

1 **Title:**

2 **Metabarcoding, direct stomach observation and stable isotope analysis reveal a highly**
3 **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.

24 **Abstract**

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

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

43 **1. Introduction**

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

49 organisms related to each other by consumer-resource relationships; their network structure
50 influences community dynamics and stability (Bascompte 2009, Thébault and Fontaine 2010). The
51 ways biological invasions impact food webs are complex and context-dependent as there are
52 various direct and indirect mechanisms by which an invasive species can affect other taxa
53 (Thomsen et al. 2011a, Thomsen et al. 2011b). Preying upon a native species is a direct mechanism
54 that usually ends up with population decrease, or even species loss (Bruno et al. 2005, David et al.
55 2017). Exploitative and apparent competition are indirect mechanisms that can also cause the
56 decline of native populations (White et al. 2006, David et al. 2017). On the other hand invaders can
57 display positive effects for resident species. In fact, for species at higher trophic positions, invaders
58 can increase food availability and new habitats (Rodriguez 2006, Thomsen et al. 2010, Thomsen et
59 al. 2014). The invaders' impact depends on the food web's invasibility which is ultimately related to
60 network structure (Hui et al. 2016). Earlier work has shown that food webs with higher diversity are
61 more resistant to invasions; however, on the flip side highly diverse communities generally support
62 more invasive species (Fridley et al. 2007, Tilman et al. 2014, David et al., 2017). By using
63 simulations, Romanuk et al. (2017) have shown that trophic cascades are more frequent when
64 invaders become secondary consumers with abundant prey and only few other predators.
65 Consequently, if we want to accurately predict possible impacts of a newly arrived invasive species,
66 we need to understand its trophic interactions at the invaded ecosystem.

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

75 or faeces using DNA sequencing or immunoassays. DNA-based techniques allow the
76 characterization of feeding interactions at high taxonomic resolution and are very useful when
77 visual recognition is not possible (Clare 2014, Unger et al. 2020). In cases of omnivorous species,
78 where a mixture of DNA from different species is present in gut content, the most effective
79 approach is metabarcoding (Taberlet et al. 2012, Pompanon et al. 2012, Nielsen et al. 2018).
80 Metabarcoding couples DNA barcoding (Hebert et al. 2003) with high-throughput sequencing of
81 community samples (i.e. gut samples) (Nielsen et al. 2018). It not only provides information on
82 ingested organisms, it also has the advantage of providing complementary information such as the
83 gastrointestinal parasites of a predator. Aside from such direct methods, indirect tracers of diet
84 composition are robust and commonly used tools. They include the characterization of biomarkers
85 such as fatty acids, sterols or pigments present in consumer tissues and the determination of the
86 isotopic compositions of C, N and S in specific tissues (e.g. Galloway et al. 2015, Stock et al.,
87 2018). Stable isotope analysis (SIA) is based on predictable differences between the isotopic
88 signature of an organism and its food resources. It allows for the quantitative estimation of fluxes in
89 food webs, the determination of a species' trophic level and insights into the patterns of resource
90 allocation (Boecklen et al. 2011). All these approaches are largely complementary as direct methods
91 can identify the prey which is ingested, while indirect methods provide information on what is
92 assimilated. In addition, indirect methods such as SIA provide information at longer time scales
93 while DNA or visual analysis register consumption within short time periods (typically 6-48h)
94 (Albaina et al. 2010, Hayden et al. 2014). Majdi et al. (2018) highlighted the potential of combining
95 direct and indirect methodologies and noted the lack of this kind of studies in current trophic
96 ecology research.

97 However, there are method-specific biases. Direct visual stomach content analysis provides
98 indispensable first-hand information, allows quantifying the ingested amount, and for certain cases
99 distinguishes between secondary predation and random ingestion; however, digestion of prey
100 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.

