31° South: Dietary niche of an arid-zone endemic passerine

Ângela M. Ribeiro[1§], Ben Smit[2,4] and M. Thomas P. Gilbert[1,3]

[1] Natural History Museum of Denmark, University of Copenhagen, Copenhagen, Denmark

[2] Department of Zoology, Nelson Mandel Metropolitan University, Port Elizabeth, South Africa

[3] Norwegian University of Science and Technology, University Museum, Trondheim, Norway

[4] *Current address:* Department of Zoology and Entomology, Rhodes University, Grahamstown, South Africa

[§] E-mail: ribeiro.angela@gmail.com

Running title: Metabarcoding for dietary analysis of a passerine

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ABSTRACT

Balancing energy budgets is thought to be challenging for birds living in arid ecosystems because food supplies are low and unpredictable, and climatic conditions extreme. Thus, to ensure they obtain sufficient energy to fuel daily energetic budgets, birds may need to adjust their diets and become less selective (generalist) as conditions become harsher. To test this hypothesis, we used DNA metabarcoding to characterize both the prey availability (from pitfall traps) and the dietary content (from fecal samples) of several conspecific populations of a semi- and arid-endemic insectivorous bird, the Karoo-scrub-robin (*Cercotrichas coryphaeus*) across a climatic gradient. Our results showed that Coleoptera, Hymenoptera, Orthoptera, and Lepidoptera were the main prey. When accounting for their presence as available prey, Coleoptera and Hymenoptera were preferred in all regions, whereas robins avoided Orthoptera and Lepidoptera in all but the most arid region. Although the different populations live in regions that vary with regards to productivity and thermoregulatory demands, we found that the dietary niche breadth (Bs) of the three populations was intermediate to low, and did not differ significantly. As a whole, our findings show that regardless of environmental harshness these insectivores have similar dietary niches, suggesting that large dietary plasticity is fundamental for their survival in energy-depauperated ecosystems.

Keywords: birds, arid ecosystems, dietary niche, arthropods, DNA-barcoding, high-throughput sequencing

INTRODUCTION

Food acquisition is a basic need of all animals: it provides the energy and nutrients required to sustain life in any given environment. Because of the intricate relationship of animals with the environment (where food is obtained from), there has long been an interest in describing patterns of dietary variation in order to predict the underlying causes. The classical Optimal Foraging Theory model (MacArthur and Pianka 1966) (Levins and MacArthur 1969) (Schoener 1971) predicts that animals seek to maximize energy intake, so as to meet their daily energy costs. Consequently, both prey availability in the environment and animal physiological status can affect diet variation (e.g.: (Burgar et al. 2014).

Endotherms living in landscapes characterized by dramatic change in rainfall and temperature face two main problems: firstly, frequent changes in food webs; and secondly, thermal challenges that require them to adjust their physiology (energetically expensive tasks) to maintain body temperatures within tolerable limits. As a consequence of both, some populations may undergo dietary niche shifts in order to maximize fitness (Roches et al. 2016). In response to limited resources, selection can either lead to generalist strategies such as dietary niche flexibility to use the panoply of resources available (Grant et al. 2008), or drive the evolution of specialization so as to exploit underused resources (Martin and Pfennig 2009). Moreover, if resources vary greatly through time or space, being a generalist may allow fitness to be maintained (Stephens and Krebs 1986).

Arid-zone endemic birds are good models for testing the role of environmental temperature and energetic physiology in dietary strategies, because they face continual challenges from their energy-depauperated ecosystems, while seeking to fulfill the dietary requirements needed to fuel the physiological processes associated with thermoregulation. In this study we molecularly characterized both the diet and available arthropod prey community (Pompanon et al. 2012) (Bohmann et al. 2011) of conspecific populations of the Karoo-scrub robin (*Cercotrichas*

coryphaeus; hereafter: robin), a ground-feeding insectivore (Roberts et al. 2005). This southern African passerine provides a good system with which to explore the avian dietary strategy to ensure adequate energy intake, because its range spans an environmental gradient of primary productivity variation (Fig. 1A). Primary productivity ultimately determines arthropod biomass in the regions, which in turn affects the food available for insectivores (Maclean 1969) (Lloyd 1999). Furthermore, these robins also face dramatic temperature changes, which lead thermoregulatory challenges that the birds must adjust their rates of energy expenditure to (Fig. 1B).

Our objectives were therefore two-fold. Firstly, to determine whether environmental heterogeneity resulted in differential prey availably. And secondly, to assess whether this prey availably and physiological status lead to differences in the birds' niche breadths, a proxy for their dietary flexibility and prey selectivity. In particular, we hypothesized that populations resident in the area with the lowest productivity and highest metabolic expansibility (capacity to increase metabolism so as to deal with any energetic demand, for instance cold), should exhibit the most generalist feeding strategy, as their means for coping with the harshness of the arid-conditions.

2. MATERIAL AND METHODS

2.1 Study sites, arthropods and fecal sample collection

The study was conducted along a gradient of (i) annual temperature amplitude (T_{amp}) and (ii) primary productivity (measured as normalized difference vegetation index – NDV) (Fig. 1). We selected these two metrics with the rationale that: wide ambient temperature range implies increased energy expenditure so as to deal with extreme climatic conditions (cold and hot), and consequent incremental intake of energy sources (Bicudo et al. 2010); and that NDVI is a good predictor of plant biomass in the arid-zone of southern Africa (Borer et al. 2012).

We used the GPS coordinates of our pitfall grid to obtain T_{amp} and NDVI. T_{amp} was estimated using data from WorldClim2 at 2.5min resolution (Fick and Hijmans 2017) as: maximum temperature in December – minimum temperature in July. NDVI data, the surrogate for primary productivity, was extracted from eMODIS dataset hosted by NASA-LPDAAC (USGS, USA). Beside environmental variables, we gathered physiological phenotypic data (Ribeiro et al. *in prep*) for populations inhabiting the same localities. We used these metrics to define three operational regions: *Coastal, Central,* and *Inland.*

We sampled available prey arthropods using 27 pitfall traps left open for seven consecutive days, at each of three locations (Fig. 1) in the summer of 2015 and winter of 2016. We selected pitfall traps because scrub-robins are mostly ground-feeders. The pitfall traps were placed within an area we previously checked to be used by robins, and then set them in three grids. Each grid comprised of nine traps forming a square (3m x 3m), with a distance of one meter between individual traps, and a distance of 100 m between grids. The traps were made from two nested transparent plastic cups of 8 cm diameter, and 250 ml of volume; we partially filled them with a solution of water and soap to reduce superficial tension (Cheli and Corley 2010). We collected the arthropods in the traps on the fourth and seventh (last) day of sampling. All arthropods caught per site were pooled to represent the arthropod community of the region in the given season. Post sampling, arthropods were washed with water and ethanol to remove debris, and stored in 96% ethanol until further use.

