern part of the system (i.e. Murray Estuary and North Lagoon regions) and nearby wetlands (e.g. Lower Lakes and Morella Basin) where data remain limited. Furthermore, information on the diets of longer-billed shorebirds (e.g. curlew sandpiper, red-necked avocet and banded stilt) in the Coorong during current conditions is required because existing knowledge is based on low sample sizes obtained during a period of extensive macrophyte cover (Paton 1982). Within the timeframe of our study, insufficient scat samples of red-necked avocet were collected to enable quality assessment of diet. The data obtained for short-billed shorebirds in the current study are not applicable for these species because of their different feeding morphologies and behaviour.

4.3 Management implications

Dietary knowledge is critical for understanding a species' ecology and their key food resource requirements, which ultimately informs species and ecosystem management. The internationally significant Coorong estuary and associated wetland habitats support resident species and are important non-breeding sites for migratory shorebirds of the East Asian–Australasian flyway. While all target waterbird species are abundant in the Coorong, it is estimated that the Coorong wetlands support up to 21, 14 and 21% of the global

populations of sharp-tailed sandpiper, red-necked stint and chestnut teal, respectively (Paton et al. 2009). During their overwintering time (Northern Hemisphere) in the Coorong, commonly between November and April, migratory shorebirds need to accumulate enough energy reserves through feeding to meet the high energy requirements of their pre-breeding return flight (Swift et al. 2020). Poor habitat and food resource quality at stopover sites can consequently affect global flyway populations (Aharon-Rotman et al. 2016). Gerwing et al. (2016) further highlights the importance of food quality at stopover sites along migratory routes for birds, where essential energy needs to be accumulated to allow the next leg in the migration to be completed successfully. Information on the diets of key waterbirds and fish in the Coorong is therefore important for informing management of shorebird populations at local and international scales. This information is particularly critical given the declines of shorebird populations worldwide (Clemens et al. 2016, Szabo et al. 2016, Studds et al. 2017) and their dependence on the foraging habitats in the Coorong.

More broadly, the knowledge of diets contribute to understanding trophic interactions between species and the functioning of food webs. Restoring or maintaining a functioning and resilient food web and abundant food resources is critical to the ecological character of the Coorong and is a key goal of the HCHB program. The dietary data collected in this study will be used as an input into integrated quantitative food web models under *Activity 4* of T&I *Component 3*. These food web models aim to assess food web responses to various conditions, such as management actions and interventions, and provide a tool to inform management decision-making to optimise ecological outcomes and provide abundant food resources for key biota at risk in the Coorong.

Amphipods are a key food resource for many small-bodied fish (e.g. lagoon goby, this study; smallmouth hardyhead and Tamar goby, Geddes and Francis 2008, Silvester 2011, Hossain et al. 2017; and sandy sprat *Hyperlophus vittatus*, Hossain et al. 2017) and juveniles of large-bodied species (e.g. longsnout flounder, this study; greenback flounder, Geddes and Francis 2008, Earl 2014; yellow-eye mullet *Aldrichetta forsteri*, Giatas 2012; and mulloway *Argyrosomus japonicus*, Giatas and Ye 2015). While lagoon goby or longsnout flounder have not been specifically recorded in the diets of piscivores in the Coorong (i.e. in part due to difficulty in differentiation from similar gobiids and pleuronectids when degraded) they are considered to be prey for large-bodied fish (Giatas and Ye 2015) and other piscivores (Goldsworthy et al. 2019) as they are morphologically and behaviourally similar to other co-occurring species.

Our results suggest that amphipods are also a key food resource of short-billed shorebirds in the Coorong, including migratory species (specifically sharp-tailed sandpiper) that depend on these food resources to meet energy reserve demands for their return-flight migration, consistent with other studies (Thomas and Dartnall 1971, Hicklin and Smith 1984, Novcic et al. 2016). Our study shows that amphipods are currently consumed by shorebirds primarily where they are most available in the North Lagoon. Amphipods are a keystone primary consumer taxon that facilitate the transport of energy from the basal food web to higher trophic levels. Their salinity tolerance (i.e. 0–50 ppt, Ye et al. 2020a) and association with higher freshwater discharge into the Coorong (Dittmann et al. 2015) highlights the importance of maintaining freshwater inflows and preventing hypersaline conditions predominating in the North Lagoon. The prevalence of chironomids in shorebird diets in the South Lagoon suggests that they are also an important food resource under present conditions, where high salinities prevent establishment of more diverse macroinvertebrate prey assemblages. Foraging habitat quality for shorebirds in the South Lagoon is impacted by several factors including salinity, water levels and filamentous algae blooms (Paton et al. 2018b). Extended periods of high water levels could severely limit available foraging habitat for shorebirds, while filamentous algae blooms can suppress chironomid emergence and reduce the extent of bare mudflat available (Lewis et al. 2014, Green et al. 2015, Paton et al. 2018b). Promoting conditions that favour: (1) an abundant and diverse benthic invertebrate prey assemblage that includes amphipods and chironomids; and (2) for shorebirds, an increased extent of suitable foraging habitat (e.g. bare mudflat) where benthic prey are accessible, will benefit shorebird and fish populations that use the Coorong.

In our study, teal showed wider foraging and a more diverse diet compared to earlier findings by Delroy (1974) in the Coorong. Earlier work, however, was conducted during a period of extensive cover of submergent vegetation (e.g. *Ruppia tuberosa, R. megacarpa*, *Althenia cylindrica*, *Lamprothamnium papulosum*) throughout the Coorong (Geddes 1987). Therefore, it is difficult to determine whether our study reflects an inadequate biomass and distribution of these waterfowl food resources in the Coorong. Additionally, uncertainty about the relative proportions of animal to plant food items in the diets of omnivorous waterfowl further complicate our interpretations of our data.

4.4 Conclusions

This was the first study to document the diets of lagoon goby and sharp-tailed sandpiper in the Coorong. This work builds on investigations by Delroy (1974) and Paton (1982) and used a modern molecular technique to quantify the relative contribution of food items to the diets of red-necked stint, red-capped plover, chestnut teal and grey teal. Increased knowledge of the diets of these key species has improved our understanding of the foraging ecology of waterbirds in the Coorong and, more broadly, temperate Australia. Future research should be directed towards understudied aspects of waterbird foraging ecology. This includes the diets of long-billed shorebirds not studied here (e.g. curlew sandpiper, red-necked avocet and banded stilt), all shorebird diets in the northern part of the Coorong (i.e. Murray Estuary and North Lagoon regions) and nearby wetlands (e.g. Lower Lakes and Morella Basin), and the minimum prey requirements (e.g. diversity, abundance and distribution) needed to support healthy waterbird populations. Our study improves understanding of food web interactions in the Coorong and provides vital data for quantitative food web models of the HCHB program, which ultimately aim to inform management decision-making in the Coorong to optimise ecological outcomes.

