Title:

Metabarcoding, direct stomach observation and stable isotope analysis reveal a highly diverse diet for the invasive green crab in Atlantic Patagonia

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DEG, GB, ML, and GC conceived and designed this study. DEG, GB, and GC took the samples. GC conducted the visual analysis and prepare the samples for stable isotope analysis. GC and DEG analyzed direct stomach observation and stable isotope data. ML contributed to preparing the metabarcoding samples and analyzed metabarcoding data. EV visited as an invited researcher the Centre for Biodiversity Genomics to obtain the metabarcoding data. EV, BT, DAL, and DS contributed to the generation of metabarcoding data, molecular and bioinformatic analyses. All authors contributed to manuscript writing.

Abstract

The European green crab *Carcinus maenas* and, its sister species, *C. aestuarii* are highly invasive species causing damages to coastal ecosystems and severe economic losses worldwide. *C. maenas* was detected at the Atlantic Patagonian coast twenty years ago. In this work, we studied the diet of the green crab in a recently invaded location in Nuevo Gulf, using three complementary techniques: direct stomach observation, stable isotope analysis, and metabarcoding of the gut content. Direct stomach observation and metabarcoding showed that green crabs have a broad omnivorous diet, ingesting most of the phyla present in the study area. Gut content metabarcoding allowed a detailed description of algae diversity and revealed other taxa that went unnoticed in the visual stomach analysis. Stable isotope analysis showed that the major contribution to crabs' diet was from the phytoplankton chain (by bivalve consumption) and not directly from algae. This study approach combining three complementary techniques allowed us to detect some differences in the diet between sexes, which suggests that male and female crabs are not as ecologically equivalent as previously thought. Besides, we detected sequences corresponding to *C. aestuarii* suggesting that the green crab Patagonian population is a hybrid of both sister species. Finally, we highlight possible direct and indirect interactions of the green crab with the native species that can trigger negative effects throughout the entire food web.

Keywords: *Carcinus maenas*, *Carcinus aestuarii*, e-DNA, Rocky shore ecology, Trophic interactions

1. Introduction

Invasive species, particularly invasive predators are one of the main causes of worldwide species decline and biodiversity loss (Clavero and García-Berthou 2005, Doherty et al. 2016). Once invasive species arrive at a new location, they establish new ecological interactions with resident species and these new interactions can have both positive or negative effects on native species or the entire native food web (David et al. 2017). Food webs represent energy and matter flows among

organisms related to each other by consumer-resource relationships; their network structure influences community dynamics and stability (Bascompte 2009, Thébault and Fontaine 2010). The ways biological invasions impact food webs are complex and context-dependent as there are various direct and indirect mechanisms by which an invasive species can affect other taxa (Thomsen et al. 2011a, Thomsen et al. 2011b). Preying upon a native species is a direct mechanism that usually ends up with population decrease, or even species loss (Bruno et al. 2005, David et al. 2017). Exploitative and apparent competition are indirect mechanisms that can also cause the decline of native populations (White et al. 2006, David et al. 2017). On the other hand invaders can display positive effects for resident species. In fact, for species at higher trophic positions, invaders can increase food availability and new habitats (Rodriguez 2006, Thomsen et al. 2010, Thomsen et al. 2014). The invaders' impact depends on the food web's invasibility which is ultimately related to network structure (Hui et al. 2016). Earlier work has shown that food webs with higher diversity are more resistant to invasions; however, on the flip side highly diverse communities generally support more invasive species (Fridley et al. 2007, Tilman et al. 2014, David et al., 2017). By using simulations, Romanuk et al. (2017) have shown that trophic cascades are more frequent when invaders become secondary consumers with abundant prey and only few other predators. Consequently, if we want to accurately predict possible impacts of a newly arrived invasive species, we need to understand its trophic interactions at the invaded ecosystem.

Determining the diet of a particular species is a key requirement to understand its trophic ecology, but it is far from being a simple achievement. There are a series of conceptual and methodological aspects that must be taken into account (e.g Traugott et al. 2013). It is not the same to document which organisms an animal ingests as to list which organisms are targets of a predator or a scavenger. Similarly, the ingestion of a certain item does not assure its digestion and assimilation. A variety of direct and indirect methods are currently used to analyze diet composition (Majdi et al. 2018). Methods include visual observation of feeding events and regurgitations; the inspection of faeces, pellets and stomach contents; and the molecular profiling of stomach contents

or faeces using DNA sequencing or immunoassays. DNA-based techniques allow the characterization of feeding interactions at high taxonomic resolution and are very useful when visual recognition is not possible (Clare 2014, Unger et al. 2020). In cases of omnivorous species, where a mixture of DNA from different species is present in gut content, the most effective approach is metabarcoding (Taberlet et al. 2012, Pompanon et al. 2012, Nielsen et al. 2018). Metabarcoding couples DNA barcoding (Hebert et al. 2003) with high-throughput sequencing of community samples (i.e. gut samples) (Nielsen et al. 2018). It not only provides information on ingested organisms, it also has the advantage of providing complementary information such as the gastrointestinal parasites of a predator. Aside from such direct methods, indirect tracers of diet composition are robust and commonly used tools. They include the characterization of biomarkers such as fatty acids, sterols or pigments present in consumer tissues and the determination of the isotopic compositions of C, N and S in specific tissues (e.g. Galloway et al. 2015, Stock et al., 2018). Stable isotope analysis (SIA) is based on predictable differences between the isotopic signature of an organism and its food resources. It allows for the quantitative estimation of fluxes in food webs, the determination of a species' trophic level and insights into the patterns of resource allocation (Boecklen et al. 2011). All these approaches are largely complementary as direct methods can identify the prey which is ingested, while indirect methods provide information on what is assimilated. In addition, indirect methods such as SIA provide information at longer time scales while DNA or visual analysis register consumption within short time periods (typically 6-48h) (Albaina et al. 2010, Hayden et al. 2014). Majdi et al. (2018) highlighted the potential of combining direct and indirect methodologies and noted the lack of this kind of studies in current trophic ecology research.

However, there are method-specific biases. Direct visual stomach content analysis provides indispensable first-hand information, allows quantifying the ingested amount, and for certain cases distinguishes between secondary predation and random ingestion; however, digestion of prey material can limit its detection strength, favoring certain taxonomic groups such as shell-bearing

101 organisms. Moreover, it largely depends on the skills of taxonomists involved (Majdi et al. 2018,
102 Traugott et al. 2020). Molecular results can contain errors associated with contamination, biases
103 associated with primer coverage, sequencing errors, as well as bioinformatics errors (Braukmann et
104 al. 2019). On the other hand, metabarcoding is typically regarded as a powerful, high-resolution
105 method (Unger et al. 2020) although it sometimes can lack taxonomic resolution due to gaps in
106 reference databases, particularly for poorly described environments. As for indirect methods, a
107 potential confounding factor of SIA is the variability of trophic discrimination factors (TDF) and
108 turnover rates that may depend on environments, diet, taxon and tissue (Pinnegar and Polunin 1999,
109 Philippsen and Benedito 2013, Hussey et al. 2014, Lefebvre and Dubois 2016). Isotope
110 compositions are highly variable through space and time (Hyndes et al. 2013, Mackey et al. 2015).
111 Therefore, these two factors combined with TDF should be carefully considered when stable
112 isotopes are used in diet studies.