In the same above-mentioned periods we collected 49 bird fecal pellets ($N_{Coastal}$= 16, $N_{Central}$= 16, N_{Inland}= 17) using single use filter-paper bags lining cloth bags in which captured birds were placed. Birds were caught using baited flat traps, in place from early morning to dusk in all three regions. Upon collection, the fecal pellets were placed in tubes containing beads and lysis buffer as provided in the Soil/Fecal DNA MiniPrep kit (ZymoResearch, USA), homogenized by vortexing at 2700rpm (45HZ) for 1 min, and stored at 4˚C until further processing in the lab. The

98 sampling periods were selected to avoid the breeding season; in fact, we found no sign of
99 breeding activity while in the field.
100
101 **2.2 DNA extraction**
102 Prior to DNA extraction, arthropods collected from pitfall traps were sorted by size into three
103 categories: small (<1cm), medium (1-3cm) and large (>3cm). The rational was two fold: first,
104 large-sized arthropods could be easily identified morphologically (Appendix Table S1); and
105 second, we wanted to extract DNA from several arthropods at a time (mixed sample), hence it is
106 crucial that the animals in the same pool have similar sizes so not to biases the proportion of
107 DNA in the final volume. Arthropod samples were drained and let dried at 56˚C to remove all
108 ethanol. In order to maintain the arthropod exoskeletons intact, we extracted DNA from
109 arthropod samples following (Gilbert et al. 2007) and (Nielsen 2015). Briefly, this consisted of
110 immersing the arthropods in a digestion buffer with incubation overnight at 56˚C with gentle
111 agitation, after which the digest was purified using the QiaQuick Purification kit (Qiagen, USA).
112 Extracted DNA was stored at -20°C until further use.
113 We homogenized fecal samples prior to DNA extraction using a tissue lyser (Qiagen Tissue
114 Lyser II, USA) for 30s at 30Hz, followed by 30s at 20Hz. DNA extractions used the Soil/Fecal
115 DNA MiniPrep kit (ZymoResearch, USA), with one minor change to the manufacturer's
116 guidelines: after homogenization, we added Proteinase K and incubated the samples at 60 °C for
117 30 min. The DNA extracted was stored at -20°C until further use. We specifically chose this kit
118 because it contains beads in a bead beating tube, a feature that may help crush both the
119 exoskeleton fragments of arthropods and seeds in fecal pellets, hence increase the yield of DNA.
120 Negative controls were included in each extraction to check for potential contamination. These
121 negative controls proceeded in the workflow (from PCR to sequencing) as with all other extracts.
122

123 **2.3 Metabarcoding and sequencing**

124 To maximize detection and identification of arthropods we used two different primers targeting
125 different portions of the COI gene in the mitochondrial genome of arthropods: i) ZBJ-
126 ArtF1c/ZBJ-ArtR2c (Zeale et al. 2011) and, ii) FormiF/FormiR. The latter set was included
127 because the ZBJ-Art primers have been reported to poorly detected ants and termites (Hamad et
128 al. 2014) (Brandon-Mong et al. 2015). Thus we specifically designed this set to ensure detection
129 of ants (Appendix Table S2), by first downloading all arthropod COI sequences from South
130 Africa archived on GenBank in January 2016, then designing them by hand in Geneious 7.0.6
131 (Biomatters, New Zealand) to meet the following criteria: a) 20-22 bp length; b) produce a
132 fragment of 150-210 bp (including primers); and c) C/G as the first two 3' end bases (alignment
133 provided as Appendix).

134 In addition, because robins have been seen eating berries (P. Lloyd, pers. comm.), we targeted the
135 chloroplast rbcL gene using primers rbcL-h1aF/rbcL-h2aR (Poinar et al. 1998). The ZBJ-
136 ArtF1c/ZBJ-ArtR2c primers amplify a 211 bp region in the 5' section of COI gene, while the
137 FormiF/FormiR primers target a 168bp region in the 3' of same gene. All primers were modified
138 to include a unique 6 to 8 nucleotide sequence (nucleotide multiplex identifiers; MIDs) on the 5'
139 end to allow individual identification following (Binladen et al. 2007). MIDs were designed with
140 a custom script using R (R version 3.2.1): we generated random sequences of 6-8 nucleotides,
141 where the probability of each base per site was 0.25, and selected those with pairwise differences
142 >50% as our MIDs.

143 Prior to the metabarcoding PCR amplifications, we compared the efficiency of ZBJ-ArtF1c/ZBJ-
144 ArtR2c and FormiF/FormiR primers, and generated pilot data to help decide the clustering
145 thresholds needed to obtain the most accurate taxonomic richness, by performing PCRs on an
146 artificial positive control. This sample contained a mixture of DNA derived from four species
147 covering a broad taxonomic breadth (1 Coleoptera: Carabidae, 1 Diptera: Sarcophagida, 2

148 Hymenoptera: *Tetramorium sp* and *Camponotus fulvopilosus*).

149 PCR amplifications (25 µl) contained 1-3 µl of template DNA, 25 nM $MgCl_2$ (stock 25nM), PCR
150 buffer (stock 1x), 2 nM each dNTP, 0.6 nM each primer, 0.2U AmpliTaqGold DNA polymerase;
151 thermal cycling conditions for arthropods amplifications (primers Formi and ZBJ) were: 95 °C
152 for 5 min, followed by 33 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s. Thermal
153 cycling conditions for plant amplifications were 95 °C for 5 min, followed by 38 cycles of 95 °C
154 for 30 s, 50.0 °C for 30 s and 72 °C for 30 s. A final extension step of 72 °C for 5 min was
155 included in all reactions. The optimal number of PCR cycles was established using a real-time
156 PCR assay in a subset of samples as the inflection point in the amplification curve and it
157 represents a compromise to obtain sufficient prey DNA while reducing clonality and consequent
158 bias in prey diversity. Real time PCR was performed in 20ul reaction containing 1-2 µl of
159 template, 1x buffer, 2.5 nM MgCl2, 2 nM each dNTP, 0.5U Amplitaq Gold, 1 nM of each
160 primer, and 1 µl SYBR Green/Rox mix (Invitrogen, USA). PCR amplifications were detected by
161 gel electrophoresis on 2% agarose gel stained with Gelred. All PCR preparations used aerosol
162 resistant filter tips, and were prepared in UV-sterilized laminar flow hoods. We note that
163 arthropods and fecal samples were processed independently, and the same applied to summer and
164 winter samples. This differential processing of samples was implemented as to avoid cross-
165 contamination.

166 All PCR products were visualized on a gel to confirm amplification success, and then pooled at
167 an approximately equimolar ratio into 11 pools (5 for summer and 6 for winter). We included the
168 extraction blanks (from both fecal and arthropod pitfall extractions) in the pools, using 5 µl,
169 despite no actual product visualized in agarose.