List of shortened forms and glossary

Activity	Activity refers to the specific task within a Component of the T&I project.
AGRF	Australian Genome Research Facility.
Amphibious vegetation	Plants situated in the transition zone between aquatic and terrestrial environments. They are adapted to areas with fluctuating water levels.
Amplicon	DNA product that is generated by polymerase chain reaction amplification.
Amplification	The process of making millions of copies of a DNA fragment.
Assay	An investigative procedure for testing samples, reagents and conditions.
Benthic	Of or associated with the sediment at the bottom of an estuarine or marine system. 'Epibenthic' specifically refers to organisms that occupy the area on the surface of the sediment.
Bentho-pelagic	An organism that uses both demersal (benthic) and open water (pelagic) zones of a water body.
Blocking primer	Primer specifically designed and added to a polymerase chain reaction mix to block predator DNA amplification.
BOLD	Barcode of Life; publicly available sequence reference database.
Brackish	Water with salinity greater than freshwater but lower than that of sea water, i.e. 0.5 to 35 parts per thousand (ppt) or grams per litre (g/L).
COI	cytochrome *c* oxidase subunit 1 mitochondrial gene.
Component	Component refers to the area of investigation (e.g. Food Webs) of the T&I project.
Detritivore	Feeds predominantly on detritus.
DNA metabarcoding	Targeting and amplifying short, highly-variable DNA regions, which are then are simultaneously sequenced using fast and cost-effective high-throughput methods such as next-generation sequencing.
Emergent vegetation	Aquatic plants that emerge above the water's surface.
Extraction	A routine procedure using physical and/or chemical methods to isolate DNA from cells.
Food web	The interconnection of organisms in an environment by their food relationships, often represented by links or chains, showing 'what eats what'.
Food web model, quantitative	Data supported model based on multiple data sources to provide a plausible food web based upon different scenarios of ecosystem drivers (e.g. barrage flows).
Gene	The basic physical and functional unit of heredity comprising a region of DNA that encodes function.
Gel electrophoresis	A common laboratory method used to separate DNA fragments according to their size and charge.
Halophyte	A salt tolerant plant that occurs in soil or water with high salinity.
Hypersaline	Water with salinity greater than sea water, i.e. over 40 parts per thousand (ppt) or grams per litre (g/L).
HCHB	Healthy Coorong, Health Basin, a program committed to restoring a healthy Coorong.
Intertidal	The area of the shore between the low and high water level that is regularly submerged and exposed by rising and falling tides. This term is used broadly, also for regions in the Coorong that are not influenced by tide and are wetted episodically through wind seiching and water level changes.
Macroinvertebrate	Invertebrate fauna that are retained on sieve mesh size greater than 0.5 mm.

Marker	A DNA sequence within a gene with a known location on a chromosome that can be used to identify individuals or species.
Mastermix	A mix containing all the necessary ingredients (e.g. DNA polymerase, dNTPs, $MgCl_2$, buffers and primers) required for polymerase chain reaction.
Metazoan	A group of multicellular animals with cells differentiated into tissues and organs and usually a digestive cavity lined with specialised cells.
Millennium Drought	An Australian drought which impacted the Murray-Darling Basin over the period 1996-2010, and substantially impacted the Coorong over the period 2001-2010.
Mudflat	Areas of mud that are exposed during lower water levels, which may be driven by tide or wind.
NCBI	National Centre of Biotechnology Information; publicly available sequence reference database.
NGS	Next-generation sequencing; a high-throughput DNA sequencing technology that uses parallel sequencing to simultaneously sequence millions of small fragments per run.
Omnivore	Feeds on animal items and vegetation or detritus.
OTU	Operational Taxonomic Unit; clustering of similar sequences into discreet units to classify groups of closely related individuals.
PCR	Polymerase chain reaction; a widely-used laboratory technique to rapidly amplify DNA sequences.
ppt	Parts per thousand, a measure of salinity in water.
Primer	A short, single-stranded nucleic acid sequence providing the starting point for DNA synthesis.
rbcL	Ribulose-bisphosphate carboxylase Large; protein-coding chloroplast gene.
Reference library/database	Database of sequences that are publicly available, or have been generated in-house for specific taxonomic groups, which are used to classify the taxonomic identification of next-generation sequence data.
Samphire	Succulent salt-tolerant (halophytic) plants that often occur in association with water.
Scat	Faecal sample used to explore dietary constituents through DNA metabarcoding.
Submergent vegetation	Aquatic plants that are submerged below the water's surface.
Subtidal	A spatial zone that describes a nearshore area of habitat that is always underwater, i.e. below the low water mark.
Taxon (taxa: plural)	A unit of rank (e.g. Phylum, Class, Order, Family, Genus, Species) designating an organism or a group of organisms.
Terrestrial vegetation	Plants that occur on land, above areas that are frequently inundated by water.
Trophic	Feeding and nutrition of plants and animals and where they fit into niches and levels of the food web. Trophic 'dynamics' specifically refers to the transfer of energy through the food web.
T&I	Trials and Investigations project, part of the HCHB program.
Zoobenthivore	Feeds predominantly on benthic invertebrates.

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Appendix A – Longsnout flounder diet composition

Four juvenile (39–91 mm TL) longsnout flounder were retained for gut content analysis from fish sampling in the Coorong (see Section 2.2.1); two from Long Point in March 2020 and two from Godfreys Landing in June 2021. One gut was empty and excluded from the dataset. Amphipods and the polychaete *Simplisetia aequisetis* were the most important prey items by volume (%V = 47.9% and 8.1%, respectively) (Table A1). A large portion (43.9% by volume) of the diet was unidentified material. No feeding selectivity or statistical analyses were considered for longsnout flounder due to low sample size.

Table A1. Percentage contribution of prey items in the guts of longsnout flounder (*n* = 3) by volume (%V) and number (%N). Gammaridae was observed to be the most dominant Family of amphipods. Unid. = unidentified.