113 The European green crab *Carcinus maenas* (Linnaeus, 1758) is an invasive species native to
114 the North Atlantic Coast. To this day it has reached a worldwide distribution (Carlton and Cohen
115 2003). Not only *C. maenas* have invaded new locations, but its sister species *C. aestuarii*, or
116 hybrids of both, have also been detected at invaded sites (Darling 2011). Concerning its feeding
117 ecology, green crab is considered an aggressive invader due to its voracity and predation on other
118 invertebrates (McDonald et al. 2001). It has been shown that portunid crabs are scavengers and
119 predators, and that green crab is an active carnivore feeding especially on mollusca and crustacea
120 (Ebling et al. 1964, Elner 1981, Grosholz and Ruiz, 1995, McDonald et al. 2001, Chen et al. 2004).
121 It was first detected at the Patagonian coast in 2001, and twelve years later it was registered in the
122 Nuevo Gulf, 250 km north of the point of the first observation (Torres and González-Pisani 2016).
123 The coast of Argentina currently bears a total of 129 introduced and 72 cryptogenic marine species,
124 and it has been estimated that every 178 days a new invasion is detected (Schwindt et al. 2020). The
125 interaction of the green crab population with native as well as introduced species could have
126 unpredictable consequences for the local trophic structure, as well as for ecosystem functioning.

Very little is known about green crab ecology at the Argentine coasts, in particular with regards to its local trophic interactions (Hidalgo et al. 2007, Young and Elliott 2020). Moreover, the observation that feeding behaviour is more aggressive in invasive than in native populations (Howard et al. 2018), leads to further uncertainties on how this predator could affect the invaded ecosystem.

In this study we aim to shed a light on the diet of the green crab in Atlantic Patagonian coastal environments by using three complementary techniques that have proven useful for diet tracing: visual stomach content inspection, stable isotope analysis, and amplicon sequencing of a gene fragment from stomach content DNA (metabarcoding). We address the following questions to understand possible impacts on the trophic structure of the affected coastal communities in this coastal environment: What are green crabs eating in the affected coastal environments of Atlantic Patagonia? Are there differences in diet between female and male green crabs? What changes can green crabs trigger in local food webs? Does the use of a multidisciplinary approach contribute to a more complete picture?

2. Materials and Methods

2.1 Sample collection

Green crabs were collected at Punta Este beach in the Nuevo Gulf, Patagonia, Argentina (42°46 ′ S, 64°57 ′ W). Sampling was done from November 2018 to February 2019 in shallow waters with depths of about 2-3 m at high tide. Crabs were captured manually by SCUBA diving, resulting in a total of 223 specimens. Individuals were separated into two groups. one for traditional visual analysis (183) and the other for metabarcoding (40). After sampling, all specimens were transported to the laboratory and immediately frozen at -20 °C. Crab sizes were registered by measuring carapace width (CW) in cm. Subsequently, crabs were sexed and dissected. For specimens destined for visual analysis, stomach content was extracted and kept in 70% ethanol. For specimens destined for metabarcoding, stomach content was manually collected from frozen

152 samples by letting samples thaw and carefully extracting contents with a plastic pipette tip through
153 the mouth of the crab. Contents were placed in 100% ethanol (Merck) and stored at -80°C until
154 DNA extraction. Muscle from crab chela was extracted and frozen for stable isotope analysis. In
155 addition, five individuals of the mussel *Mytilus edulis* and five of the herbivorous snail *Tegula*
156 *patagonica* were collected to establish isotopic baselines. This was done under the assumption that
157 both the mussel and the herbivorous snail are primary consumers feeding mostly on micro- and
158 macroalgae, respectively.

159 **2.2 Visual analysis of gut contents**

160 The visual analysis of gut contents was done using a stereomicroscope (Zeiss Stemi, model
161 2000c). Prey was identified to the lowest possible taxonomic level following catalogues and keys of
162 the local fauna (Fauchald 1977, Gosztonyi and Kuba 1996, Boschi et al. 1992, Spivak et al. 2019),
163 as well as through expert consultation and by comparisons with fresh material. Presence of prey
164 items in each stomach was recorded and the frequency of occurrence ($FO_{(vis)}$%) calculated per prey
165 item as the percentage of non-empty stomachs in which the item was recorded. For statistical
166 analysis, we grouped prey using major groups (Annelida, Arthropoda, Mollusca, Chordata,
167 Echinodermata and Algae) and estimated the confidence interval of the FO by a bootstrap procedure
168 (Tirasin and Jorgensen 1999); using the functions implemented in the R package 'boot' (Ripley
169 2005).

170 **2.3 Metabarcoding analysis of gut content**

171 *DNA extraction and COI amplification*

172 All molecular analyses were carried out under clean-room conditions at the Centre for
173 Biodiversity Genomics (University of Guelph, Canada). First, the ethanol was evaporated from the
174 gut content samples in a fume hood over night, then the remaining pellets were lysed and the DNA
175 extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following
176 manufacturers' instructions except for an additional centrifugation step (14000 rpm) for removing

the buffer AW2 from the silica membrane and for elution in two steps using 100 µl of Buffer AE per each. A fume hood control and extraction control were processed along the stomach content samples to check for cross-contamination. The DNA extracts were quantified using Qubit dsDNA HS Assay Kit and a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, MA, USA). For the amplification of gut content DNA, we used a two-step PCR protocol following Elbrecht et al. (2019). PCRs were carried out using the Multiplex PCR Master Mix Plus (Qiagen, Hilden, Germany), 0.5 mM of each primer (IDT, Skokie, Illinois), and molecular grade water (HyPure, GE, Utha, USA) for a total volume of 25 ml. For the first PCR step, amplification of cytochrome oxidase I gene was performed using primers BF3/BR2 (5' CCHGAYATRGCHTTYCCHCG 3' / 5' TCDGGRTGNCCRAARAAYCA 3') and 4 µl of DNA extract. For the second PCR step, 1 µl of the product obtained from the first PCR was used and amplification was performed using fusion primers with individual tags per sample (see Electronic Supplemental Material, Online Resource 1, section 2.3) (Elbrecht and Leese 2017, Elbrecht et al. 2019, Elbrecht and Steinke 2019). Thermocycling conditions for both PCR steps included an initial denaturation at 95°C for 5 minutes, 24 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 50 seconds; and a final extension at 68°C for 10 minutes. After the second PCR, amplification success and band strength were checked on 1.5% agarose gels. For several samples which showed no or only very weak amplification, the two PCRs were repeated, but with 35 cycles instead of 24 in the first PCR. These replicates received individual tag combinations and were prepared for sequencing on an Illumina MiSeq platform along with the extraction controls and seven PCR negative controls:

First, normalization of the second PCR products was performed using the SequalPrep Normalization Plate Kit (Invitrogen, CA, USA), then samples were pooled in one tube and cleaned using SPRIselect following the left side size protocol with a sample-to-volume ratio of 0.76X (Beckman Coulter,CA, USA). Library quantification was performed using Qubit dsDNA HS Assay Kit and a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, MA, USA).

Sequence processing

Sequences were preprocessed using JAMP (https://github.com/VascoElbrecht/JAMP). Briefly, sequences were demultiplexed, and forward and reverse paired-end reads were merged. Primer sequences were removed, and sequences between 380bp and 440 bp (+/- 30bp of expected fragment length) were kept in the dataset, in order to preserve the most of the possible diversity present in the sample.

The resulting files were processed in two ways: i) the reads were quality filtered and taxonomy was assigned using the Multiplex Barcode Research And Visualization Environment of the Barcode of Life platform (mBRAVE/BOLD; Ratnasingham, and Hebert, 2007), and ii) quality filtering was carried out in JAMP and the resulting haplotypes were grouped into OTUs and blasted against the NCBI nucleotide database (for details see Supplementary Methods).