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

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

141 **2. Materials and Methods**

142 **2.1 Sample collection**

143 Green crabs were collected at Punta Este beach in the Nuevo Gulf, Patagonia, Argentina
144 (42°46 ' S, 64°57 ' W). Sampling was done from November 2018 to February 2019 in shallow
145 waters with depths of about 2-3 m at high tide. Crabs were captured manually by SCUBA diving,
146 resulting in a total of 223 specimens. Individuals were separated into two groups. one for traditional
147 visual analysis (183) and the other for metabarcoding (40). After sampling, all specimens were
148 transported to the laboratory and immediately frozen at -20 °C. Crab sizes were registered by
149 measuring carapace width (CW) in cm. Subsequently, crabs were sexed and dissected. For
150 specimens destined for visual analysis, stomach content was extracted and kept in 70% ethanol. For
151 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

177 the buffer AW2 from the silica membrane and for elution in two steps using 100 µl of Buffer AE per
178 each. A fume hood control and extraction control were processed along the stomach content
179 samples to check for cross-contamination. The DNA extracts were quantified using Qubit dsDNA
180 HS Assay Kit and a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, MA, USA). For the
181 amplification of gut content DNA, we used a two-step PCR protocol following Elbrecht et al.
182 (2019). PCRs were carried out using the Multiplex PCR Master Mix Plus (Qiagen, Hilden,
183 Germany), 0.5 mM of each primer (IDT, Skokie, Illinois), and molecular grade water (HyPure, GE,
184 Utha, USA) for a total volume of 25 µl. For the first PCR step, amplification of cytochrome
185 oxidase I gene was performed using primers BF3/BR2 (5' CCHGAYATRGCHTTYCCHCG 3' / 5'
186 TCDGGRTGNCCRAARAAYCA 3') and 4 µl of DNA extract. For the second PCR step, 1 µl of
187 the product obtained from the first PCR was used and amplification was performed using fusion
188 primers with individual tags per sample (see Electronic Supplemental Material, Online Resource 1,
189 section 2.3) (Elbrecht and Leese 2017, Elbrecht et al. 2019, Elbrecht and Steinke 2019).
190 Thermocycling conditions for both PCR steps included an initial denaturation at 95°C for 5
191 minutes, 24 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and
192 extension at 72°C for 50 seconds; and a final extension at 68°C for 10 minutes. After the second
193 PCR, amplification success and band strength were checked on 1.5% agarose gels. For several
194 samples which showed no or only very weak amplification, the two PCRs were repeated, but with
195 35 cycles instead of 24 in the first PCR. These replicates received individual tag combinations and
196 were prepared for sequencing on an Illumina MiSeq platform along with the extraction controls and
197 seven PCR negative controls:

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

203 *Sequence processing*

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

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

214 *Bioinformatic and statistical analysis of molecular data*

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

230 material is usually at various states of digestion and the sequence counts are less likely to contain a
231 meaningful quantitative signal compared to e.g. faecal matter (Nakahara et al. 2015, Deagle et al.
232 2019). Although green crab inhabits the intertidal zone, there had been no previous evidence of
233 terrestrial intakes (Cohen et al. 1995). Therefore, terrestrial taxa such as most insects in the
234 metabarcoding data were not taken into account for further analyses. Complete tables of taxonomic
235 assignments are provided in Electronic Supplemental Material (Online Resource 2: Table A1 and
236 Online Resource 3: Table A2).

237 **2.4 Statistical analysis of diet description**

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

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

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

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

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

272 **2.5 Stable isotope analysis**

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

282 We estimated the trophic position (TP) of the green crabs using the two baselines (i.e. $\delta^{13}\text{C}$
283 and $\delta^{15}\text{N}$ values of mussels and snails) and the Bayesian approach proposed by Quezada-Romegialli
284 et al. (2018) (See equations in ESM: Online Resource 1: eq. A1, eq. A2 and eq. A3). The
285 discrimination factors used for TP estimations were $1.80 \pm 0.40 \text{‰}$ ($\delta^{13}\text{C}$) and $2.35 \pm 1.26\text{‰}$ ($\delta^{15}\text{N}$)
286 following published values for omnivorous crustaceans (Parker et al. 1989, Vanderklist and Ponsard
287 2003, Rudnick and Resh 2005, Yokoyama et al. 2005). For TP estimations sex was taken into
288 account (i.e. independent estimations were undertaken for female and male crabs). We performed
289 pairwise comparisons of posterior estimated TP as the probability of TP_A to be less than or equal to
290 TP_B ($\text{TP}_A \leq \text{TP}_B$) for comparisons between sexes. We similarly performed pairwise comparisons of
291 the proportional contribution of each baseline (α). The Bayesian probability was considered to be
292 significant when $p > 0.95$ as an analogous of the frequentist p-value. Estimations of TP and α were
293 done using the ‘TwoBaselinesFull’ model in the R package ‘tRophic Position’ (Quezada-Romegialli
294 et. al. 2018).