PREY ITEM	%V	%N
Crustacea	**47.9**	**83.3**
Amphipoda		
Annelida	**8.0**	**8.3**
Polychaeta		
Simplisetia aequisetis		
Mollusca	**0.2**	**8.3**
Gastropoda unid.		
Unid. material	**43.9**	**N/A**

Appendix B – Detailed description of DNA techniques

COI blocking primer design for each bird species

Scat samples contain both degraded prey DNA and more abundant high-quality DNA from the predator itself, therefore in the absence of appropriate control measures, predator co-amplification when using universal metazoan (animal) gene markers (e.g. *COI*) may prevent or bias prey recovery, leading to high proportions of the sequences obtained mapping back to the predator itself instead of the intended prey (Pompanon et al. 2012, Bowser et al. 2013, Leray et al. 2013a). As such, four predator species-specific annealing blocking primers were designed (i.e. to sharp-tailed sandpiper, red-necked stint, red-capped plover and teal spp.) using Geneious Prime 2019.1.3 (https://www.geneious.com) to help in the reduction of predator DNA amplification from scat samples while simultaneously allowing for amplification of prey DNA. Each blocking primer was designed to overlap with the universal forward primer binding site and extended into the predator specific sequence. As recommended by Vestheim and Jarman (2008), the blocking primer also contained two priming regions separated by a polydeoxyinosine linker and terminated with a C3 spacer to prevent elongation without affecting annealing properties (Table B1). Following the *in silico* design, the four newly designed bird species-specific blocking primers were tested *in situ* as described below.

Table B1. Location of the four bird species-specific blocking primers designed and used in this study. Blocking primers were designed to overlap the *COI* universal forward primer (red) and comprises two priming regions (blue and green) separated by a polydeoxyinosine linker (black) and terminated in a C3 spacer (yellow). The location of the blocking primer within each bird species *COI* sequence region is shown.

COI PRIMER/SEQUENCE NAME	*COI* NUCLEOTIDE SEQUENCE
Universal* COI *primer (Leray et al. 2013b)	
mICOIintF	GGWACWGGWTGAACWGTWTAYCCYCC
Bird sequence and blocking primer	
Sharp-tailed sandpiper (*Calidris acuminata*) BROM281-06	GGCACAGGATGAACAGTATACCCTCCACTTGCCGGCAACCTAGCCCATGCCGGAGCTTCTGTAGACCTTGCTATCTTCTCC…
*COI*_STSPBlk_C3	AGTATACCCTCCACTTGCCGGCAACCTAGCCCATGCCGGAIIIIITGTAGACCTTG3
Red-necked stint (*Calidris ruficollis*) KF009527	GGTACAGGATGGACAGTATACCCCCCACTTGCTGGCAACTTAGCCCATGCCGGAGCTTCTGTAGACCTAGCTATCTTCTCC…
*COI*_RNSBlk_C3	AGTATACCCCCCACTTGCTGGCAACTTAGCCCATGCCGGAIIIIITGTAGACCTAG3
Red-capped plover (*Charadrius ruficapillus*) BROM496-07	GGTACAGGATGAACCGTATACCCACCCCTAGCCGGTAACTTAGCCCACGCCGGAGCTTCGGTAGACCTGGCCATCTTCTCT…
*COI*_RCPBlk_C3	CGTATACCCACCCCTAGCCGGTAACTTAGCCCACGCCGGAIIIIIGGTAGACCTGG3
Chestnut teal (*Anas castanea*) NZCOI520-09	GGTACAGGTTGAACCGTGTACCCACCCCTAGCAGGCAACCTGGCCCACGCCGGAGCCTTCAGTAGACCTGGCCATCTTCTC…
Grey teal (*Anas gracilis*) MK261992	GGTACAGGTTGAACCGTGTACCCACCCCTAGCAGGCAACCTGGCCCACGCCGGAGCCTTCAGTAGACCTGGCCATCTTCTC…
*COI*_TealBlk_C3	CGTGTACCCACCCCTAGCAGGCAACCTGGCCCACGCCGGAIIIIICAGTAGACCTG3

Validation of the three round *COI* PCR assay and blocking primer design and optimisation

Prior to cataloguing the metazoan prey composition of the four target bird species, two test Polymerase chain reaction (PCR) amplifications were performed. The first test PCR was to confirm the chosen universal COI primer set will amplify predator and prey DNA (PCR 1). The second test PCR was to (1) validate the specificity of each *COI* bird species-specific blocking primer in targeting predator DNA without compromising amplification of likely prey species, (2) determine the optimum concentration to add each *COI* bird species specific blocking primer to the PCR mastermix to reduce amplification of predator DNA while still allowing for amplification of prey DNA, and (3) verify the three round COI PCR design will generate amplicons of the expected size (PCR 2). Ethanol preserved tissue samples (liver) of each bird species were acquired from the South Australian Museum (SAM) Australian Biological Tissue Collection (ABTC) (Table B2). Genomic DNA (gDNA) was extracted from each sample using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions, with a final elution volume of 100 µl in Buffer AE. Extracts were quantified using a NanoDrop 2000 spectrophotometer before being stored at -20 °C prior to use in the test PCRs. Ethanol-preserved tissue samples of the 16 prey species (chosen due to their occurrence in the Coorong and likelihood as dietary constituents) had previously been sourced from the ABTC and extracted using the same method described above (Table B2).

Table B2. List of prey and predator samples obtained for DNA extraction and validation of the *COI* gene assay *in situ*. Taxonomic assignments were verified using the Atlas of Living Australia (https://www.ala.org.au).

PREY/PREDATOR SPECIES	ORDER	FAMILY	SPECIMEN SOURCE
Prey – Fish (6 orders, 9 families, 13 species)			
Murray River rainbowfish (*Melanotaenia fluviatilis*)	Atheriniformes	Melanotaeniidae	SAM ABTC77420
Smallmouth hardyhead (*Atherinosoma microstoma*)	Atheriniformes	Atherinidae	SAM ABTC18975
Unspecked hardyhead (*Craterocephalus fulvus*)	Atheriniformes	Atherinidae	SAM ABTC20084
Bony herring (*Nematalosa erebi*)	Clupeiformes	Clupeidae	SAM ABTC18476
Goldfish (*Carassius auratus*)	Cypriniformes	Cyprinidae	SAM ABTC91339
Common carp (*Cyprinus carpio*)	Cypriniformes	Cyprinidae	SAM ABTC122656
Australian smelt (*Retropinna semoni*)	Osmeriformes	Retropinnidae	SAM ABTC20085
Flathead gudgeon (*Philypnodon grandiceps*)	Perciformes	Eleotridae	SAM ABTC20086
Dwarf flathead gudgeon (*Philypnodon macrostomus*)	Perciformes	Eleotridae	SAM ABTC20092
Carp gudgeon complex (*Hypseleotris* spp)	Perciformes	Eleotridae	SAM ABTC18566
Golden perch (*Macquaria ambigua*)	Perciformes	Percichthyidae	SAM ABTC18569
Silver perch (*Bidyanus bidyanus*)	Perciformes	Terapontidae	SAM ABTC89912
Freshwater catfish (*Tandanus tandanus*)	Siluriformes	Plotosidae	SAM ABTC21531
Prey – Crustacea (1 order, 3 families, 3 species)			
Freshwater prawn 1 (*Macrobrachium australiense*)	Decapoda	Palaemonidae	SAM ABTC78089
Freshwater prawn 2 (*Macrobrachium australiense*)	Decapoda	Palaemonidae	SAM ABTC78090
Australian Paratya 1 (*Paratya australiensis*)	Decapoda	Atyidae	SAM ABTC118162
Australian Paratya 2 (*Paratya australiensis*)	Decapoda	Atyidae	SAM ABTC118163
Yabby 1 (*Cherax destructor*)	Decapoda	Parastacidae	SAM ABTC66630
Yabby 2 (*Cherax destructor*)	Decapoda	Parastacidae	SAM ABTC66630