Bioinformatic and statistical analysis of molecular data

Taxonomic assignments obtained by the two previous pipelines (mBRAVE/BOLD-based dataset and blast/NCBI-based dataset) were manually curated. First, genus level taxonomic tables were screened for locally occurring species and, in the case of the mBRAVE dataset, the quality of the BOLD references (BINs) was determined to define the most reliable taxonomic level. For instance, genera which are not described for Argentina, not known from the marine environment, and/or whose BIN included multiple genera or could be considered unreliable (e.g. composed by only one sequence) were considered as “unidentified” members of that family/class. The NCBI dataset was curated at the OTU level, based on % similarity of the first hit to OTU representative sequences. Only hits with pairwise identity >85% and alignment coverage >75% were kept for analysis. Of these, representative sequences were assigned to: species (at least 98% similarity with the first hit), genus (95%), family (90%), and order (85%) (Elbrecht et al. 2017). For analysis of taxa occurrence, both datasets were combined into one that contained BOLD assignments plus NCBI assignments of taxa not detected in BOLD. The results were used to calculate the frequency of occurrence ($FO_{(met)}$) at different taxonomic levels in the same way as $FO_{(vis)}$ (see section 2.2). We decided to use occurrence data and not quantitative data such as read count because gut content

material is usually at various states of digestion and the sequence counts are less likely to contain a meaningful quantitative signal compared to e.g. faecal matter (Nakahara et al. 2015, Deagle et al. 2019). Although green crab inhabits the intertidal zone, there had been no previous evidence of terrestrial intakes (Cohen et al. 1995). Therefore, terrestrial taxa such as most insects in the metabarcoding data were not taken into account for further analyses. Complete tables of taxonomic assignments are provided in Electronic Supplemental Material (Online Resource 2: Table A1 and Online Resource 3: Table A2).

2.4 Statistical analysis of diet description

To test if sample size was sufficient to obtain a representative description of the diet, we constructed cumulative trophic diversity curves using the quadrats methods following Koen Alonso et al. (2002) for both datasets (i.e. visual and metabarcoding analyses). We calculated trophic diversity as the Brillouin index (Hz) and considered diversity curves as asymptotic if the two previous values (i.e. n-1 and n-2) were in the range Hz ± 0.05 Hz for phylum and genus classification (Pielou 1966, Koen Alonso et al. 2002). Once this condition was met the number of stomachs in our study ($n_{(vis)}$ = 183 and $n_{(met)}$ = 40) was considered sufficient to describe the green crab diet.

To test differences between male and female green crab diet, we performed a distance-based multivariate Welch t-test (Wd∗-test) both for the metabarcoding (met) and the visual (vis) analyses. The Wd∗-test is a robust alternative to PERMANOVA when heteroscedasticity or imbalanced data are present (Alekseyenko et al. 2016, Hamidi et al. 2019). The tests were performed using a Bray-Curtis similarity matrix implemented in vegan and the MicEco R package (Oksanen et al. 2019, Russel 2020).

Calculation of alpha and beta diversity metrics, ordinations, and other statistical analyses based on OTUs were performed using only the NCBI dataset, using the R packages vegan and phyloseq (McMurdie and Holmes, 2013, Oksanen et al. 2019). Briefly, OTU richness was calculated with the specnumber function in vegan, and a Kruskal-Wallis non-parametric test was

used to analyze whether there were differences in prey richness between sexes. For beta diversity analyses, a similarity matrix based on OTUs was built using the Unifrac similarity index, a phylogenetic-based method which introduces the genetic distance between OTUs to estimate dissimilarity between any two communities (Lozupone and Knight 2005). A phylogenetic tree based on genetic distance was built in QIIME™ from the OTU representative sequences, using the tool phylogeny align-to-tree-mafft-fasttree. This command uses FastTree version 2, which constructs approximately maximum-likelihood trees from large datasets and calculates local support values using the Shimodaira-Hasegawa test (Price et al. 2010) . This tree was then used in phyloseq to calculate the index for each pair of samples, which is defined as the fraction of the branch length of the tree leading to OTUs unique to a particular sample over total branch length (Lozupone and Knight 2005). The index was used in its unweighted version, which does not take into account OTU abundances for index calculation. From the Unifrac pairwise distance matrix, NMDS ordinations were constructed. The whole process is wrapped in phyloseq with the command ordinate (physeq, method="NMDS", distance="unifrac"). In addition, the tree with support values based on 1,000 resamples was used to visualize the shared OTUs between males and females.

All analyses were carried out in R (R Core Team, 2020).

2.5 Stable isotope analysis

Muscle samples of both green crabs and the two baseline species (*M. edulis* and *T. patagonica*) were dried for 48 h at 60 °C, ground into a fine powder and ≈1.25 µg per sample was sent to the Stable Isotopes Center at the University of New Mexico for C and N stable isotope determination. Internal standards used in the laboratory were blue grama, buckeye, cocoa powder, casein, tuna muscle, graphite, whey, elemental protein, serine and IAEA 1N. Standard deviations (SDs) of reference materials were 0.05‰ for $\delta^{13}C$ and 0.07‰ $\delta^{15}N$. A total of 36 muscle samples were sent, of which 26 corresponded to green crabs (10 males, 11 females and 5 duplicates), five to *M. edulis* and five to *T. patagonica*. The error estimated through duplicate samples for green crabs muscle was 0.14‰ for both $\delta^{13}C$ and $\delta^{15}N$ values.

We estimated the trophic position (TP) of the green crabs using the two baselines (i.e $\delta^{13}C$ and $\delta^{15}N$ values of mussels and snails) and the Bayesian approach proposed by Quezada-Romegialli et al. (2018) (See equations in ESM: Online Resource 1: eq. A1, eq. A2 and eq. A3). The discrimination factors used for TP estimations were 1.80 ± 0.40 ‰ ($\delta^{13}C$) and 2.35 ± 1.26‰ ($\delta^{15}N$) following published values for omnivorous crustaceans (Parker et al. 1989, Vanderklist and Ponsard 2003, Rudnick and Resh 2005, Yokoyama et al. 2005). For TP estimations sex was taken into account (i.e. independent estimations were undertsaken for female and male crabs). We performed pairwise comparisons of posterior estimated TP as the probability of TP_A to be less than or equal to TP_B ($TP_A <= TP_B$) for comparisons between sexes. We similarly performed pairwise comparisons of the proportional contribution of each baseline (α). The Bayesian probability was considered to be significant when $p > 0.95$ as an analogous of the frequentist p-value. Estimations of TP and α were done using the 'TwoBaselinesFull' model in the R package 'tRophic Position' (Quezada-Romegialli et. al. 2018).

The trophic niche of green crabs was described in terms of isotopic niche characteristics following the standard ellipse approach (Jackson et al. 2011). We calculated the standard ellipse area for males and females separately considering 40% of the data and corrected by sample size (SEAc) and the Bayesian standard ellipses area (SEAb) using the 'SIBER' and "rjags" packages on R (Jackson et al. 2011). Subsequently, we calculated the percentage of overlap between females and males SEA as the area at the interception divided by the SEA of male or female respectively. To test differences between SEAb we calculated 100,000 posterior iterations of SEAb based on our data set and estimated the probability that the bigger SEAb was higher than the smaller SEAb (i.e. we estimated the probability of male SEAb > female SEAb) (Jackson et al. 2011).

3. Results

What are green crabs eating in coastal environments of Patagonia Argentina?