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

304 **3. Results**

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

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

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

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

323 Metabarcoding analysis of gut contents showed a similar picture to the visual analysis (i.e. a
324 very wide omnivorous diet) but detected a higher number of phyla and provided a more detailed
325 description at the genus level. In general, FO_(met) were higher than FO_(vis), indicating that the level of
326 detection using metabarcoding was higher than through traditional analysis. Here, Arthropoda was
327 the dominant phylum in the crab's gut, with a FO_(met) of 94.28%, followed by Ochrophyta (i.e.
328 Phaeophyta) and Mollusca. The main prey items were amphipods of the Gammaridae family
329 (82.86%), an unidentified species of Heterodonta (45.71%) and algae (*Dictyota* and an unidentified
330 member of Ralfsiales, both with 40.00%) (Table 1).

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

332 We could observe only slight differences in prey items between males and females,
333 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-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 $4.7 \pm$
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. 4A). 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 ($\alpha_{males} = 0.74$, $\alpha_{females} = 0.88$

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

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

367 4. Discussion

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

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

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

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

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

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

410 **What changes can green crabs trigger in local food webs?**

411 Green crabs from newly established populations have shown to be superior foragers to long-
412 established invaders (Rossong et al. 2011). Therefore, we expect that the most dramatic changes in
413 local food webs will occur in the early stages of the invasion until a new equilibrium state with new
414 relative abundances has been reached. However, such a new food web state can bring serious
415 consequences for native populations and even lead to extirpation of some species. The potential
416 impact of green crabs as predators at the study site (rocky shore of Punta Este) can be illustrated by
417 comparing results of an earlier survey that was part of the Census of Marine Life, NAGISA project.
418 In the mentioned study, a total of 35 species of benthic invertebrates and 29 macroalgae were
419 recorded (Rechimont et al. 2013), while in the present study we found 31 benthic invertebrates and
420 23 macroalgae in the stomachs of green crabs. Hidalgo et al. (2007) have observed that green crabs
421 are bigger and feed with greater voracity than native predators on Patagonia rocky shores. They
422 have provoked negative impacts worldwide such as damaging populations of *M. edulis*, American
423 oysters (*Crassostrea virginica*), or rock crabs (*Cancer irroratus*), among others. They have even
424 caused major economic losses for different fisheries (DeGraaf and Tyrrel 2004, Miron et al. 2005,
425 Sigurdsson and Rochette 2013, Leignel et al. 2014, Young and Elliot 2020). In addition, bird mass
426 mortality has been attributed to the transmission of parasites by green crabs (Camphuysen et al.,
427 2002). Coastal birds at Patagonia are already incorporating green crabs to their diet, a fact
428 confirmed by a recent study on the kelp gull (*Larus dominicanus*) that showed green crabs as one of
429 their prey items (Yorio et al. 2020). Carcinophagus fish (e.g. *Mustelus schmitti*) are likely to
430 incorporate green crabs into their diet as well, although this has not been confirmed yet (Belleggia
431 et al. 2012, Alessandra Pasti pers. comm. July 2020). The fact that the nemertean parasite
432 *Carcinonemertes* was observed in one of our samples highlights the possible risk of dispersion of
433 these parasites, which had not been reported before for Argentina (Bigatti and Signorelli 2018), in
434 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.

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477 **Ethical approval**

478 All applicable institutional and/or national guidelines for the care and use of animals were
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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

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

491 **Conflict of Interest**

492 The authors declare that they have no conflict of interest.

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755 (*Carcinus maenas*). *Fishes*, 5(1), 4.

756 **Table 1.** List of prey taxa at green crabs stomach content determinate by metabarcoding and visual
 757 analyzes. Frequency of occurrence (FO%) for metabarcoding (met) and visual inspection (vis) of each
 758 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
						<i>Siphonaria lessoni</i>	-	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.	-	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
		<i>Monocorophium insidiosum</i>	-	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
			<i>Tanais dulongii</i>	-	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	-
			<i>Oithona similis</i>	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
			<i>Pseudechinus magellanicus</i>	-	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
	Asciacea		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	-
		<i>Undaria pinnatifida</i>	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

759 **Figure Legends**

760 **Figure 1.** Frequency of occurrence (FO) boxplots of mayor prey groups in green crab diet from
761 Patagonia, Argentina for: A) visual and B) Metabarcoding analyses. The lower and upper hinges
762 correspond to the first and third quartiles (the 25th and 75th percentiles). Points are outliers (data >
763 $1.5 \times \text{IQR}$) and horizontal lines are medians. Annelida: “Ann”, Arthropoda: “Artr”, Mollusca: “Mol”,
764 Chordata: “Chor”, Echinodermata: “Echin”, Cnidaria: “Cnid”, Porifera: “Por”, Nemertea: “Nem”,
765 Chlorophyta: “Chlo”, Ochrophyta: “Orch”, Rhodophyta: “Rhod”, Unidentified: “Unid”.