PREY/PREDATOR SPECIES	ORDER	FAMILY	SPECIMEN SOURCE
Predator species - Bird			
Sharp-tailed sandpiper (*Calidris acuminata*)	Charadriiformes	Scolopacidae	SAM ABTC68063
Sharp-tailed sandpiper (*Calidris acuminata*)	Charadriiformes	Scolopacidae	SAM ABTC150556
Red-necked stint (*Calidris ruficollis*)	Charadriiformes	Scolopacidae	SAM ABTC139058
Red-necked stint (*Calidris ruficollis*)	Charadriiformes	Scolopacidae	SAM ABTC139920
Red-capped plover (*Charadrius ruficapillus*)	Charadriiformes	Charadriinae	SAM ABTC139379
Red-capped plover (*Charadrius ruficapillus*)	Charadriiformes	Charadriinae	SAM ABTC142768
Chestnut teal (*Anas castanea*)	Anseriformes	Anatidae	SAM ABTC139457

For PCR 1, a 35-cycle screening PCR using the universal *COI* primer set mICOIintF (GGWACWGGWTGAACWGTWTAYCCYCC; forward) and jgHCO2198 (TAIACYTCIGGRTGICCRAARAAYCA; reverse), which target a 313 base pair (bp) highly variable fragment of the mitochondrial *COI* gene (Leray et al. 2013b), was used to amplify DNA from the 16 prey species and four predator bird species. PCR amplification was performed in a total volume of 25 µl with 1.25 µl of 10 µM of each universal forward and reverse primer, 2.5 µl of 10X TaKaRa Hot Start (HS) buffer, 2 µl of 2.5 mM dNTPs, 0.13 µl of TaKaRa *taq* HS Polymerase 5 U/µl (TaKaRa Bio USA), 1 µl of 2 ug/ µl Bovine Serum Albumin and 50 ng of DNA extract. PCR amplification was performed under the following conditions: 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 30 s, followed by a single cycle of 72 °C for 5 min. A negative (no template) control was included in the PCR reaction, with samples visualised via gel electrophoresis.

For PCR 2, DNA from each bird species and one prey was amplified across three rounds of PCR. The first round of amplification (20 cycles) included the additional of the predator blocking primer (Table B1) at six concentrations (1:2, 1:3, 1:4, 1:5, 1:8, 1:10 of universal *COI* primer : predator blocking primer) as well as a control of no blocking primer addition. One microlitre of the first round PCR reaction mixture was used as a template in a second round PCR (20 cycles) for incorporating individual 6 nucleotide (nt) barcodes and Illumina specific adapters. One microliter of this reaction mixture served as a template in the third round PCR reaction (15 cycles) for incorporating the Illumina multiplexing sequencing and index primers, with reaction mixtures and cycling conditions as described above, although the third round amplification was performed in a final volume of 50 µl with reagent volumes doubled. A negative (no template) control was included in the three rounds of PCR amplification, with samples visualised via gel electrophoresis.

Validation of the three round *rbcL* PCR assay

The diet of the bird species is also expected to include plant material. A list of the aquatic and terrestrial plant species in the Coorong that are likely to be key dietary constituents was generated and the availability of published sequences to four candidate plant gene markers was assessed from searching the GenBank sequence database (Table B3). The only gene marker with coverage across the complete list of plant species was the protein-coding chloroplast gene, *rbcL*. This marker has been suggested as being optimal by other researchers in terms of species discrimination, sequence quality, recoverability and completeness of the reference database (e.g. CBOL Plant Working Group 2009; Coghlan et al. 2021). As such, a second assay targeting a 178 bp fragment of the *rbcL* gene was designed and two test *rbcL* PCR amplifications were performed to 1) confirm the chosen (and modified) *rbcL* primer set will amplify anticipated plant prey material and 2) verify the three round rbcL PCR design will generate amplicons of the expected size (PCR 2). Testing material was collected fresh from three plant species found in the Coorong region (designated Ruppia_1, Algae_1 and Ulva_1) and stored immediately at -20 °C. DNA was extracted from each sample using the DNeasy PowerBiofilm kit (Qiagen) following the manufacturer's protocol, with samples disrupted via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 6.0 for 30 s. DNA was eluted in a final volume of 50 µl of Buffer EB, which was passed through the spin column twice to

concentrate each sample. Extracts were quantified using a NanoDrop 2000 spectrophotometer before being stored at -20 °C prior to use in the test PCRs.

Table B3. Availability of published sequence data to four candidate plant gene markers for aquatic and terrestrial plant species found in the Coorong that are likely dietary constituents for sharp-tailed sandpiper, red-capped plover and teal spp.. Red font indicated key potential prey, * indicates SE wetland species, and the colouring system indicates the availability of a reference sequence (green – sequence to the species listed available, orange – sequence to the Genus listed available, red – no sequence available).