We considered the number of stomachs analyzed ($n_{(vis)}$ =183 and $n_{(met)}$ = 40) sufficient to provide a good description of the diet, because the trophic diversity curves were asymptotic and all

(n-1) and (n-2) iterations were within (Hz – 0.05) range (Fig. A1). Overall, 18.58% of the visually analyzed stomachs were empty and 28.41% contained material that was impossible to identify due to its high degree of digestion. In the metabarcoding analysis, one sample did not result in high-quality reads and four samples did not have any DNA that could be assigned to prey (i.e. they only resulted in host/green crab DNA). Therefore, the first sample was excluded from the analysis and the other four were considered as empty stomachs resulting in 10.25% of empty guts for metabarcoding.

The visual analysis of the gut content showed that green crabs at Nuevo Gulf waters had a very broad diet, with 25 prey items (Table 1). Identifications were possible due to prey remains (typically hard parts) found in gut contents. Polychaetes were identified by jaws and mollusks by opercle and radula. We also found some well preserved organisms such as tanaidaceans and amphipods, but finding an almost fully intact organism was very unusual (see Fig. A2). We detected the presence of different prey taxa such as mollusks, algae, arthropoda, annelida, fishes and echinodermata. The main prey items were Mytilidae (bivalves) with a $FO_{(vis)}$ of 35.57% followed by the gastropod *T. patagonica* (12.75%).

Metabarcoding analysis of gut contents showed a similar picture to the visual analysis (i.e. a very wide omnivorous diet) but detected a higher number of phyla and provided a more detailed description at the genus level. In general, $FO_{(met)}$ were higher than $FO_{(vis)}$, indicating that the level of detection using metabarcoding was higher than through traditional analysis. Here, Arthropoda was the dominant phylum in the crab's gut, with a $FO_{(met)}$ of 94.28%, followed by Ochrophyta (i.e. Phaeophyta) and Mollusca. The main prey items were amphipods of the Gammaridae family (82.86%), an unidentified species of Heterodonta (45.71%) and algae (*Dictyota* and an unidentified member of Ralfsiales, both with 40.00%) (Table 1).

Are there differences in diet between females and males in Patagonia Argentina?

We could observe only slight differences in prey items between males and females, evidenced by the overlapping of CIs of the simulated $FO_{(vis)}$. The main differences were a higher

334 frequency of arthropods and a lower frequency of molluscs in the female gut contents compared to
335 male contents (Fig. 1A). However, these differences were not significant ($Wd^*_{(vis)}$ = 3.14 and p-
336 $value_{(vis)}$ = 0.058). The $FO_{(met)}$ showed less differences between male and female main prey groups
337 (Fig. 1B); and we did not find significant differences in their diets ($Wd^*_{(met)}$ = 1.84 and $p\text{-}value_{(met)}$
338 = 0.098). Similarly, ordinations based on OTUs and phylogenetic distances showed a high degree of
339 overlap between males and females, although some grouping can be seen in the plot (Fig. 2A).

340 The main difference between males and females was in their trophic diversity.
341 Metabarcoding OTU richness was significantly higher in males than in females (7.4 ± 3.4 and 4.7 ±
342 2.2 for males and females, respectively; Kruskal-Wallis chi-squared = 6.208, p-value = 0.013) (Fig.
343 2B). From a total of 70 OTUs considered prey in the NCBI dataset, only 19 were shared between
344 males and females (27%) (Fig. 2C). These corresponded roughly to the most frequently occurring
345 OTUs, explaining the lack of clear grouping in ordinations. However, a great proportion (53%, 37
346 OTUs) were male-only OTUs while only 14 (20%) were female-only (Fig. 2C). These
347 corresponded to a variety of different OTUs with low frequency, but they are responsible for the
348 higher richness in male diet when they are taken together.

349 Stable isotope analysis (SIA) results were in accordance with metabarcoding analysis. Males
350 showed a broader trophic niche than females, being SEAc bigger for males ($SEAc_{males}$ = 1.03 and
351 $SEAc_{females}$ = 0.51, Fig. 4.A). The Bayesian reconstruction of the SEA showed that the probability of
352 this difference was p = 0.05 (Fig. 4B). However, it should be noted that some overlap at standard
353 ellipses between males and females was present (11.89% of male SEA and 24.59% of female SEA)
354 (Fig 4A and Fig. A3 in ESM: Online Resource 1). With respect to trophic position (TP), female
355 crabs showed a posterior estimated TP that was slightly higher than that for males (TP_{males} = 3.02,
356 $TP_{females}$ = 3.16 and $TP_{overall}$ = 3.10, Fig 3A) but pairwise comparisons did not result in significant
357 differences for TP or for alpha (α) posterior estimates (Table A3). The posterior estimates of the
358 proportional contribution of each baseline showed the phytoplankton pathway of energy to be more
359 important than the macroalgae pathway in the nutrition of green crabs (α_{males} = 0.74, $\alpha_{females}$ = 0.88

and $\alpha_{overall} = 0.86$, Fig 3B). This result is in accordance with bivalves (filter feeders) being the main prey item.

The metabarcoding analysis also showed other complementary results that are not diet-related but also relevant. First, sequences from both *C. maenas* and *C. aestuarii* were detected in most samples, highlighting the possibility of the presence of a hybrid crab population in Patagonian coastal waters. Second, we detected sequences assigned to the genus *Carcinonemertes*, a nemertean parasite known to infect populations of this crab (Torchín et al 1996).

4. Discussion

What are green crabs eating in the coastal environments of Atlantic Patagonia?

Green crabs in the study site at the Patagonian Atlantic coast display a broad omnivorous diet, consuming the majority of phyla present at the study area. The most representative phyla reported for the sampling site (Mollusca, Arthropoda and Phaeophyceae, Rechimont et al. 2013) were also the prey items with the highest occurrences in our dietary samples. Rhodophyta and Annelida were also found frequently but with a lower occurrence rate. These results are consistent with previous studies in other areas, where green crabs have been classified as opportunist omnivorous, feeding on a wide range of prey species (Elner 1981, Singh 1991, Pardal et al. 2006). Other green crab populations, both native and invasive, have shown a strong preference for mollusks (bivalves and gastropods) (Young and Elliot 2020). In this study, mollusks were among the main prey items based on visual examinations, whilst metabarcoding indicated species of amphipods of the Gammaridae family, which were present in more than 80% of the samples, as main prey item. Metabarcoding also revealed the presence of other prey items such as Nemertea, Porifera and Ascidiacea, which were not detected in visual analysis of stomach content.

The molecular method also provided a detailed description of algal diversity in the green crab diet. Although algae have been previously detected, the description achieved by visual analysis

was usually very poor (Cohen et al. 1995, Pardal et al. 2006, Wilcox and Rochette 2015). This is due to the little amount of intact algae that is usually found and the difficulties associated with taxonomic recognition. DNA analysis clearly represents an improvement for the identification of taxa that are notoriously difficult to recognize in degraded samples such as gut contents (Smith et al. 2005, García-Robledo et al. 2013, Taberlet et al. 2018). Although algae were frequently detected in our crabs' diet, stable isotope analysis revealed that the major dietary contribution stemed from phytoplankton chains (i.e. canalized by bivalve consumption). Green crabs at Atlantic Patagonia feed in an opportunist manner, with bivalves and amphipods being their preferred prey; in this manner they also ingest some algal material, but this does not significantly contribute to their nutrition.

Are there differences in diet between female and male green crabs?