170 We removed unspecific fragments and primer dimer using Ampure XP SPRI beads, following a
171 double-sided protocol. Shortly, by adjusting the ratio of beads to DNA (0.7x) we first captured
172 the fragments larger than 300bp, keep the supernatant, which was in turn used to sequester

173 fragments larger than 120bp (beads:DNA ratio = 1.8x). The concentration of the purified pools
174 was determined in Qubit and efficiency of purification evaluated in and 2% agarose gel stained
175 with Gelred.
176 The PCR pools were converted into sequencing libraries using the NEBNext 6070 blunt end
177 library build kit following the manufacture's protocol. Index PCRs were carried out in 25 µl
178 reaction volumes, and each library was amplified in three replicates to maximize amplicon
179 diversity. Each indexing PCR consisted of 2.5 µl of library, 1x Gold buffer, 2.5 U AmpliTaqGold
180 DNA polymerase, 2.5 nM MgCl2 and 2 nM each dNTP, 0.2 µM forward indexed primer
181 (InPE1.0), and 0.2 µM reverse index primer such that library contained a different reverse index.
182 PCR was performed at 95˚C for 5min, followed by 8 cycles at 95˚C for 30 sec, 60˚C for 30 sec,
183 72˚C for 30 sec, and final extension at 72˚C for 5 minutes. We combined the three replicates of
184 each library and purified them using the QiaQuick columns (Qiagen, USA). To determine the
185 quality and quantity (concentration) of our 11 libraries, we used the TapeStation System (Agilent
186 Technologies, USA). We equalized the amplified library concentrations, then sequenced them on
187 an Illumina MiSeq platform using PE250 chemistry and spiked with 30% of PhiX.
188 Metabarcoding amplifications were performed in triplicate in order to enable us to: i) discard
189 potential errors (i.e. remove sequences only present in one PCR), and ii) optimize diversity (i.e.
190 keep sequences only if they were present in at least two PCRs). All PCRs included one negative
191 control to check for contamination and one positive control (DNA from an ant or plant species
192 not present in southern Africa) to ascertain amplification. PCR products were incorporated into
193 sequencing libraries (details in Appendix) for generation of metabarcoding data using an Illumina
194 MiSeq platform with PE250 chemistry.
195

196 **2.4 Sequence processing and taxonomic assignment**

197 Post sequencing, the raw reads were processed with TRIMMOMATIC-0.36 (Bolger et al. 2014)

198 to first remove adapters (ILLUMINACLIP module), then bases with low quality. The quality of
199 the surviving reads (mean of surviving reads across pools: 93%) was checked with FASTQC
200 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then the overlapping paired-end
201 reads were merged into contigs using PEAR (Hebert et al. 2003). These contigs were then
202 demultiplexed and filtered with DAMe (Zepeda-Mendoza et al. 2016) to only retain fragments
203 that matched the following criteria: i) present in at least two PCR replicates, ii) conform to the
204 expected size after primer trimming, and iii) present in at least 25 copies (abundance cut-off),
205 thus removing low abundance contigs that could be potential artefacts.
206 Clustering of sequences (within sample) into molecular operational taxonomic units (MOTUs)
207 was carried out with USEARCH (Edgar 2010) and followed the 3% sequence divergence criteria.
208 This clustering criteria was selected taking into account our empirical distribution of pairwise
209 percentage of identity between arthropod sequences (Appendix Fig. 1S) as well as the reported
210 variability in insect populations (Hebert et al. 2003). The MOTUs were aligned to the National
211 Center for Biotechnology Information nucleotide sequence database (NCBI; website:
212 http://www.ncbi.nlm.nih.gov/; accessed July 2017) the software Basic Local Alignment Search
213 Tool (BLAST 2.2.6+; (Camacho et al. 2009)). The high-scoring hits obtained with BLASTn (e-
214 value < 1e-30) were then parsed with MetaGenome Analyzer software (MEGAN v6.0; (Huson et
215 al. 2007)) to assigning the MOTUS to their lowest common ancestor (LCA) in the NCBI
216 taxonomic tree, as to meet the stringency criteria: min score = 130 for COI and 100 for rbcL, max
217 expected = 0.01, min percent identity = 0, top percent = 10, min support percent = 0 (off), min
218 support = 1, min complexity filter = 0.01. The MOTUs for which the LCA taxonomic assignment
219 fell below the thresholds or for which a match was not found in the NCBI database was
220 categorized as "unknown". Not all MOTUs could be assigned to species level due to the
221 incompleteness of the reference database for our study area. Therefore we present results
222 identified to family-level. We contend this still provides a good grasp of the dietary components

223 as well as the arthropod communities.

224

225 **2.5 Statistical Analyses**

226 All analyses were implemented in R v3.3.2 (R Development Core Team). We estimated
227 Shannon-Wiener's (H) and Simpon's (1-D) indices to quantify both consumed and available prey
228 diversity using *vegan* package (Oksanen 2017). Diversity indices were compared among sites
229 using the non-parametric Kruskal-Wallis test, as the data was not normally distributed (Shapiro-
230 Wilk test, $p < 0.05$). Changes in composition of prey were inspected using a non-metric
231 multidimensional scaling (NMDS) with Bray-Curtis dissimilarity. To test whether region
232 (*Coastal, Central, Inland;* proxy for primary productivity and energetic demands) is shaping
233 diversity in available and consumed prey we used a Mantel test (Mantel 1967) with 999 random
234 permutations as implemented in *vegan* (Oksanen 2017). Briefly, we compared the observed
235 dissimilarity matrix (Bray-Curtis dissimilarity) against a conceptual matrix for regional effects,
236 coded as zero for same region comparisons and one for different region comparisons. We
237 highlight that we did not test for seasonal effects. Although we contend that seasonal variation in
238 weather and a bird´s life-stage (breeding vs non-breeding) may have implications relating to
239 available and consumed prey, respectively, and despite our effort to catch birds, the non-balanced
240 sample sizes hinder a formal analysis.

241 Trophic niche breadth was estimated using a standardized Levin's index (Bs; (Levins 1968).
242 Standardized Levin's index (Bs) was estimated as: $Bs=(1/\sum p_i^2)-1/n-1$, where p_i is the proportion
243 of individuals consuming arthropod family *i* and n is the total number of families available. This
244 index measures the uniformity of distribution of individuals (birds) among resource states
245 (arthropod families in each region) and it is closer to zero when diets are dominated by few items
246 (specialist) and closer to one for a generalist diet. We created Bs 95% confidence intervals for
247 each region by bootstrapping with 10,000 replicates. In addition, to test whether the observed Bs

values were significantly different from a scenario of "no regional difference" we used a permutational approach. All occurrences (p*i*) were pooled regardless of their regional origin, then we randomly sampled *n* arthropod families (from the observed range: 37 – 45) and finally estimated Bs_random. This procedure was repeated 9999 times to generate the null distribution. The estimated probability of obtaining a result that exceeded the observed value under the null hypotheses of "no region difference" was estimated as p = [(number Bs_random > Bs_observed)/total number randomizations].

For the most common arthropod MOTUs in the diet of robins, we tested whether selectivity of prey changed among regions, by calculating Ivlev's electivity index (Ivlev 1961) for each region as implemented in *selectapref* package. This index varies from "-1" (avoidance of prey) to "+1" (total reliance on prey), and "0" indicates prey selection is proportional to availability.