	PLANT MARKER			
PLANT SPECIES	RPOC1	RPOB	MATK	RBCL
Aquatic *Althenia cylindrocarpa*	Y	Y	Y	Y
Lamprothamnium papulosum	N	N	Y	Y
Ruppia tuberosa	Y	Y	Y	Y
Ulva spp.	Y	Y	N	Y
Ceratophyllum demersum	Y	Y	Y	Y
* *Chara* spp.	Y	Y	Y	Y
* Lemna spp.	Y	Y	Y	Y
* *Lepilaena australis*	Y	Y	Y	Y
* *Lepilaena patentifolia*	Y	Y	Y	Y
* *Myriophyllum amphibium*	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.
* *Myriophyllum integrifolium*	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.
* *Myriophyllum muelleri*	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.
Myriophyllum salsugineum	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.	Y	Y – as *Myriophyllum* sp.
* *Myriophyllum simulans*	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.	Y	Y – as *Myriophyllum* sp.
* *Myriophyllum verrucosum*	Y – as *Myriophyllum* sp.	Y – as *Myriophyllum* sp.	Y	Y – as *Myriophyllum* sp.
* *Nitella* spp.	Y	Y	Y	Y
Potamogeton crispis	Y – as *Potamogeton* sp.	Y – as *Potamogeton* sp.	Y – as *Potamogeton* sp.	Y – as *Potamogeton* sp.
Potamogeton pectinatus	Y – as *Potamogeton* sp.	Y – as *Potamogeton* sp.	Y – as *Potamogeton* sp.	Y – as *Potamogeton* sp.
* *Potamogeton tricarinatus*	Y – as *Potamogeton* sp.	Y – as *Potamogeton* sp.	Y – as *Potamogeton* sp.	Y – as *Potamogeton* sp.
* *Ruppia polycarpa*	Y	Y	Y	Y
* *Ruppia megacarpa*	Y	Y	Y	Y
Vallisneria australis	N	Y – as *Vallisneria* sp.	Y – as *Vallisneria* sp.	Y – as *Vallisneria* sp.
Terrestrial *Chenopodium* spp.	Y	Y	Y	Y

For PCR 1, a 35 cycle screening PCR using the *rbcL* primers from Aziz et al. (2017) was used to amplify DNA from the three plant species extracts and four testing teal scat samples, although these primers were modified slightly to make them more redundant for targeted amplification of the likely list of plant prey species shown in Table B3. An alignment of the plant species sequences in Table B3 was generated, and the forward modified primer rbcL-357F_mod (CATTGTRGGTAAYGTWTTTGG) and reverse modified primer rbcL-556R_mod (ACATTCATAWACHGCWCKACC) were designed. PCR amplification was performed in a total volume of 25 µl with 1.25 µl of 10 µM of each universal forward and reverse primer, 2.5 µl of 10X TaKaRa Hot Start (HS) buffer, 2 µl of 2.5 mM dNTPs, 0.13 µl of TaKaRa *taq* HS Polymerase 5 U/µl (TaKaRa Bio USA), 1 µl

of 2 ug/ µl Bovine Serum Albumin and 50 ng of DNA extract. PCR amplification was performed under the following conditions: 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 49 °C for 30 s and extension at 72 °C for 30 s, followed by a single cycle of 72 °C for 5 min. A positive (*Chlorella vulgaris* DNA; algae culture provided by the Algae and Biofuels Facility, SARDI Aquatic Sciences) and negative (no template) control was included in the PCR reaction, with samples visualised via gel electrophoresis.

For PCR 2, DNA from one bird species scat sample and the positive control (*Chlorella vulgaris* DNA) was amplified across three rounds of PCR. The first round of amplification (20 cycles) was performed as described above. One microlitre of the first round PCR reaction mixture was used as a template in a second round PCR (20 cycles) for incorporating individual 6 nt barcodes and Illumina specific adapters. One microliter of this reaction mixture served as a template in the third round PCR reaction (15 cycles) for incorporating the Illumina multiplexing sequencing and index primers, with reaction mixtures and cycling conditions as described above, although the third round amplification was performed in a final volume of 50 µl with reagent volumes doubled. A negative (no template) control was included in the three rounds of PCR amplification, with samples visualised via gel electrophoresis.

Bird scat and sediment scraping samples – DNA extraction, amplification and sequencing

DNA was extracted from bird scat samples using the QIAamp Fast DNA Stool Mini Kit, human protocol (Qiagen), following the manufacturer's instructions. Prior to extraction, the bird scat sample was vortexed for 10 s, centrifuged briefly to pellet the scat sample and the RNA*later*™ (ThermoFisher®) removed by pipetting. The scat sample was then weighed and the appropriate volume of Inhibitex buffer added (1 mL for smaller scat samples with weights up to 220 mg (predominately sharp-tailed sandpiper, red-necked stint and red-capped plover scats) and 10 mL for larger scat samples with weights of 1 g (predominately teal scats)). Scat samples were eluted in a final volume of 100 µl in Buffer ATE. The yield and purity of the DNA scat extracts were assessed using the NanoDrop 2000 Spectrophotometer (Thermo Scientific). The scats were concentrated by ethanol precipitation using standard procedures, with precipitated extracts assessed a second time using the Nano-Drop 2000 spectrophotometer. All samples were then stored at -20 °C prior to downstream library preparation. Three sediment scraping samples were also collected from the mudflats at Parnka Point, South Lagoon, to assess the ambient metazoan community composition present in the environment and to screen scat samples for potential contamination. These samples were extracted using the Fast DNA Spin Kit for Soil (MP Biomedicals), following the manufacturer's instructions. Samples were assessed using the NanoDrop 2000 Spectrophotometer (Thermo Scientific), before being stored at -20 °C prior to downstream library preparation.

For an insight into the metazoan community composition, DNA extracts from 188 bird scat samples and three sediment scraping samples were PCR amplified using the universal *COI* primer set described above (Table 4). The predator species-specific blocking primer was added at an optimum concentration of 1:8 (universal primer : blocking primer) for sharp-tailed sandpiper, 1:2 for red-necked stint, 1:3 for red-capped plover and 1:5 for teal. Additionally, to assess the plant dietary component of sharp-tailed sandpiper, red-capped plover and teal, 61 selected scat samples from these three bird species were PCR amplified with the *rbcL* modified primer set (Table 4). A summary of the collection localities and collection dates for the bird species and environmental samples included in the final *COI* and *rbcL* datasets post filtering is shown in Table B4.

Table B4. Summary of collection localities and collection dates for each bird species and environmental sample that were included in the final dataset post filtering for *COI* and *rbcL*.

BIRD SPECIES/ENVIRONMENTAL SAMPLE	COLLECTION LOCALITY	COLLECTION MONTH-YEAR	FINAL NO. SAMPLES IN DATASET POST FILTERING
COI			
Sharp-tailed sandpiper	Long Point, NL	Mar-21	10
	Seven Mile Road, NL	Feb-21	1
	Parnka Point, SL	Jan-21	15
	Parnka Point, SL	Feb-21	2
Red-necked stint	Parnka Point, SL	Feb-21	5
	Hack Point, SL	Jan-21	12
Red-capped plover	Parnka North, SL	Mar-21	10
	Parnka North, SL	Jun-21	11
	Villa del Yumpa, SL	Mar-21	7
	Villa del Yumpa, SL	Jun-21	8
	Salt Creek, SL	Mar-21	10
	Salt Creek, SL	Jun-21	13
Teal	Pelican Point, ME	Jun-21	3
	Long Point, NL	Jun-21	5
	Seven Mile Road, NL	Jun-21	4
	Hack Point, SL	Jun-21	10
	Hack Point, SL	Jan-21	16
Sediment scraping	Parnka Point, SL	Jan-21	3
rbcL			
Sharp-tailed sandpiper	Parnka Point, SL	Jan-21	7
	Parnka Point, SL	Feb-21	1
Red-capped plover	Parnka North, SL	Mar-21	5
Teal	Pelican Point, ME	Jun-21	4
	Long Point, NL	Jun-21	5
	Seven Mile Road, NL	Jun-21	5
	Hack Point, SL	Jun-21	10
	Hack Point, SL	Jan-21	10

Abbreviations: ME, Murray Estuary; NL, North Lagoon; SL, South Lagoon.