766 **Figure 2. A)** Ordination of samples based on COI metabarcoding data (OTUs, NCBI-based
767 assignments), depicted by sex. Distance measure: unweighted Unifrac. **B)** Distribution of OTU
768 richness (number of different OTUs) by sex. Letters "a" and "b" indicate differences between sexes
769 ($p < 0.05$; Kruskal-Wallis rank-sum test). **C)** Phylogenetic tree of OTU-representative COI
770 sequences. The final, curated taxonomic assignment of each OTU is shown at the dendrogram tips.
771 Occurrence by sex is depicted in circles (red: females, black: males). Local support values over
772 1,000 resamplings are shown at the nodes.

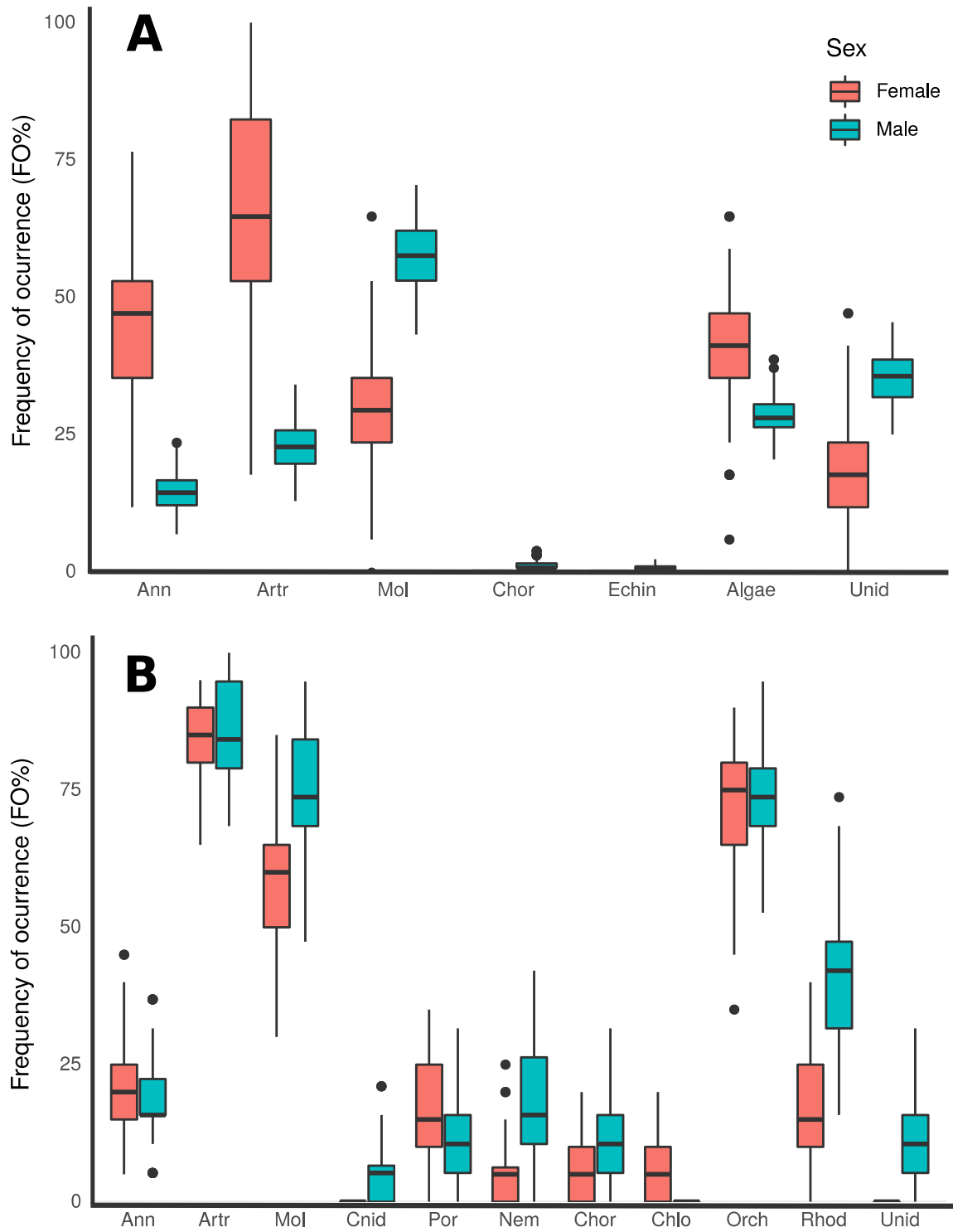
773 **Figure 3. A)** Trophic position (TP) posterior estimates for female and male green crabs **B)** Alpha
774 (α) posterior estimates for female and male green crabs at Nuevo Gulf, Patagonia Argentina. Means
775 are indicated by dotted lines (males: blue, female: pink and overall: black).