2.6 Ethics and Permits

Capture permits were issued by Cape Nature (0056-AAA008-00057) and the Department of Environment and Nature Conservation (1611/2015) in South Africa. Ethical approval was obtained from the Animal Research Ethics Committee at Nelson Mandela University (A15-SCI-ZOO-005).

3. RESULTS

3.1 Arthropod availability

Our survey of potential Arthropoda prey availability across the gradient revealed 519 MOTUs, with the majority (83%) identified to class Insecta (Appendix Table 3S). Coleoptera, Diptera, Hymenoptera and Lepidoptera were the most abundant orders (Fig. 2). The most diverse community was found in the *Central* region, the area with largest annual primary productivity (192 MOTUS, 45 Families, Shannon-Wiener's H = 3.44, Simpson's 1-D = 0.95; Table 1),

273 whereas the in region with lowest NDVI and highest temperature amplitude, *Inland,* the
274 arthropod community was least diverse (157 MOTUs, 42 Families, Shannon-Wiener's H = 3.30,
275 Simpson's 1-D = 0.94; Table 1). However, when these observations were tested statistically, we
276 were unable to reject the null hypothesis that there is no regional effect on arthropod community
277 ($Mantel_{region}$, p = 0.430).
278
279 **3.2 Arthropods and plants consumed**
280 After data filtering criteria, 82% of fecal samples provided arthropod MOTUs (170). Insecta was
281 the richest Arthropoda class (127 MOTUs) with the orders Coleoptera, Hymenoptera,
282 Lepidoptera and Orthoptera being the most abundant (Fig. 2, Appendix Table 3S). Although the
283 Karoo scrub-robin is viewed as insectivorous, we detected plant DNA in 94% of the fecal
284 samples. Overall, 86 MOTUs were assigned to Spermatophyta (seed Plants) with Solanales and
285 Aspargales being the most abundant (Appendix Table 4S).
286 Our data showed no evidence that dietary composition was associated with region whether
287 considering only arthropod prey (Shannon-Wiener's H: Kruskal-Wallis X^2 = 1.855, df = 2, p =
288 0.396; Simpson´s 1-D: Kruskal-Wallis X^2 = 1.885, df = 2, p = 0.390; $Mantel_{region}$, p = 0.072; Fig.
289 3) or combing arthropod prey and plant items (Shannon-Wiener's H: Kruskal-Wallis X^2 = 0.745,
290 df = 2, p = 0.689; Simpson's 1-D: Kruskal-Wallis X^2 = 0.422, df = 2, p = 0.810; $Mantel_{region}$ p =
291 0.433; Fig. 3).
292
293 **3.3. Trophic niche and prey selection**
294 When accounting for available arthropod prey, we found that birds living in the *Inland* region -
295 which was least productive and exerted high energetic demands on the robins – appeared to have
296 the narrowest dietary niche breadth (Bs = 0.372, 95% CI = 0.241 – 0.677, Fig. 3A). This contrasts

297 with the *Coastal* population, whose individuals presented the widest niche (Bs = 0.479, 95% CI =
298 0.328 – 0.667; Fig. 3A). However, again when statistically tested, we found that the observed Bs
299 values for each of the three study regions was not significantly different from the values expected
300 under the null hypothesis of no regional difference (mean Bs_random = 0.445, 95% CI = 0.247 -
301 0.592, $p_{Coastal}$= 0.658, $p_{Central}$ = 0.568, p_{Inland} = 0.738; Appendix Fig. 2S).
302 An electivity analysis (that measures utilization of food items) restricted to the five most
303 abundant potential prey items (Coleoptera, Diptera, Hymenoptera, Lepidoptera and Orthoptera),
304 showed that robins from all regions positively selected for Hymenoptera:Formicidae (Ivlev´s
305 electivity > 0; Fig. 4). Electivity was, however, largest in the *Inland* population, with 55% of
306 sampled birds consuming ants. Although in *Central* region relatively more Coleoptera families
307 were potentially available as prey (Appendix Fig. 3S), robins did not positively selected on this
308 prey type (Fig. 4). We also found that Dipterans, another widely available potential prey source,
309 were negatively selected on in all regions (Ivlev´s electivity < 0; Fig. 4)
310
311 **4. DISCUSSION**
312 Species living in spatially and temporally heterogeneous environments must adjust their
313 behavior and physiology in order to persist. Dietary plasticity may thus be an advantageous
314 trait in energy-depauperated ecosystems as switching from alternative resources allows them to
315 deal with environmental change (e.g.: (Varner and Dearing 2014). Here, we investigated dietary
316 patterns among conspecific Karoo scrub-robin populations living in regions with different
317 productivity and thermoregulatory demands. Overall our findings revealed that: i) although
318 Karoo scrub-robins are generalist insectivores, some plant items seem to be included in its diet;
319 ii) dietary composition was not associated with region; iii) when accounting for available
320 arthropod prey the dietary niche breadth of each population was not different from a scenario of
321 no regional effect (i.e., similar productivity and thermoregulatory demands); iv) Formicidae

322 (ants) were positively selected in the three populations, yet *Inland* robins showed the largest
323 electivity.
324
325 **Dietary niche breadth**
326 The concomitant assessment of available and consumed prey in conspecific populations revealed
327 that the foraging behavior of robins is not simply based on encounter rate: Coleopterans,
328 Hymenopterans and Orthopeterans were preferred prey. While coleopterans represent less than
329 16% of available prey (7%, 16% and 11% of MOUTUs in *Coastal, Central* and *Inland*,
330 respectively) they were clearly preferentially preyed upon (representing 33% of total dietary
331 MOTUs in the *Coastal* region, 23% in the *Central* region and 20% in the *Inland* region). A
332 similar pattern of preference was found for Hymenopterans in the *Central* and *Inland* regions:
333 Hymenopetans were consumed at a higher rate than their relative availability (comprising 11%
334 and 19% of the dietary MOTUs ,*vs* 7% and 11% of available MOTUs). The exception was found
335 in the area with intermediate primary productivity and temperature amplitude – the *Coastal*
336 region – where Hymenopetans were only 4% of available prey and 5% of consumed MOTUs. In
337 contrast, Diptera and Lepidoptera were consumed at a rate inversely proportional to their
338 presence, i.e. they were avoided by the birds. These results are in accordance to prey descriptions
339 based on Karoo scrub-robins stomach contents (invasive method) sampled throughout the year in
340 a region East of our *Inland* site (Free State; (Oatley 1970)). Specifically, Oatley (1970) revealed
341 that ants (Hymenoptera) and beetles (Coleoptera) were present in 55% and 14% of the stomachs,
342 respectively. We also noted that while terrestrial arthropods are the bulk of robin's prey, they
343 appear to supplement their diets with plant matter (94% of samples yielded plant DNA).
344 Although, this finding could in theory simply indicate that robins are plant secondary consumer
345 through herbivore arthropods, we contend the latter is unlikely given both the anticipated
346 degradation to the plant DNA that would be incurred through two rounds of ingestion and

degradation, and perhaps more importantly, two additional lines of evidence. Firstly, there are unpublished observations (P. Lloyd, pers. comm.) of birds foraging on berries and plant shoots, and secondly, seeds and berries were detected in the stomachs contents of robins (Oatley 1970). Thus together, it suggests that that plant matter is not simply a serendipitous food item, but may in fact be selected for.