As described above, three rounds of PCR amplification were performed for each gene marker. In brief, for the first PCR reaction, the intended target was amplified using the universal primers. One microlitre of the first round PCR reaction mixture was used as a template in a second PCR for incorporating individual 6 nt barcodes and Illumina specific adapters. One microliter of this reaction mixture served as a template in the third PCR reaction for incorporating the Illumina multiplexing sequencing and index primers. Reaction mixtures and cycling conditions were as described above for each barcoding gene PCR assay. All PCR reactions included positive (*COI* – common galaxias, *Galaxias maculatus*; *rbcL* – algae culture, *Chlorella vulgaris*) and negative (no template) controls. Amplicons generated from the positive control samples were included within each pooled library as a sequencing control.

Samples were visualised via gel electrophoresis with products of the expected size purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified in duplicate using the Quant-iT Picogreen dsDNA kit (Life Technologies) following the manufacturer's instructions before being pooled in equimolar ratios and sequenced in two libraries of ~100 pooled samples each on the MiSeq platform (Illumina, San Diego, CA, United States) using 250 nt paired-end sequencing chemistry through the Australian Genome Research Facility (AGRF, North Melbourne, Victoria, Australia).

Reference library samples – DNA extraction, amplification and sequencing

Coorong-specific *COI* and *rbcL* sequence reference libraries were generated to assess the taxonomic classification of otherwise unclassified OTUs. DNA from the invertebrate prey collected and listed in Table 3 was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions, with a final elution volume of 100 µl in Buffer AE. DNA from the plant species was extracted using the DNeasy PowerBiofilm Kit (Qiagen), with samples disrupted via bead-beating using the FastPrep-24™ 5G instrument (MP Biomedicals) at an intensity of 6.0 for 30 s. DNA was eluted in a final volume of 50 µl of Buffer EB, which was passed through the spin column twice to concentrate each sample. All prey DNA extracts were quantified using a NanoDrop 2000 spectrophotometer before being stored at -20 °C.

DNA was amplified in a single round of PCR using the universal *COI* (invertebrate DNA) or *rbcL* (plant DNA) forward and reverse primers (e.g. *COI* – mICOIintF/jgHCO2198, *rbcL* - rbcL-357F_mod/rbcL-556R_mod) and cycling conditions described above (35 cycles). All PCR reactions included positive (*COI* – red-necked stint, *Calidris ruficollis*; *rbcL* – algae culture, *Chlorella vulgaris*) and negative (no template) controls. Samples were visualised via gel electrophoresis with products of the expected size sent to AGRF for PCR clean-up, Sanger sequencing in the forward and reverse direction, and sequencing clean-up. Sequences were then edited and aligned using Geneious Prime 2019.1.3 (https://www.geneious.com).

Appendix C – Rarefaction plots

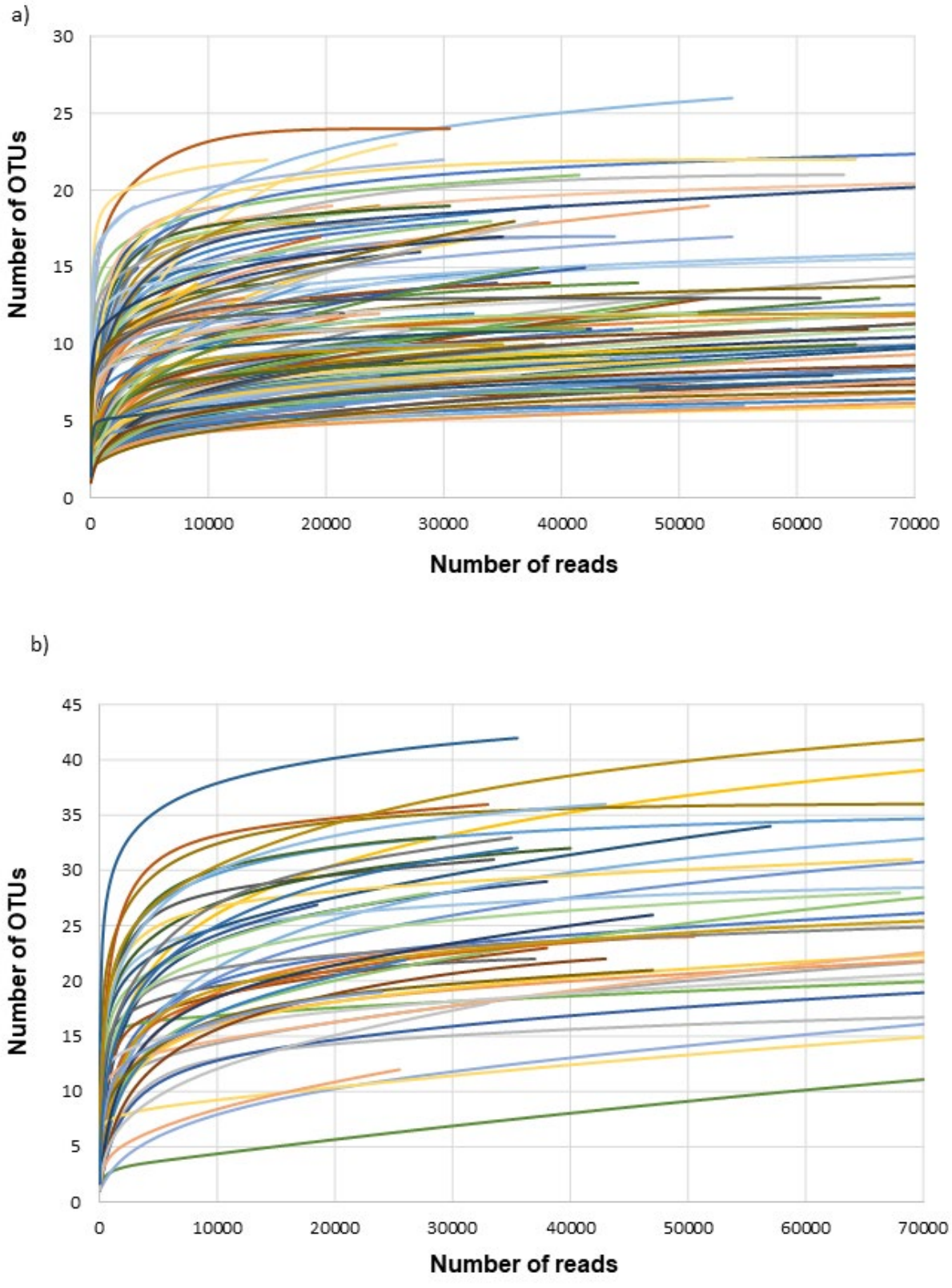

Figure C1. Rarefaction curves portraying the number of resolved Operational Taxonomic Units (OTUs) (with a sample contribution > 0.01%) against sequencing depth for each bird scat sample in the a) *COI* dataset and b) *rbcL* dataset.