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

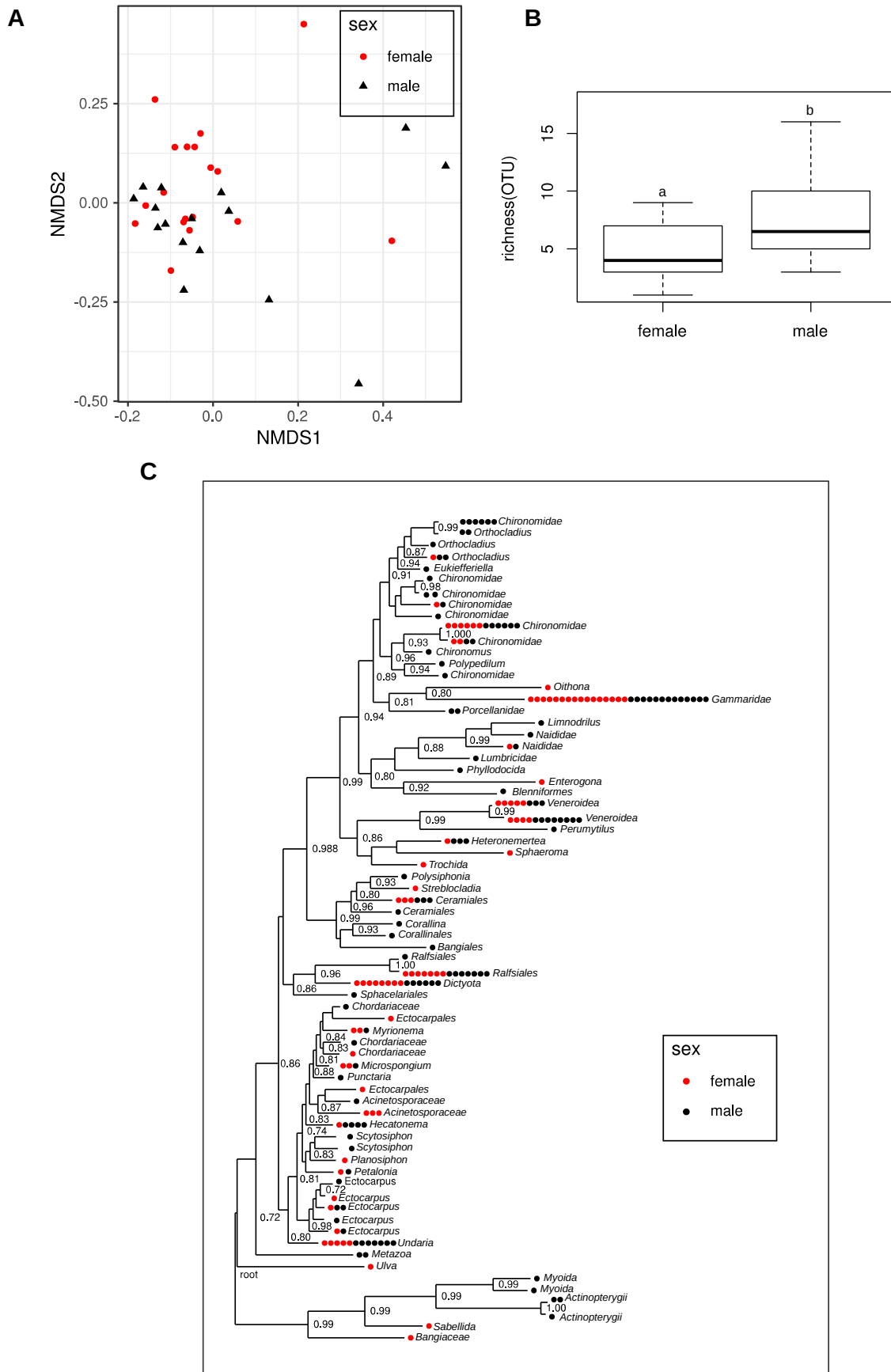
779 **Figure 5.** Diagram representing the different techniques and their contribution to the analysis of
780 trophic ecology (top left). R: Richness, QC: Quantitative Contribution, TL: Trophic Level, TP:
781 Trophic Pathway. Line width represents the relative importance of each technique. The orange
782 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.