Various studies have shown that there are no differences between male and female foraging, as well as no differences in prey handling time and that therefore the two sexes are ecological equivalents (Spooner et al. 2007, Young and Elliot 2020). However, we observed a more complex situation in Patagonian waters. Males and females displayed similar diets without differences in trophic position (TP) but males had a broader diet than females (i.e. higher trophic diversity). This difference was evidenced by both the isotopic niche and the metabarcoding OTU richness. The fact that males have a higher trophic diversity is probably related to differences in behavior between the sexes, e.g. females are usually found in deeper waters (Klassen and Locke 2007). Crab's chela could also be playing a role in this process, as it was previously shown and later confirmed by modeling that master chela size was a good predictor of feeding rate and energy intake (Elner 1980, Howard et al. 2018). Such higher feeding efficiency could be indirectly influencing prey diversity in males, and suggests that females and males may not be ecological equivalents after all. However, dietary overlap between the sexes remains high and the differences are rather subtle. New dietary studies focusing on seasonality and the reproductive season will be very helpful in exploring these differences in Atlantic Patagonia.

What changes can green crabs trigger in local food webs?

Green crabs from newly established populations have shown to be superior foragers to long-established invaders (Rossong et al. 2011). Therefore, we expect that the most dramatic changes in local food webs will occur in the early stages of the invasion until a new equilibrium state with new relative abundances has been reached. However, such a new food web state can bring serious consequences for native populations and even lead to extirpation of some species. The potential impact of green crabs as predators at the study site (rocky shore of Punta Este) can be illustrated by comparing results of an earlier survey that was part of the Census of Marine Life, NAGISA project. In the mentioned study, a total of 35 species of benthic invertebrates and 29 macroalgae were recorded (Rechimont et al. 2013), while in the present study we found 31 benthic invertebrates and 23 macroalgae in the stomachs of green crabs. Hidalgo et al. (2007) have observed that green crabs are bigger and feed with greater voracity than native predators on Patagonia rocky shores. They have provoked negative impacts worldwide such as damaging populations of *M. edulis*, American oysters (*Crassostrea virginica*), or rock crabs (*Cancer irroratus*), among others. They have even caused major economic losses for different fisheries (DeGraaf and Tyrrel 2004, Miron et al. 2005, Sigurdsson and Rochette 2013, Leignel et al. 2014, Young and Elliot 2020). In addition, bird mass mortality has been attributed to the transmission of parasites by green crabs (Camphuysen et al., 2002). Coastal birds at Patagonia are already incorporating green crabs to their diet, a fact confirmed by a recent study on the kelp gull (*Larus dominicanus*) that showed green crabs as one of their prey items (Yorio et al. 2020). Carcinophagus fish (e.g. *Mustelus schmitti*) are likely to incorporate green crabs into their diet as well, although this has not been confirmed yet (Belleggia et al. 2012, Alessandra Pasti pers. comm. July 2020). The fact that the nemertean parasite *Carcinonemertes* was observed in one of our samples highlights the possible risk of dispersion of these parasites, which had not been reported before for Argentina (Bigatti and Signorelli 2018), in green crabs and other species of native crabs.

435 Community structure at intertidal Patagonian rocky shores is driven by harsh physical
436 conditions to which organisms are exposed to, rather than by consumer pressure (Bertness et al.
437 2006, Hidalgo et al. 2007). Invasive green crabs could have a negative impact on the community
438 structure not only by the increase in predation pressure, but also by interfering with processes that
439 maintain biodiversity such as facilitation (Silliman et al. 2011). That means green crabs could
440 trigger both direct and indirect effects on Patagonian food webs. We predict that changes will begin
441 with their preferred prey: mollusks, especially bivalves, as well as crustaceans, and then scale up to
442 the entire food web through a cascade effect (White et al. 2006, Davies et al. 2017) (Figure 5). We
443 also predict that high predation on foundation species such as mitylids will constitute a loss of
444 refuge for other intertidal species, which could result in a decline in biodiversity (Hidalgo et al.
445 2007). Changes at Nuevo Gulf coasts have already been observed such as a dramatic decrease of
446 Perumytilus purpuratus populations and Brachidontes rodriguezii (Mendez et al., 2019), however,
447 the causes for this mass mortality are unknown and if it relates to the green crab invasion or
448 environmental factors is currently unknown. In addition, other species of common native crabs are
449 much more difficult to find at the intertidal since green crabs arrival, suggesting that green crabs
450 could have a negative interaction with other species of local crabs.

451 **Does the use of a multidisciplinary approach contribute to a more complete picture?**

452 The inclusion of new techniques to trophic ecology such as stable isotope analyses and
453 metabarcoding can add new results that would otherwise go unnoticed when using traditional
454 techniques such as visual inspection of gut contents. Both visual and metabarcoding analysis
455 contribute to the knowledge of diet richness and provide quantitative information about it. Stable
456 isotope analysis allows for estimates of species trophic levels and trophic pathways (i.e. which
457 sources contribute the most to species nutrition). The conjunction of techniques used in our work
458 provided information on different aspects of trophic ecology, such as interspecific prey-predator
459 interactions and intrapopulation differences (Figure 5). In particular, we observed slight differences
460 between male and female diets related to niche breadth and we achieved a more detailed taxonomic
461 classification of certain taxa due to the use of various techniques. The methodology used in this

462 work allowed us to better understand the trophic relations of the green crab in a recently invaded
463 habitat, highlighting the importance of monitoring coastal ecosystems to detect changes that can
464 affect these marine habitats, which provide very important ecosystem services.

465 **Acknowledgements**

466 We would like to thank Florencia Ríos, Manuela Funes, Juan Pablo Livore, and Maria
467 Martha Mendez for their fieldwork support. We would like to thank Agustina Ferrando, Cynthia
468 Ibarra, Pablo Macchi and, German Cheli for expert assessment on taxonomic identifications. We
469 would like to thank Antonela Leiva for facilitating the *Larus dominicanus* photograph.

470 **Funding**

471 This research was supported by Consejo Nacional de Investigaciones Científicas y Técnicas
472 (CONICET, Argentina) and Sistema Nacional de Datos Genómicos (SNDG, Argentina). Fieldwork
473 campaigns and stable isotope analysis were funded by Fondo para la Investigación Científica y
474 Tecnológica (FonCyT) under the grants PICT 2017-2403, PICT 2018-0903 and PICT 2018-0969.
475 The metabarcoding analysis was funded by the Natural Sciences and Engineering Research Council
476 of Canada and the Canada First Research Excellence Fund.

477 **Ethical approval**

478 All applicable institutional and/or national guidelines for the care and use of animals were
479 followed. Permissions for sample collection were granted from the Secretaría de Pesca and
480 Secretaría de Conservación de Áreas Protegidas from Chubut Province. Exportation permissions
481 were granted in 2019 by the Dirección Nacional de Biodiversidad (Secretaría de Ambiente y
482 Desarrollo Sustentable) under ANEXO II, resolution number: IF-2019-40388897-APN-DNBI#SGP.

483 **Author Contributions**

484 DEG, GB, ML, and GC conceived and designed this study. DEG, GB, and GC took the
485 samples. GC conducted the visual analysis and prepare the samples for stable isotope analysis. GC

and DEG analyzed direct stomach observation and stable isotope data. ML contributed to preparing the metabarcoding samples and analyzed metabarcoding data. EV visited as an invited researcher the Centre for Biodiversity Genomics to obtain the metabarcoding data. EV, BT, DAL, and DS contributed to the generation of metabarcoding data, molecular and bioinformatic analyses. All authors contributed to manuscript writing.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1. List of prey taxa at green crabs stomach content determinate by metabarcoding and visual analyzes. Frequency of occurrence (FO%) for metabarcoding (met) and visual inspection (vis) of each prey taxa are shown.