Appendix D – Percentage contribution of animal and plant food items to waterbird diet

Table D1. Percentage contribution of prey items in the animal diet portion for waterbirds in the Coorong during 2021. Taxa presented in this table include those with sequence matches to the Coorong *COI* reference library database and publicly available databases. Sites and seasons are pooled. Data are expressed as % contribution to total number of sequence reads in the *COI* dataset, for OTUs with contribution >0.01%. STSP = sharp-tailed sandpiper, RNS = red-necked stint, RCP = red-capped plover, teal = chestnut teal and grey teal.

TAXA	STSP (*n* = 28)	RNS (*n* = 17)	RCP (*n* = 59)	TEAL (*n* = 38)
Annelida	**0.30**			**0.19**
Polychaeta				
Capitella teleta				0.01
Capitellidae sp. a				0.11
Simplisetia aequisetis	0.30			0.05
Manayunkia athalassia				0.02
Arachnida			**0.13**	**0.06**
Araneae				
Lycosidae sp.				0.01
Miturgidae sp.			0.13	
Arachnida sp.				0.02
Chillopoda				
Chilopoda sp.				0.03
Crustacea	**13.24**	**0.06**	**1.33**	**19.38**
Amphipoda				
Austrochiltonia sp.				0.02
Corophiidae sp. a	12.33			5.98
Monocorophium insidiosum	0.02			
Gammaridea sp. a	0.54			0.10
Amphipoda sp.	0.01			0.02
Branchiopoda				
Chydorus brevilabris				0.01
Decapoda				
Caridina sp.				0.02
Paratya australiensis	0.01			0.05
Macrobrachium australiense	0.10	0.06	0.02	3.25
Hexanauplia				
Harpacticella sp.	0.03			0.02
Cyclopoida sp.				0.02
Harpacticoida sp.				0.02
Hexanauplia sp.	0.15		0.03	0.16
Isopoda				
Haloniscus searlei	0.03		1.28	9.44
Ostracoda				
Ostracoda sp. a				0.01
Podocopida sp.				0.06
Malacostraca sp.				0.20
Diplopoda			**0.29**	**6.99**
Julida				
Julidae sp.				0.15
Ommatoiulus sp.			0.28	3.26
Julida sp.			0.01	3.58
Insecta	**83.67**	**99.92**	**98.23**	**39.79**
Blattodea				
Blattidae sp.			0.03	
Microcerotermes sp.		0.02		
Coleoptera				
Carabidae sp.			0.80	
Sitona discoideus			0.01	
Coleoptera sp.			0.20	0.05
Diptera				
Chironomidae sp. a	74.65	89.79	83.38	36.17
Chironomus oppositus			0.04	
Cricotopus albitarsis				0.03
Dicrotendipes pseudoconjunctus			0.08	
Oscinellinae sp.			0.88	0.02
Dolichopodidae sp. a			0.02	0.33
Ephydridae sp.			0.05	

TAXA	STSP (*n* = 28)	RNS (*n* = 17)	RCP (*n* = 59)	TEAL (*n* = 38)
Hybotidae sp.				0.03
Helina sp.			0.01	
Lispe sp.		0.11	1.01	0.03
Muscidae sp.			0.17	
Sphaeroceridae sp.			0.86	
Stratiomyidae sp.			0.05	
Diptera sp.		0.08		
Diptera sp. a (Fly sp.)	0.04		2.91	0.08
Hemiptera				
Nysius sp.			0.03	
Nabis kinbergii			0.05	
Rhyparochromidae sp.			0.32	
Hemiptera sp.			0.39	
Hymenoptera				
Camponotus terebrans			0.02	
Formicidae sp.			0.06	
Ochetellus glaber				0.02
Hymenoptera sp.			0.04	
Achyra affinitalis			0.05	
Hygraula nitens				0.02
Zizina otis			0.04	
Psychidae sp.			0.05	
Ischnura heterosticta				0.02
Insecta spp.	8.97	9.92	6.77	2.91
Mollusca	**0.07**	**0.01**	**0.02**	**3.92**
Bivalvia				
Arthritica semen				0.06
Gastropoda				
Salinator fragilis	0.07			0.06
Physella acuta				0.30
Ascorhis tasmanica				0.24
Coxiella striata				3.05
Euthyneura sp.		0.01	0.02	
Hypsogastropoda sp.				0.21
Platyhelminthes	**2.73**			**4.50**
Cestoda				
Cestoda sp.				0.16
Polycladida				
Echinoplana celerrima	2.42			4.26
Rhabditophora				
Rhabditophora sp.				0.01
Trematoda				
Notocotylidae sp.				0.02
Trematoda sp.				0.01
unclassified Platyhelminthes	0.31			0.04
Teleostei				**25.17**
Atheriniformes				
Atherinosoma microstoma				8.82
Cyprinodontiformes				
Gambusia holbrooki				0.06
Mugiliformes				
Aldrichetta forsteri				0.03
Perciformes				
Philypnodon macrostomus				0.01
Acanthopagrus butcheri				12.03
Salmoniformes				
Galaxias maculatus				4.21

Table D2. Percentage contribution of food items in the plant diet portion for waterbirds in the Coorong during 2021. Taxa presented in this table include those with sequence matches to the Coorong *rbcL* reference library database and public available databases. Sites and seasons are pooled. Data are expressed as % contribution to total number of sequence reads in the *rbcL* dataset, for OTUs with contribution >0.01%. STSP = sharp-tailed sandpiper, RCP = red-capped plover, teal = chestnut teal and grey teal.