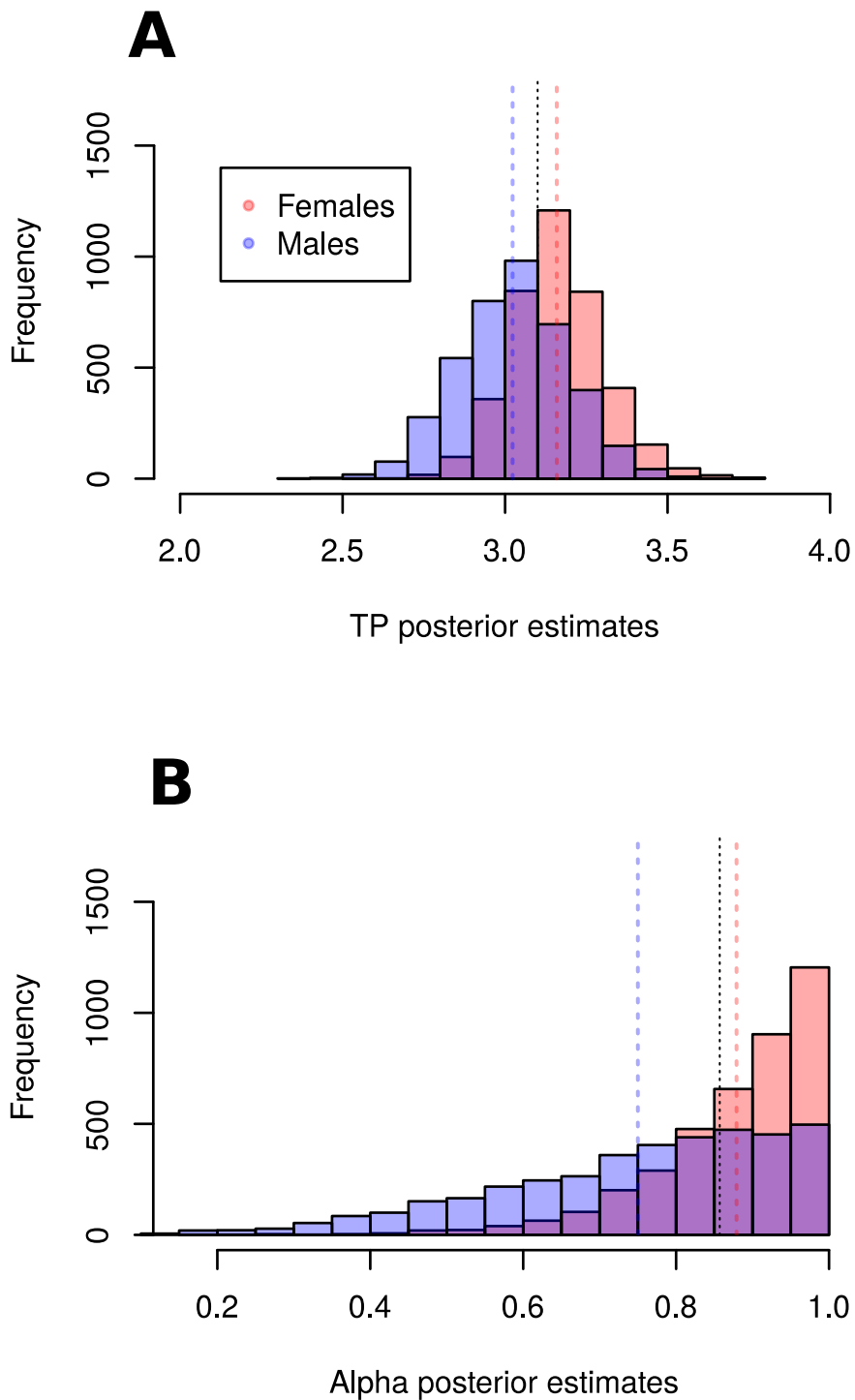
792 **Figure 1**



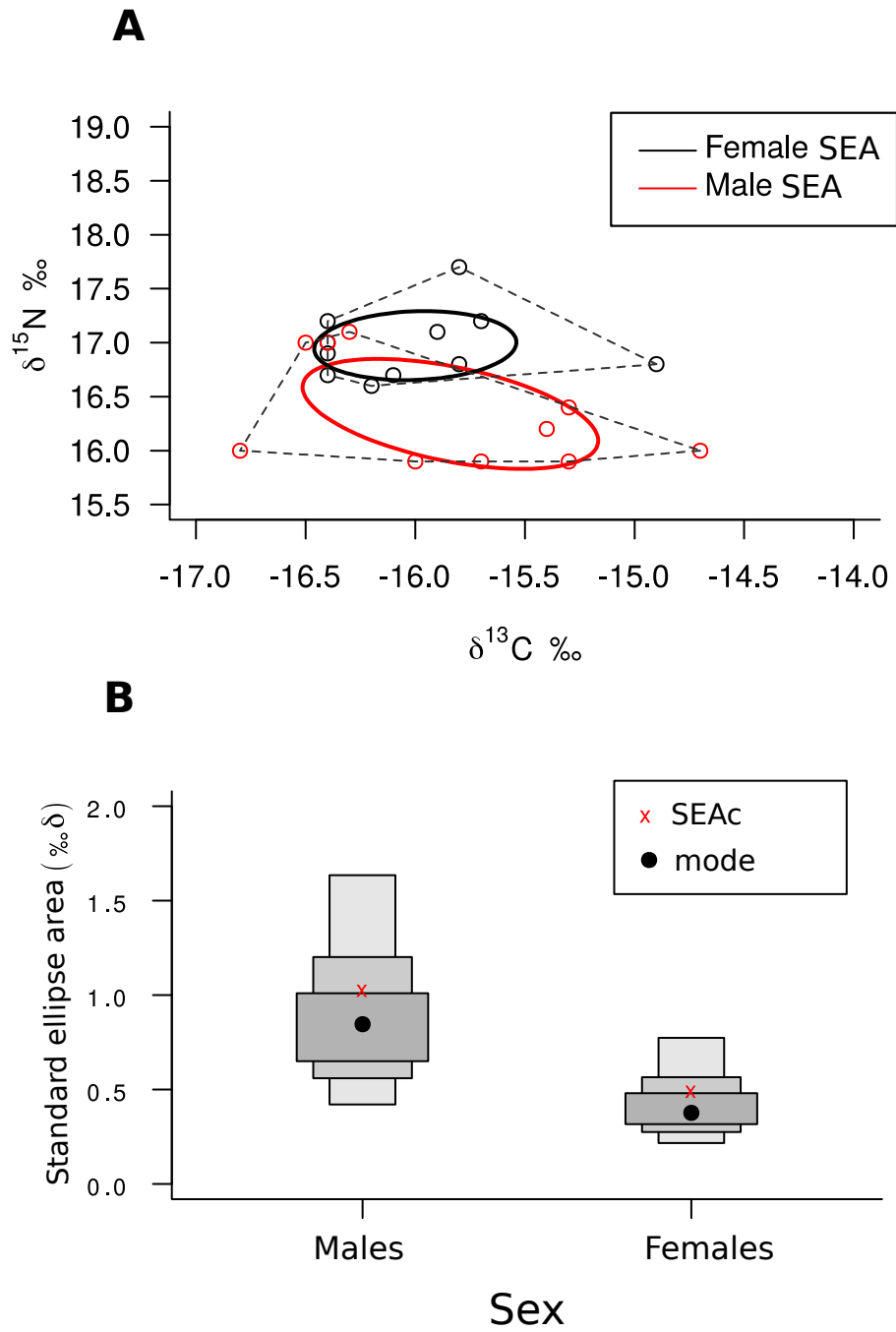
793 **Figure 2**



794 **Figure 3**



795 **Figure 4**



796 **Figure 5**