Phylum	Subphylum	Class	Subclass	Order/Subclass	Family/Suborder	Genus/Specie	FO (Met)	FO (Vis)
Mollusca							**74.28**	**46.31**
		Bivalvia					68.57	35.57
				Mytilida			17.14	35.57
					Mytilidae		17.14	35.57
						Aulacomya	2.86	-
						Brachidontes/ Aulacomya/ Mytilus	5.71	-
						Brachidontes/ Perumytilus	5.71	-
			Heterodonta				62.86	-
				Heterodonta			62.86	-
					Heterodonta			-
						Heterodonta Unid. 1	25.71	-
						Heterodonta Unid. 2	45.71	-
					Myoida		2.86	-
						Myoida Unid. 1	2.86	-
						Myoida Unid. 2	2.86	-
		Gastropoda					8.57	18.12
				Trochidae			8.57	12.75
					Tegulidae		8.57	12.75
						Tegula	8.57	12.75
				Siphonariida			-	4.03
					Siphonariidae		-	4.03
						Siphonaria lessoni	-	4.03
		Polyplacophora					-	0.67
				Chitonida			-	0.67
					Chaetopleuridae		-	0.67
						Chaetopleura	-	0.67
Annelida							**22.87**	**16.78**
		Clitellata					11.43	-
				Haplotaxida			11.43	-
					Naididae		11.43	-
						Nadididae Unid. 1	8.57	-
						Nadididae Unid. 2	2.86	-
					Haplotaxida Unid.		5.71	-

					Haplotaxida Unid. 1	2.86	-
					Haplotaxida Unid. 2	2.86	-
		Polychaeta				22.86	-
			Phyllodocida			14.28	4.70
				Nereididae			3.36
					Platynereis	5.71	-
					Nereidae Unid	-	3.36
				Phyllodocidae		2.86	-
					Eulalia	2.86	-
				Polynoidae		-	0.67
					Polynoidae Unid.	-	0.67
				Glyceridae		-	0.67
					Glyceridae Unid.	-	0.67
			Palpata			2.86	-
				Sabellida		2.86	-
					Sabellida Unid.	2.86	-
			Eunicida			-	0.67
				Eunicidae		-	0.67
					Eunicidae Unid.	-	0.67
			Terebellida			2.86	-
				Terebellidae		2.86	-
					Terebellidae Unid.	2.86	-
			Other Polychaeta Unid.		Polychaeta Unid.	-	11.41
Nemertea						**11.43**	-
		Pilidiophora				11.43	-
			Heteronemertea			11.43	-
				Heteronemertea		11.43	-
					Heteronemertea Unid.	11.43	-
Arthropoda						**94.28**	**20.80**
	Crustacea					85.71	20.13
		Malacostraca				82.86	6.03
			Amphipoda			82.86	1.34
				Gammaridae		82.86	-
					Gammaridae Unid.	82.86	-
				Corophiidae		-	1.34
					Monocorophium insidiosum	-	1.34
			Decapoda			5.71	-
				Porcellanidae		5.71	-
					Pachycheles	5.71	-
			Isopoda			5.71	0.67
				Sphaeromatidae		5.71	-
					Exosphaeroma/	2.86	-

					Pseudosphaeroma		
					Sphaeroma	2.86	-
				Isopoda Unid.		-	0.67
			Euphausiacea			-	0.67
				Euphausiacea Unid.		-	0.67
					Euphausiacea Unid.	-	0.67
			Tanaidacea			-	2.68
				Tanaididae		-	2.68
					Tanais dulongii	-	2.68
			Cumacea			-	0.67
				Cumacea Unid.		-	0.67
					Cumacea Unid	-	0.67
	Hexanauplia					2.86	-
		Copepoda				2.86	-
			Cyclopoida			2.86	-
				Oithonidae		2.86	-
					Oithona similis	2.86	-
	Cirripedia					-	1.32
			Cirripedia Unid.			-	1.32
				Cirripedia Unid.		-	1.32
					Cirripedia Unid.	-	1.32
	Other Crustacea Unid.				Crustacea Unid.	-	18.79
	Insecta					54.28	0.67
			Diptera			54.28	0.67
				Chironomidae		54.28	0.67
					Chironomus	2.86	-
					Chironomidae Unid. 1	2.86	-
					Eukiefferiella	5.71	-
					Chironomidae Unid. 2	8.57	-
					Chironomidae Unid. 3	2.86	-
					Chironomidae Unid 4.	37.14	-
					Polypedilum	2.86	-
					Tanytarsus	2.86	-
					Chironomidae Unid. 5	31.43	-
					Chironomidae Unid. 6	-	0.67
Echinodermata						-	0.67
	Echinoidea					-	0.67
			Camarodonta			-	0.67
				Temnopleuridae		-	0.67
					Pseudechinus magellanicus	-	0.67
Cnidaria						**2.86**	-

	Hydrozoa				2.86	-
		Anthoathecata			2.86	-
			Pandeidae		2.86	-
				Amphinema	2.86	-
Porifera					**14.28**	-
	Demospongiae				14.28	-
		Demospongiae Unid.			14.28	-
Chordata					**8.57**	**1.32**
	Ascidiacea				2.86	-
		Enterogona			2.86	-
			Enterogona Unid.		2.86	-
				Enterogona Unid 1.	2.86	-
	Actinopterygii				5.71	-
		Blenniiformes			2.86	-
				Blenniiformes Unid. 1	2.86	-
		Actinopterygii Unid.			5.71	-
				Actinopterygii Unid. 1	2.86	-
				Actinopterygii Unid. 2	5.71	-
	Fishes Unid.				-	1.32
Metazoa Unid.					5.71	-
				Metazoa Unid.	5.71	-
Orchrophyta					**82.86**	**2.68**
	Phaeophyceae				82.86	2.68
		Ectocarpales			48.57	-
			Acinetosporaceae		11.43	-
				Acinetosporaceae Unid. 1.	2.86	-
				Acinetosporaceae Unid. 2	8.57	
			Chordariaceae		40.00	-
				Hecatonema	14.28	-
				Myrionema	8.57	-
				Microspongium	8.57	-
				Chordariaceae Unid. 1	2.86	-
				Chordariaceae Unid. 2	2.86	-
				Chordariaceae Unid. 3	2.86	-
				Chordariaceae Unid. 4	2.86	-
			Ectocarpaceae		17.14	-
				Ectocarpus Unid. 1	14.28	-

				Ectocarpus Unid. 2	5.71	
			Scytosiphonaceae		14.28	-
				Scytosiphon	5.71	-
				Petalonia	5.71	-
				Planosiphon	2.86	-
			Ectocarpales Unid.		5.71	-
				Ectocarpales Unid. 1	2.86	-
				Ectocarpales Unid. 2	2.86	-
		Dictyotales			40.00	-
			Dictyotaceae		40.00	-
				Dictyota	40.00	-
		Ralfsiales			40.00	-
			Ralfsiales Unid.		40.00	-
				Ralfsiales Unid. 1	40.00	-
				Ralfsiales Unid. 2	40.00	-
		Sphacelariales			2.86	-
			Sphacelariales Unid.		2.86	-
				Sphacelariales Unid. 1	2.86	-
		Laminariales			34.28	-
			Alariaceae		34.28	-
				Undaria pinnatifida	34.28	-
Rhodophyta					**31.43**	**2.68**
	Florideophyceae				28.57	-
		Ceramiales			22.86	-
			Rhodomelaceae		20.00	-
				Polysiphonia	2.86	-
				Streblocladia	2.86	
				Gredgaria	17.14	
			Ceramiales Unid.		2.86	-
				Ceramiales Unid.	2.86	-
		Corallinales			5.71	-
			Corallinaceae		2.86	-
				Corallina Unid.	2.86	-
			Corallinacea Unid.		2.86	-
				Corallinacea Unid.	2.86	-
	Bangiophyceae				2.86	-
		Bangiales			2.86	-
			Bangiaceae		2.86	-
				Bangiales Unid.	2.86	-