Our study sites are embedded within the Southern African semi- and arid-regions, where seasonal pulses of rainfall are known to determine arthropod densities (Dean and Milton 1999) and subsequently food supplies for insectivores (Lloyd 1999). Surprisingly, despite the differences in primary productivity among our study sites, the dietary niche breadth (Bs) among populations was not different from a scenario of homogeneous environment (i.e., no difference in productivity or thermal demands). This plasticity in diet may be favorable in environments where prey is patchily distributed in space and time, such as the Southern African semi- and arid-regions (Dean and Milton 1999). Moreover, this finding corroborates the expectations of Optimal Foraging Theory that animals can only afford to be specialist (small niche breadth) where resources are readily available (Stephens and Krebs 1986).

Life in arid environments

Life in arid environments is harsh for endotherms in general, and in particular for ground-feeding insectivore birds as they need to fuel their typically high avian metabolic rates in energy-depauperated ecosystems. We predicted the robins living in the most demanding regions (*Inland*) would exhibit the most generalist feeding strategy, under the rationale that as this was the most energy-depauperated ecosystem and energetically demanding environment, their diet should become more generalist as ignoring potential sources of energy is not an option (Levins and MacArthur 1969; Schoener 1971). Specifically, we expected robins living in the *Inland* region to prey on the greatest diversity of ground arthropods, so as to obtain sufficient energy to fuel the

372 thermogenic demands of winter (burn energy to produce heat as a means to warm-up
373 (thermogenesis; (Hothola 2004) as well as actively cool down in summer through evaporative
374 cooling (Williams and Tieleman 2005). In contrast however, we found that the *Inland* robins prey
375 on an array of arthropods that is similar to robins living in the less demanding region (*Coastal*).
376 We also noted they exhibit a preference for ants (Formicidae), a finding that lends support to the
377 notion that dependable food sources such as ants are critical for arid endemic birds (Dean and
378 Milton 2017), yielding the necessary energy, nutrients and water (robins do not drink) to
379 overcome the challenges that the environment exerts.
380 We also hypothesize that supplementation of their diet with plant items such as berries (the most
381 common plant in diet, Solanaceae, produces berries) may provide the carbohydrates and hence
382 calories required to cover their general energetic budget, when sufficient animal protein and fat is
383 not available. Furthermore beside sugars, fruits would also provide extra water, a critical
384 component when dealing with hot temperatures: birds loose water to cool down and avoid
385 hyperthermia in the hot days. Although we did not test for seasonal differences, it is worth noting
386 that plant DNA was mostly found in samples collected in winter. We hypothesize this may be
387 related to high energy demands to deal with cold as well as short time-window to actively search
388 for arthropod prey on the ground (shorter periods of day light).
389
390 **eDNA metabarcoding in trophic ecology**
391 Investigating foraging strategies by applying molecular methods to environmental and non-
392 invasive samples is increasingly facilitating the study of highly-mobile and evasive animals (e.g.:
393 (Jedlicka et al. 2013) (Mallott et al. 2014) (Jedlicka et al. 2017). Nevertheless, there are several
394 caveats underlying inferences of the dietary components and arthropod communities based on
395 DNA recovered from fecal and environmental samples: i) data is typically qualitative allowing
396 only presence/absence inferences, ii) primer specificity may bias detection of some taxa, iii) the

397 differential digestibility of hard- *vs* soft-bodied prey may bias results, iv) incompleteness of
398 reference databases can hinder species-level taxonomic assignment, v) samples, either fecal or
399 environmental, represent a snapshot of the dietary diversity available and preyed on, and iv)
400 inferences of herbivory may be confounded if animals are actively consuming herbivorous
401 arthropods – secondary consumption. Nevertheless, we contend the dietary patterns obtained in
402 this study are robust and provide a first grasp on how arid endemic birds may be ensuring
403 sufficient energy yields in a rather challenging environment. Thus we hope our findings provide a
404 stepping-stone for further research into the role of plasticity in foraging strategies. Moreover,
405 studies like this one are both relevant for evolutionary biologists because describing the factors
406 that lead to dietary variation in natural populations is fundamental for understanding local
407 adaptation and speciation (e.g.: (Grant et al. 2008; Svanbäck and Schluter 2012), as well as for
408 conservation biologists because the on-going global change is expected to increase aridity and
409 consequently alter food-webs, which in turn affect avian communities (e.g.: (Iknayan and
410 Beissinger 2018).

411 **AUTHOR CONTRIBUTIONS**

412 AMR and MTPG conceived the study. AMR and BS collected samples. AMR performed
413 molecular work and conducted analyses. AMR, BS and MTPG wrote the paper.

414

415 **DATA ARCHIVING**

416 Data reported are available as Appendix.

417 1. Fasta file with Formi primers aligned against Formicidae.

418 2. Fasta files with available and consumed MOTUs obtained with different primer sets.

419

420

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516

517 **TABLE**

518

519 **Table 1.** Available arthropod diversity and dietary diversity (Arthropod and Spermatophyta: seed

520 plants) among populations of Karoo scrub-robin. T_{amp}: annual temperature range. In parenthesis

521 we denote relative arthropod consumption, i.e. % of prey consumed in relation to prey available.

522

	Coastal	**Central**	**Inland**
	NDVI: 0.43	NDVI: 0.50	NDVI: 0.30
	T_{amp}: 21.5 °C	T_{amp}: 27.7 °C	T_{amp}: 32.1 °C
Available Arthropod MOTUs	170	192	157
Available Families	37	45	44
Shannon-Wiener's H	3.109	3.422	3.300
Simpson's 1-D	0.930	0.953	0.943
Consumed Arthropod MOTUs	36 (21%)	57 (30%)	64 (40%)
Consumed Families	20 (54%)	25 (56%)	28 (64%)
Shannon-Wiener's H	2.880	2.706	2.947
Simpson's 1-D	0.936	0.878	0.906
Consumed Spermatophyta MOTUs	21	22	43
Consumed Families	12	14	21
Shannon-Wiener's H	3.404	3.684	3.727