TAXA	TEAL (*n* = 34)	STSP (*n* = 7)	RCP (*n* = 4)
Charophyta	**93.11**	**99.46**	**98.77**
Alismatales			
Lemna sp.	0.03		
Maundia sp.	0.02	0.02	
Althenia sp.	4.98	1.58	1.64
Potamogeton sp.	0.13	0.08	0.02
Zannichellia sp.	0.03		
Ruppia sp. a	61.84	96.90	94.38
Ruppia sp. other	0.79	0.70	0.77
Asterales			
Aster sp./*Lactuca* sp./*Sonchus* sp.	0.28		
Caryophyllales			
Alternanthera sp.	0.02		
Chenopodium sp.	0.03		
Salicornia sp./*Sarcocornia* sp./*Tecticornia* sp.	2.92		
Suaeda sp.	5.13		
Ceratophyllales			
Ceratophyllum demersum	5.72		
Ceratophyllum sp.	0.02		
Charales			
Lamprothamnium sp.	0.23		
Ericales			
Actinidia sp.			0.02
Fabales			
Medicago sp.	0.04		
Robinia sp.			1.63
Trifolium sp.	0.16		
Fagales			
Quercus sp.		0.20	
Laurales			
Litsea sp.	0.02		
Pinales			
Picea sp.			0.31
Pittosporaceae			
Pittosporum sp.	0.02		
Poales			
Hordeum vulgare	5.71		
Typha sp.	0.04		
Sapindales			
Citrus sp.	0.15		
Saxifragales			
Myriophyllum sp.	3.23		
Solanales			
Wilsonia backhousei	0.04		
Zingiberales			
Musa spp.	1.55		
Chlorophtya	**6.89**	**0.54**	**1.23**
Chaetophorales			
Chaetophoraceae sp.			0.21
Ulotrichales			
Ulothrix zonata			0.22
Ulvales			
Kornmanniaceae sp.	0.79		
Percursaria percursa	0.08		
Ulva sp. a	2.42	0.46	0.15
Ulva sp. other	1.06	0.07	0.64
Ulvaceae sp.	0.15		
Acrochaete sp.	2.29		
Ulvella sp.	0.02		
Ulvellaceae sp.	0.09		

Appendix E – PERMANOVA and SIMPER tables

Table E1. *COI* dietary items identified by SIMPER for driving the site dissimilarity in dietary composition of sharp-tailed sandpiper (STSP) and red-necked stint (RNS) in the Coorong. LP = Long Point, PaP = Parnka Point, HP = Hack Point. *Indicates that the percentage contribution of a dietary category is greater for the site in bold. The average dissimilarities (%) between groups are presented. Unclass. = unclassified.

SITE - SPECIES	STSP	RNS
LP-PaP	***89.41*** Chironomidae sp. a Corophiidae sp. a*	
PaP-HP		***13.78*** Chironomidae sp. a Unclass. insects*

Table E2. PERMANOVA test results for site and season effects on diet composition of red-capped plover from the South Lagoon of the Coorong, with pair-wise comparisons between site-season. PaP = Parnka Point, VY = Villa de Yumpa, SC = Salt Creek. After B–Y method FDR correction, α = 0.020 for comparisons between sites (six comparisons) and α = 0.027 for comparisons between seasons (three comparisons). p-values presented in bold are significant comparisons.

MAIN TEST FACTOR	DF	PSEUDO-F	P(PERM)
Site	2	6.2406	**0.0003**
Season	1	5.3315	**0.0051**
Site x Season	2	8.603	**0.0001**
Residual	53		

PAIRWISE COMPARISONS	T	P(PERM)
Between sites		
Autumn		
PaP,VY	0.95041	0.4710
PaP,SC	1.7746	**0.0149**
VY, SC	1.2078	0.1267
Winter		
PaP,VY	1.0857	0.3339
PaP,SC	4.3301	**0.0004**
VY, SC	4.3311	**0.0006**

Table E3. *COI* dietary items identified by SIMPER for driving the site dissimilarity in dietary composition of red-capped plover in the South Lagoon of the Coorong. PaP = Parnka Point, VY = Villa de Yumpa, SC = Salt Creek. *Indicates that the percentage contribution of a dietary category is greater for the site in bold. The average dissimilarities (%) between groups are presented. Results are not presented for non-significant (n.s.) comparisons. Unclass. = unclassified.

SITE-SEASON	AUTUMN	WINTER
PaP-VY	n.s.	n.s.
PaP-SC	***29.27*** Chironomidae sp. a Unclass. insects* Oscinellinae sp. *H. searlei**	***45.86*** Chironomidae sp. a* Diptera sp. a (Fly sp.)
VY-SC	n.s.	***47.31*** Chironomidae sp. a* Diptera sp. a (Fly sp.)

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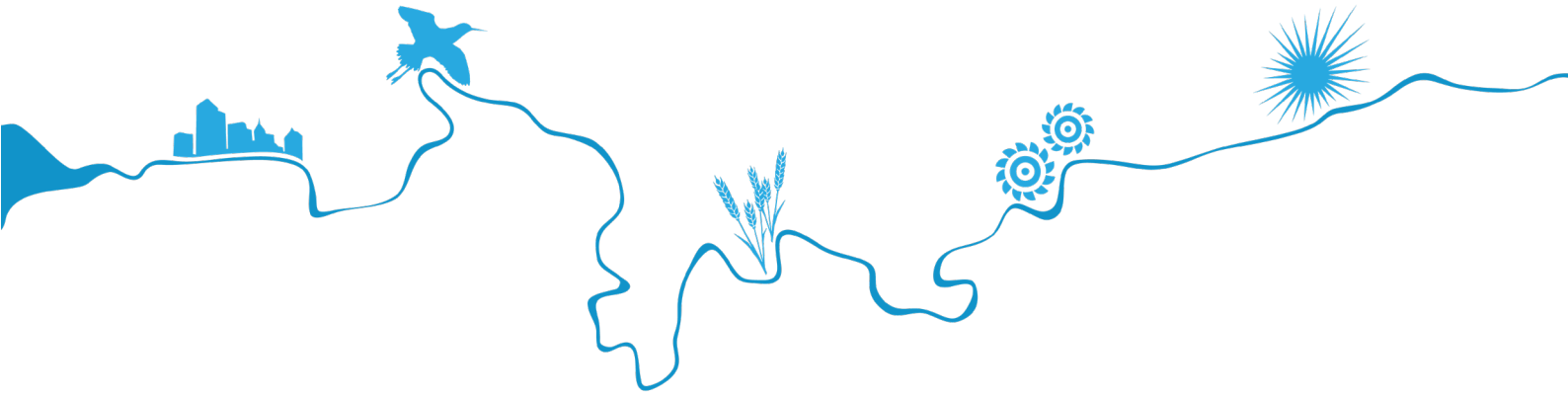

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