				Bangiaceae Unid.	2.86	
Chlorophyta					**2.86**	**0.67**
	Ulvophyceae				2.86	-
		Ulvales			2.86	-
			Ulvaceae		2.86	-
				Ulva	2.86	-
Other Algae Unid.				Algae Unid.	-	**23.49**

Figure Legends

Figure 1. Frequency of occurrence (FO) boxplots of mayor prey groups in green crab diet from Patagonia, Argentina for: A) visual and B) Metabarcoding analyses. The lower and upper hinges correspond to the first and third quartiles (the 25th and 75th percentiles). Points are outliers (data > 1.5* IQR) and horizontal lines are medians. Annelida: “Ann”, Arthropoda: “Artr, Mollusca: “Mol”, Chordata: “Chor”, Echinodermata: “Echin”, Cnidaria: “Cnid”, Porifera: “Por”, Nemertea: “Nem”, Chlorophyta: “Chlo”, Ochrophyta: “Orch”, Rhodophyta: “Rhod”, Unidentified: “Unid”.

Figure 2. A) Ordination of samples based on COI metabarcoding data (OTUs, NCBI-based assignments), depicted by sex. Distance measure: unweighted Unifrac. **B)** Distribution of OTU richness (number of different OTUs) by sex. Letters "a" and "b" indicate differences between sexes ($p<0.05$; Kruskal-Wallis rank-sum test). **C)** Phylogenetic tree of OTU-representative COI sequences. The final, curated taxonomic assignment of each OTU is shown at the dendrogram tips. Occurrence by sex is depicted in circles (red: females, black: males). Local support values over 1,000 resamplings are shown at the nodes.

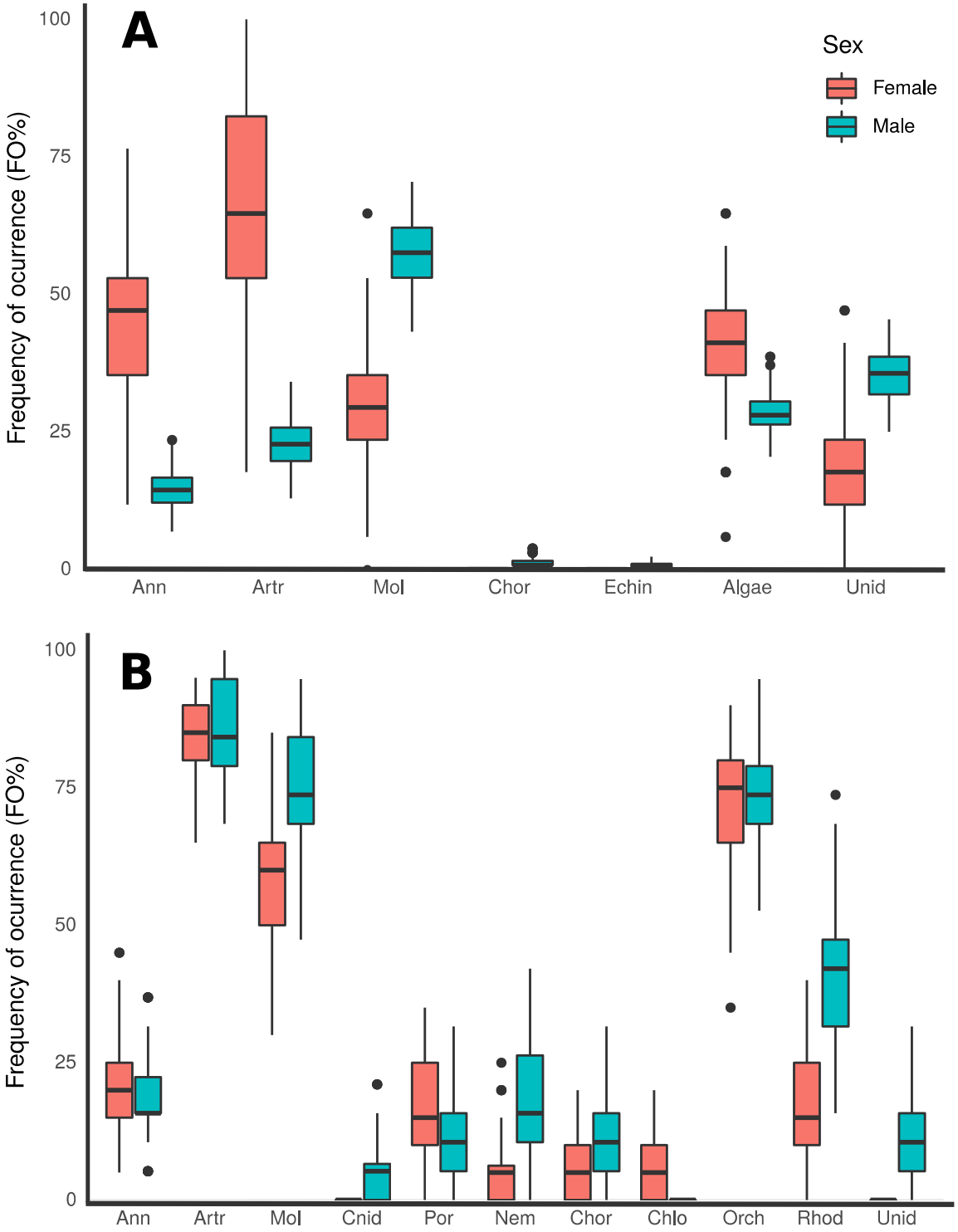
Figure 3. A) Trophic position (TP) posterior estimates for female and male green crabs **B)** Alpha (α) posterior estimates for female and male green crabs at Nuevo Gulf, Patagonia Argentina. Means are indicated by dotted lines (males: blue, female: pink and overall: black).

Figure 4. A) δ^{15}N‰ and δ^{13}C‰ values for female and male green crabs. Ellipses represent the standard ellipses for each sex corrected for sample size. **B)** Standard ellipse area (‰2) estimated by Bayesian methods (SEAb) for males and females green crabs.

Figure 5. Diagram representing the different techniques and their contribution to the analysis of trophic ecology (top left). R: Richness, QC: Quantitative Contribution, TL: Trophic Level, TP: Trophic Pathway. Line width represents the relative importance of each technique. The orange circle represents intra-population variability. The above diagram represents a simplified version of