523

FIGURES

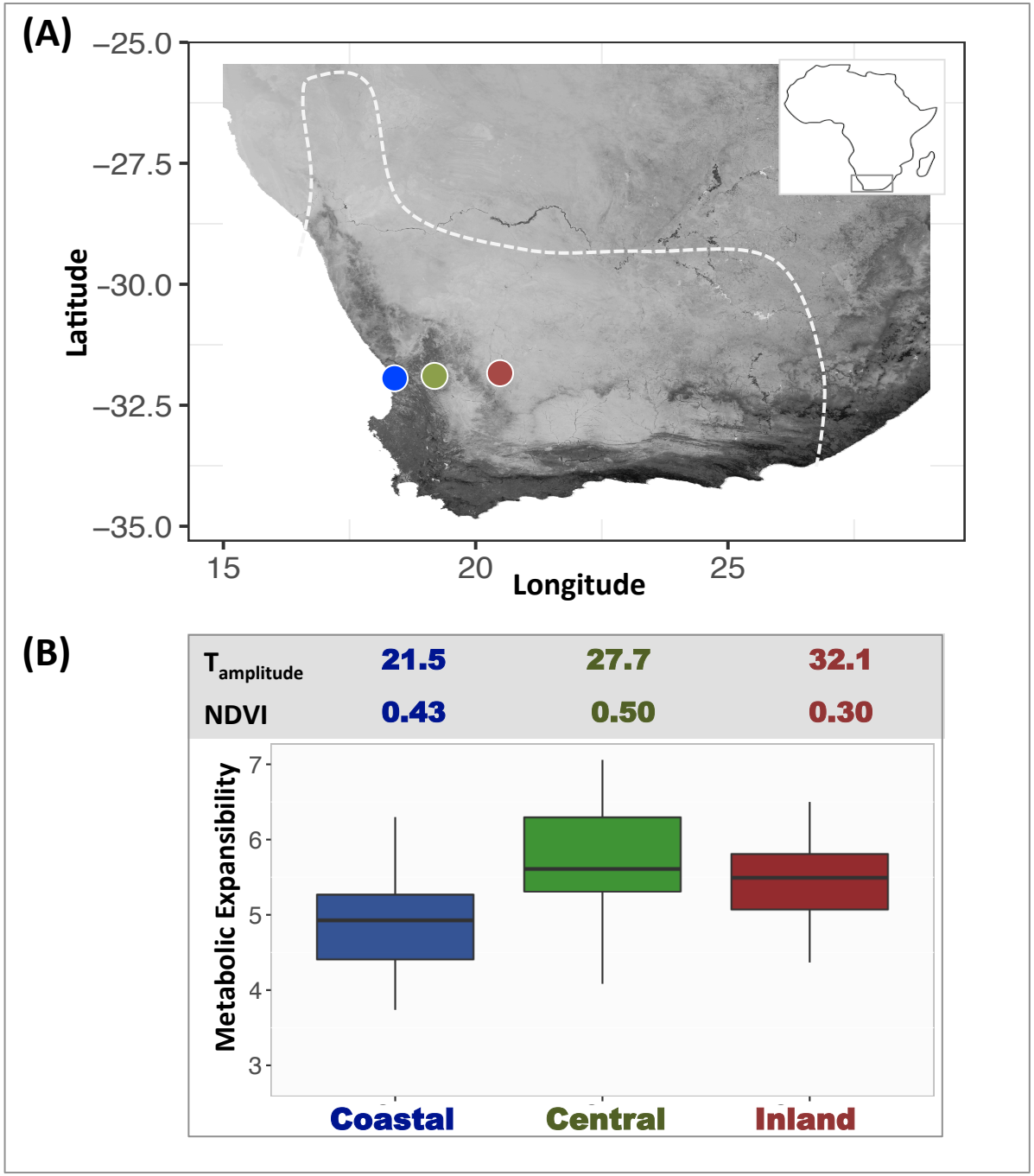
Figure 1. Energetic landscape of the three study sites and populations of Karoo scrub-robin (*Cercotrichas coryphaeus*). (A) Map of annual primary productivity (NDVI; the darker the pixel the larger the productivity) with depiction of the three study sites. Karoo scrub-robin range limit is delimited by a dashed line. (B) Primary productivity (NDVI), Temperature amplitude (ºC) and energetic physiology (metabolic expansibility) for the study populations (from Ribeiro et al unpublished data) three study sites.

Figure 2: Diversity of Arthropod MOTUs per Family in pitfalls and fecal samples at three study locations: *Coastal*, *Central* and *Inland*. Bubble size is proportional to the number of MOTUs found in each family. Arthropod Orders are annotated with initials as a prefix to Family names (e.g.: Ar_Agelenidae): Ar: Araneae, As: Astigmata, Di: Diptera, En: Entomobryomorpha, He: Hemiptera, Hy: Hymenoptera, Is: Isopetra, Od: Odonata, Or: Orthtoptera, Pl: Pleurostigmophora, Po: Polydesmida , Ps: Psocoptera, Sc: Scutigemorpha, Sp: Spiristrepida, Th: Thysanoptera,

Figure 3. Diversity in arthropods potentially available to, and consumed by, the Karoo-scrub robin. (A) Simpson's 1-D diversity index and Levin's Bs niche breadth ($Bs_{Coastal}$ 95% CI = 0.328 – 0.667, $Bs_{centrall}$ 95% CI = 0.285 – 0.659, Bs_{Inland} 95% CI = 0.241 – 0.677). (B) NMDS for arthropod prey and (C) NMDS for plant items consumed; $STRESS_{arthropods}$ = 0.17, $STRESS_{plants}$ = 0.14.

Figure 4. Ivlev's electivity index for the main arthropod orders consumed: Coleoptera (Co) Diptera (Di), Hymenoptera (Hy), Lepidoptera (Le) and Orthoptera (Or). Positive values indicate prey selection whereas negative values indicate prey avoidance.

Figure 1


(A)
−25.0
−27.5
−30.0
−32.5
−35.0
Latitude
15
20
25
Longitude
(B)
T amplitude
21.5
27.7
32.1
NDVI
0.43
0.50
0.30
Metabolic Expansibility
7
6
5
4
3
Coastal
Central
Inland

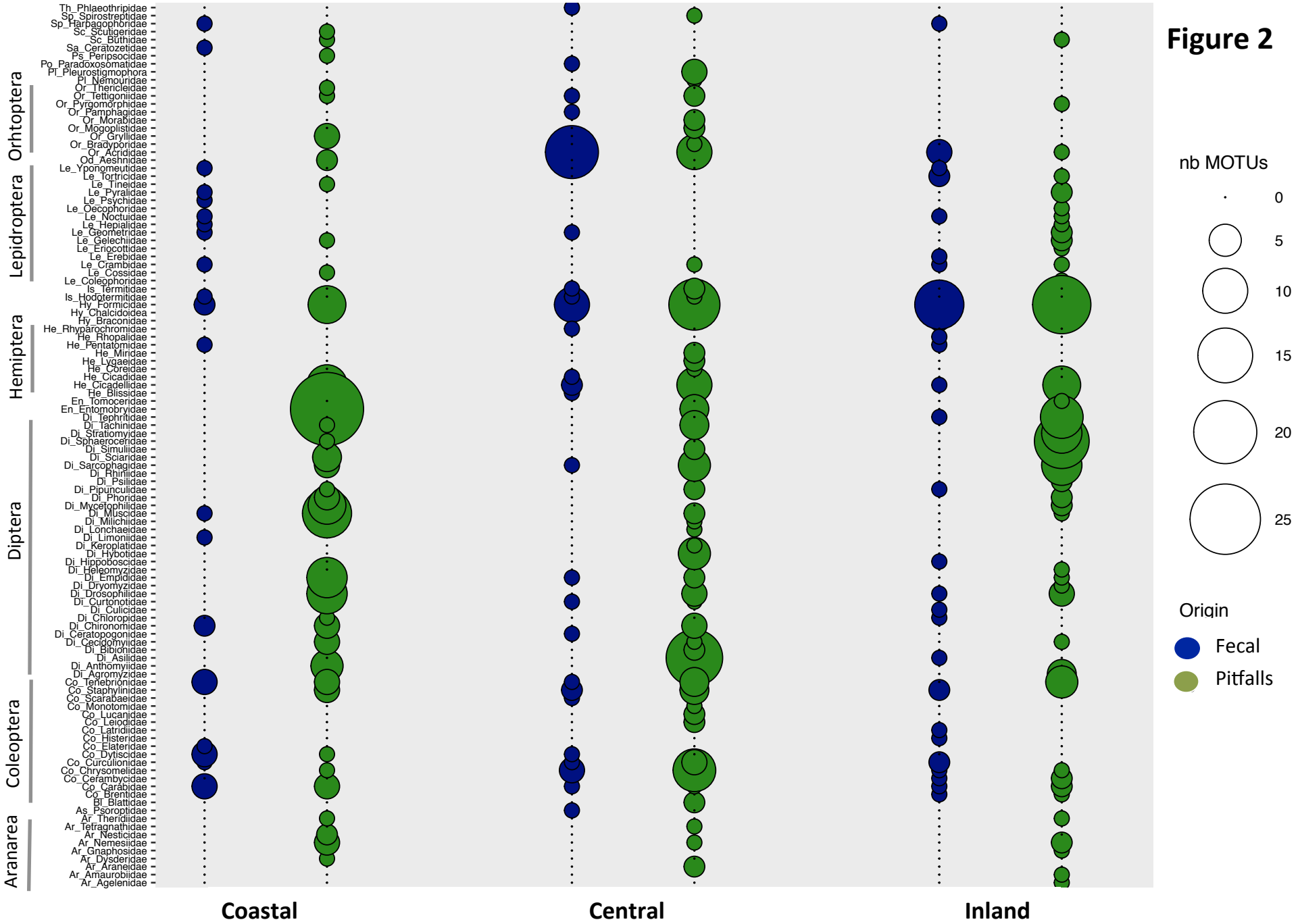


Figure 2
Th_Phlaeothripidae
Sp_Spirostreptidae
Sp_Harpagophoridae
Sc_Scutigeridae
Sc_Buthidae
Sa_Ceratozetidae
Ps_Peripsocidae
Po_Paradoxosomatidae
Pl_Pleurostigmophora
Pl_Nemouridae
Or_Thericleidae
Or_Tettigoniidae
Or_Pyrgomorphidae
Or_Pamphagidae
Or_Morabidae
Or_Mogoplistidae
Or_Gryllidae
Or_Bradyporidae
Or_Acrididae
Od_Aeshnidae
Le_Yponomeutidae
Le_Tortricidae
Le_Tineidae
Le_Pyralidae
Le_Psychidae
Le_Oecophoridae
Le_Noctuidae
Le_Hepialidae
Le_Geometridae
Le_Gelechiidae
Le_Eriocottidae
Le_Erebidae
Le_Crambidae
Le_Cossidae
Le_Coleophoridae
Is_Termitidae
Is_Hodotermitidae
Hy_Formicidae
Hy_Chalcidoidea
Hy_Braconidae
He_Rhyparochromidae
He_Rhopalidae
He_Pentatomidae
He_Miridae
He_Lygaeidae
He_Coreidae
He_Cicadidae
He_Cicadellidae
He_Blissidae
En_Tomoceridae
En_Entomobryidae
Di_Tephritidae
Di_Tachinidae
Di_Stratiomyidae
Di_Sphaeroceridae
Di_Simuliidae
Di_Sciaridae
Di_Sarcophagidae
Di_Rhiniidae
Di_Psilidae
Di_Pipunculidae
Di_Phoridae
Di_Mycetophilidae
Di_Muscidae
Di_Milichiidae
Di_Lonchaeidae
Di_Limoniidae
Di_Keroplatidae
Di_Hybotidae
Di_Hippoboscidae
Di_Heleomyzidae
Di_Empididae
Di_Dryomyzidae
Di_Drosophilidae
Di_Curtonotidae
Di_Culicidae
Di_Chloropidae
Di_Chironomidae
Di_Ceratopogonidae
Di_Cecidomyiidae
Di_Bibionidae
Di_Asilidae
Di_Anthomyiidae
Di_Agromyzidae
Co_Tenebrionidae
Co_Staphylinidae
Co_Scarabaeidae
Co_Monotomidae
Co_Lucanidae
Co_Leiodidae
Co_Latridiidae
Co_Histeridae
Co_Elateridae
Co_Dytiscidae
Co_Curculionidae
Co_Chrysomelidae
Co_Cerambycidae
Co_Carabidae
Co_Brentidae
Bl_Blattidae
As_Psoroptidae
Ar_Theridiidae
Ar_Tetragnathidae
Ar_Nesticidae
Ar_Nemesiidae
Ar_Gnaphosidae
Ar_Dysderidae
Ar_Araneidae
Ar_Amaurobiidae
Ar_Agelenidae
Orhtoptera
Lepidroptera
Hemiptera
Diptera
Coleoptera
Aranarea
Coastal
Central
Inland
nb MOTUs
0
5
10
15
20
25
Origin
Fecal
Pitfalls