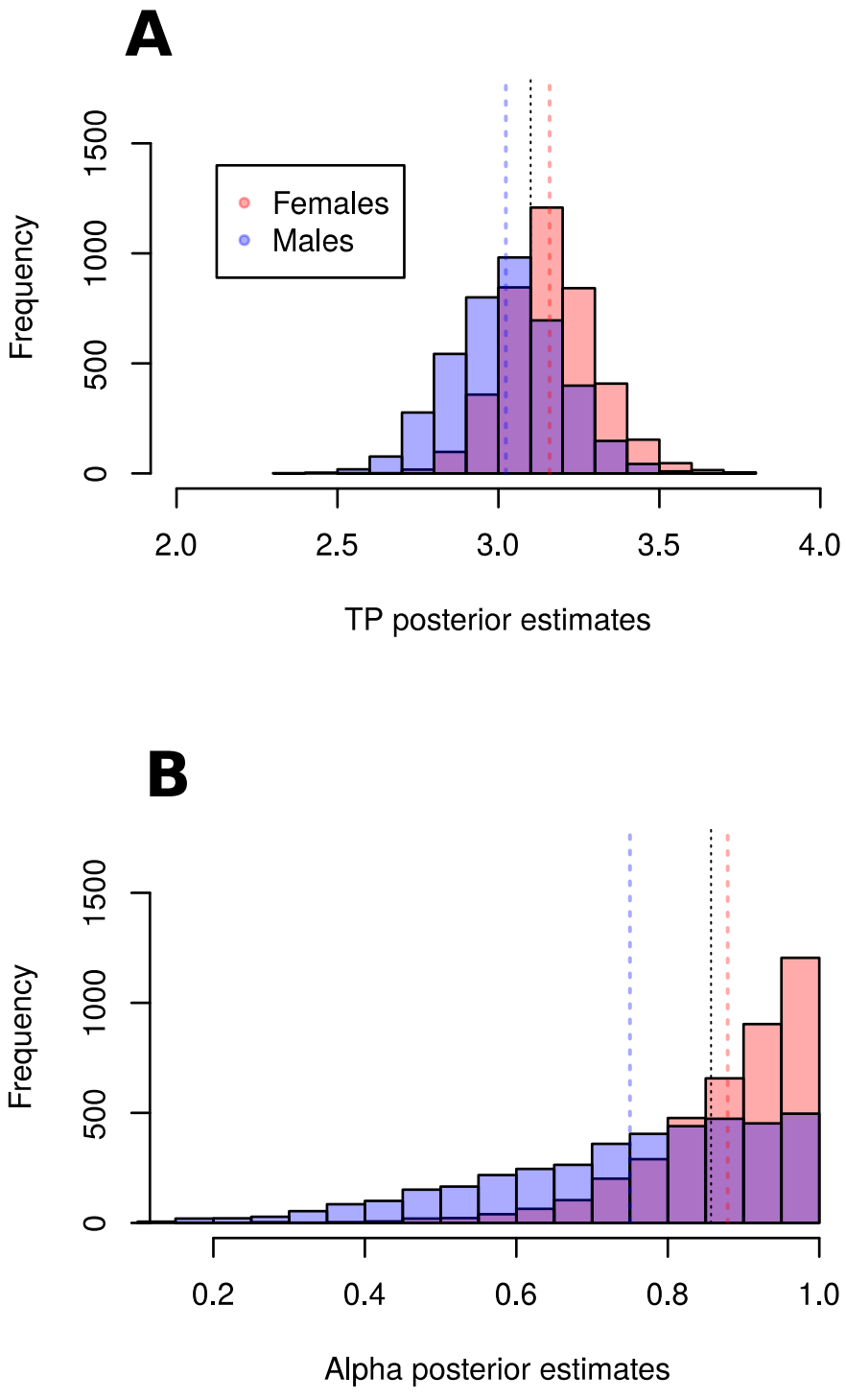
783 the Atlantic Patagonian intertidal food webs showing the possible effects of the green crab invasion.
784 The solid red lines represent a direct negative relationship (predation on resident species). The
785 dotted red lines represent an indirect negative relationship to resident species (competition or
786 parasite transfer). The solid green line represents a direct positive relationship to bird species (by
787 food facilitation). The dotted green line represents a possible positive relationship to fish (by food
788 facilitation). F: phytoplankton, M: macroalgae, D: detritus, Z: zooplankton, H: herbivores, C: native
789 crabs as *Cyrtograptus angulatus* or *C. altimanus*, B: bivalves, T: *Trophon geversianus*, L: *Larus*
790 *dominicanus*, P: fishes and GC: green crab *Carcinus maenas*. Photo credits: Antonela Leiva and
791 Gregorio Bigatti.

792 **Figure 1**

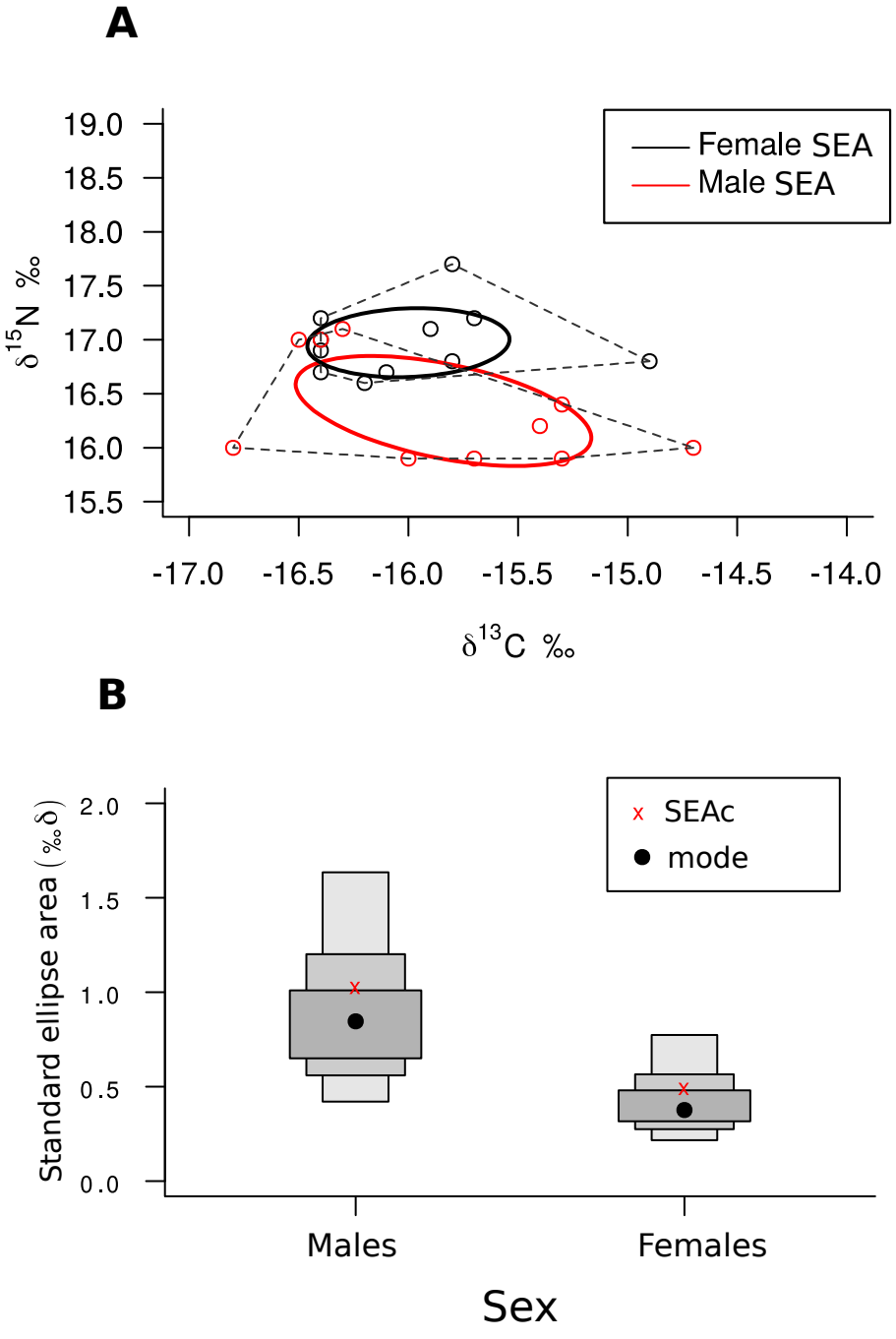


793 **Figure 2**

794 **Figure 3**



795 **Figure 4**



796 **Figure 5**