Figure 3

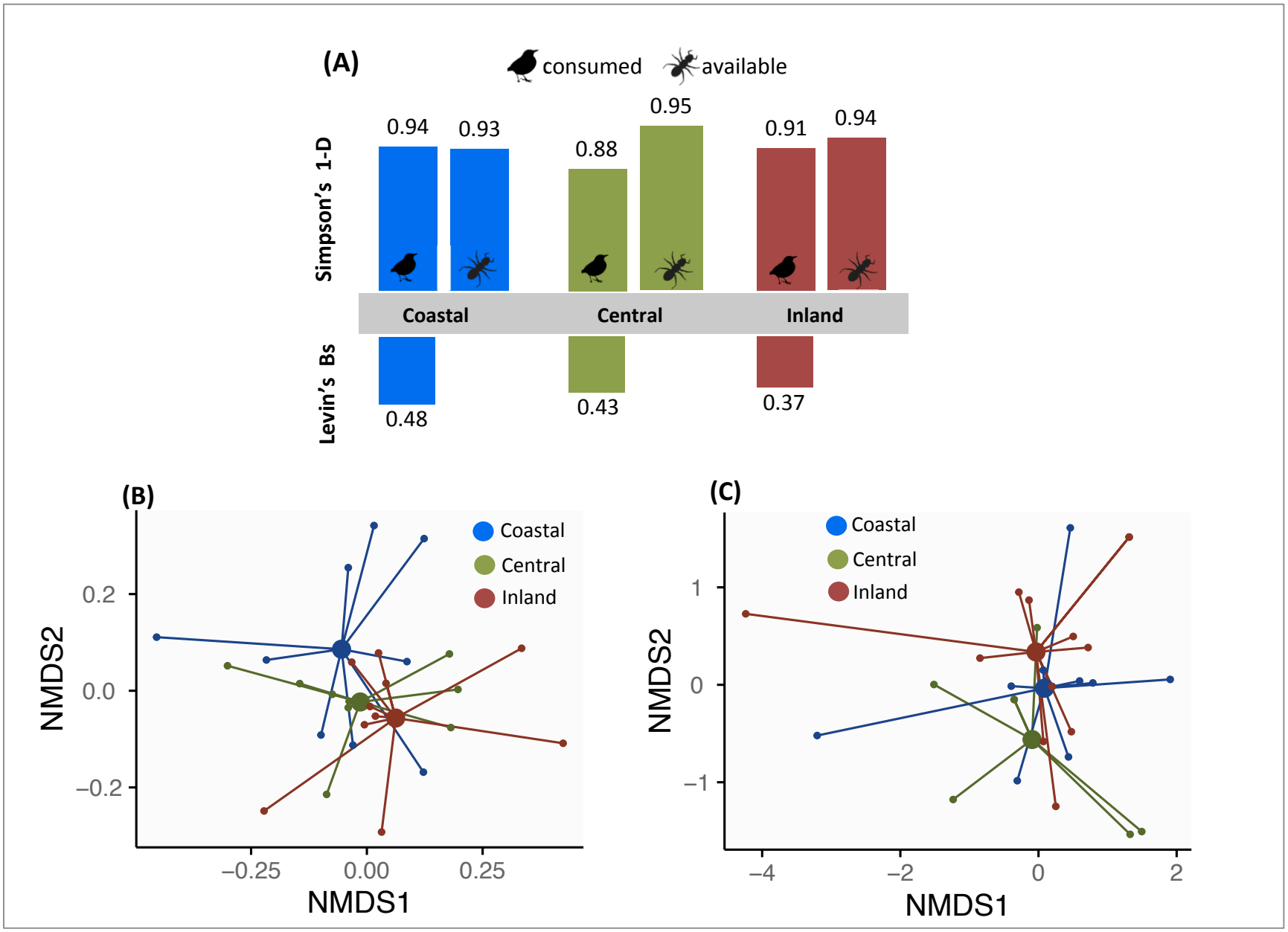
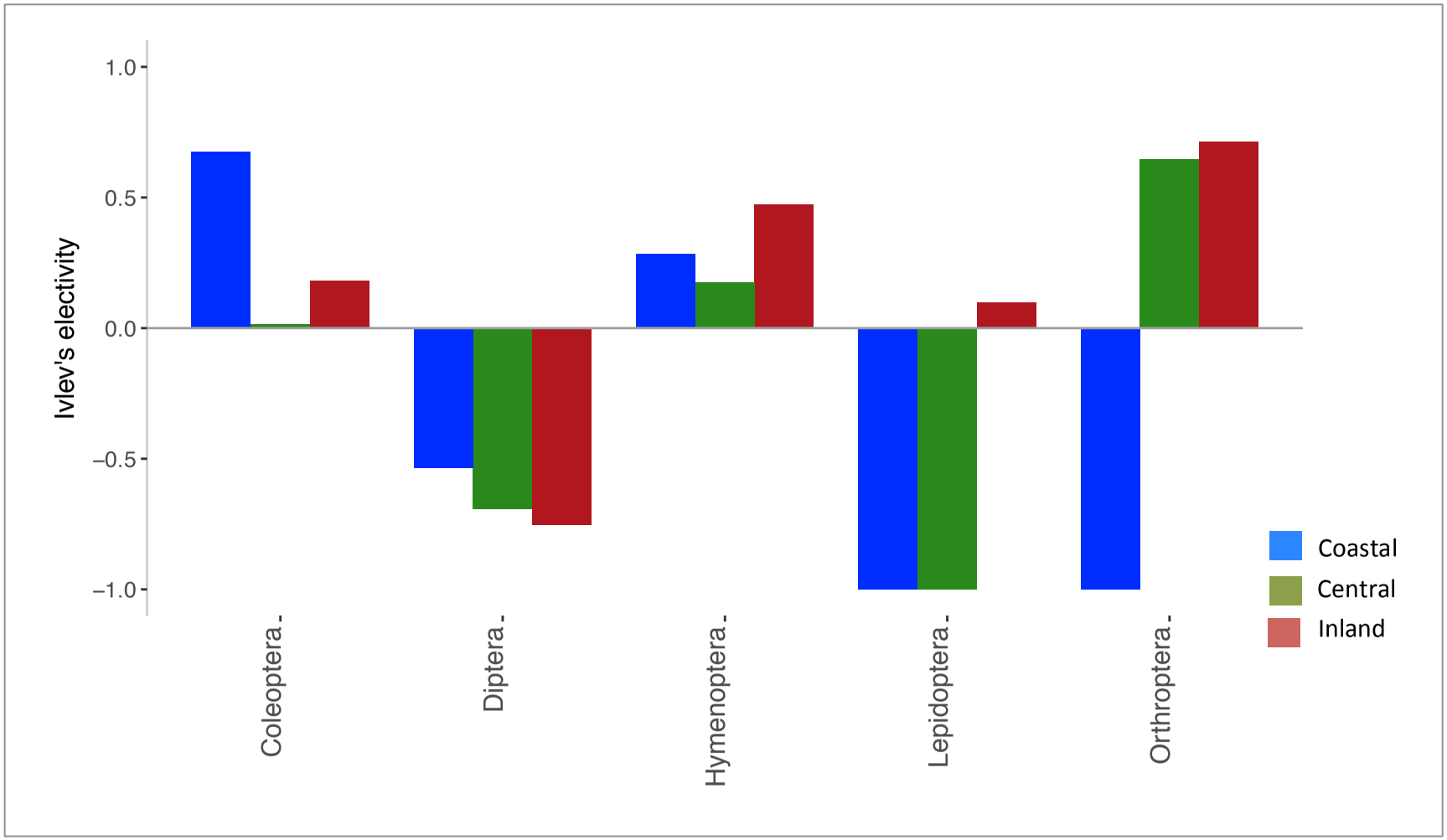

(A)
consumed
available
Simpson's 1-D
0.94
0.93
0.88
0.95
0.91
0.94
Coastal
Central
Inland
Levin's Bs
0.48
0.43
0.37
(B)
Coastal
Central
Inland
NMDS2
0.2
0.0
−0.2
−0.25
0.00
0.25
NMDS1
(C)
Coastal
Central
Inland
NMDS2
1
0
−1
−4
−2
0
2
NMDS1

Figure 4


Ivlev's electivity
1.0
0.5
0.0
−0.5
−1.0
Coleoptera
Diptera
Hymenoptera
Lepidoptera
Orthroptera
Coastal
Central
Inland