

Diet patterns of water green frogs (*Pelophylax esculentus complex*) in mixed population systems in Serbia

KATARINA V. BREKA, MILAN PLEĆAŠ, NIKOLA VESOVIĆ, KATARINA STOJANOVIĆ, BORIS
DUDIĆ AND SRĐAN Ž. STAMENKOVIĆ

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1 **Diet patterns of water green frogs (*Pelophylax esculentus* complex) in mixed**
2 **population systems in Serbia**

3

4 KATARINA V. BREKA^{1,*}, MILAN PLEČAŠ¹, NIKOLA VESOVIĆ¹, KATARINA STOJANOVIĆ¹, BORIS
5 DUDIĆ¹ AND SRĐAN Ž. STAMENKOVIĆ¹

6 ¹ *Institute of Zoology, Faculty of Biology, University of Belgrade, Studentski Trg 16, 11000*
7 *Belgrade, Serbia*

8 *Corresponding author. E-mail: katarina.breka@bio.bg.ac.rs

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12

13 **Abstract.** Population systems in which all three taxa of the *Pelophylax esculentus* complex
14 coexist are rare and in Serbia and can only be found along the Danube. Although several studies
15 describe the diet in populations with only one taxa, there is a lack of data from mixed
16 populations. We studied the diet in mixed populations of edible frogs at three sites for three
17 years. We collected gut contents from 221 adult frogs using the stomach flushing method and
18 identified 1477 prey items. The diet consisted mainly of insects (88%). For all three taxa, the
19 most frequently consumed prey groups were Hymenoptera (28%), Coleoptera (18%),
20 Lepidoptera (17%) and Diptera (11%). Larger prey (in terms of length and volume) was mainly
21 consumed by *P. ridibundus*, followed by *P. esculentus*. The smallest prey were mainly
22 consumed by *P. lessonae*. However, *P. ridibundus* consumed fewer prey items than *P. lessonae*.
23 No significant difference was found between the taxa in the staple diet, while there were
24 differences in the less abundant prey categories, especially between *P. ridibundus* and *P.*
25 *lessonae*. The narrowest trophic niche width was observed in *P. lessonae*, followed by *P.*

26 *esculentus*, and the widest in *P. ridibundus*. Our results suggest that the diet of the three taxa is
27 diverse and consists of a large number of invertebrate groups. However, the diet was locality-
28 specific, with the dominance of different prey groups in different localities depending on habitat
29 characteristics. These results confirm our expectation that the frogs of the *Pelophylax esculentus*
30 complex follow an opportunistic foraging strategy, as predicted by optimal foraging theory.

31

32 **Keywords.** European water frogs, feeding habits, diet composition, prey diversity, Danube,
33 Serbia

34

35 **Running title:** Green frogs' diet patterns in mixed population

36

37

INTRODUCTION

38 Frogs have an important position in the trophic network by regulating the populations of
39 invertebrates and other groups of organisms on which they feed (Hocking and Babbitt, 2014).
40 Larval stages feed on algae and detritus and, as primary consumers, influence ecosystem
41 structure and function by altering algal communities, patterns of primary production, and
42 organic matter dynamics in a variety of freshwater habitats (Kupferberg, 1997; Flecker et al.,
43 1999). Within trophic webs, adult frogs occupy an intermediate position and are involved in
44 aquatic and terrestrial food webs as both prey and predator of various taxa due to their complex
45 life history (McCoy et al. 2009). Frogs are reported as prey for various vertebrates and
46 invertebrates, but also as predators of invertebrates and some vertebrate species (e.g. small
47 lizards, snakes, birds, mammals and other anurans) (Toledo et al. 2007).

48 Green frogs are semi-aquatic ambush ("sit and wait") predators (Moore and Biewener,
49 2015) that feed both during the day and at night (Cogălniceanu et al., 2000). The success of this
50 strategy depends on factors such as prey density, prey mobility, and the energy requirements of

51 the predator (Moore and Biewener, 2015). According to the requirements of optimal foraging
52 theory, animals with such a strategy must be generalists across the spectrum and opportunists
53 in prey selection in order to maximize energy intake per unit time (Pianka, 2000; Glaudas et al.
54 2019).

55 Information about dietary habits and trophic interactions are the key determinants of the
56 structure and dynamics of ecological niches in coexisting species (Lunghi et al. 2022). They
57 are necessary for a basic understanding of the life history of anurans and the fluctuation patterns
58 of their populations (Anderson et al., 1999), for identifying environmental conditions and
59 habitat changes (Batista et al., 2011), and for designing and implementing management and
60 conservation strategies for species that require protection (Stuart et al. 2004). Although some
61 species among anurans are considered specialists (Simon and Toft, 1991), frogs are mainly
62 considered generalists and opportunists (e.g., Çiçek and Mermer, 2007; Almeida-Gomes et al.,
63 2007). In generalist species, aspects such as phylogeny, foraging mode, prey availability and
64 abundance, and morphological constraints to capture and ingest a particular type of prey, may
65 be related to resource partitioning among species (Lima, 1998; Vignoli et al., 2009). In addition,
66 community niche micro-differentiation of both frog taxa and prey may be influenced by
67 anthropogenic activities that can affect ecological interactions (Albrech and Gotelli, 2001).

68 Serbia is populated by all three taxa of the *Pelophylax esculentus* complex: *Pelophylax*
69 *ridibundus* (*P. rid*), *Pelophylax lessonae* (*P. les*), and their hybrid *Pelophylax esculentus* (*P.*
70 *esc*). Throughout its range, mixed populations in which all three taxa of the *P. esculentus*
71 complex coexist in the same locality are generally rare (“REL population systems”, named after
72 the initial letters of the taxa that compose them) (Suriadna et al., 2020). In Serbia, these
73 population systems are detected in a few areas along the Danube (Krizmanić and Ivanović,
74 2010). We believe that REL population systems provide the opportunity to study all three taxa
75 living in syntopy and sharing the same resources. Although they are likely to have similar

76 ecological needs, they should have measurable differences in resource use that reduce the
77 possibility and/or extent of competition between taxa and thus promote coexistence (Costa-
78 Pereira et al., 2019). Prior to the clarification of the taxonomic status of green frogs (Berger,
79 1973) and the advent of molecular identification techniques, field identification of taxa in this
80 complex was difficult (see Breka et al., 2020). We note that most trophic studies treated green
81 frogs as a single unit without considering possible dietary differentiation within the complex
82 (e.g. Löw and Török 1998; Cogălniceanu et al., 2000). Trophic studies on green frogs have
83 mostly been reported for populations with a single taxon (e.g. Šimić et al., 1992; Mollov, 2008;
84 Sas et al, 2009), while only a few have reported on diet in mixed populations where at least one
85 parental species coexists with the hybrid taxon - LE or RE population systems (Sas et al., 2007;
86 Ferenti et al., 2009), and a few on diet in REL systems (Popović et al., 1992; Paunović et al.,
87 2010; Karaica et al., 2016). More recently, field studies on green frogs have been conducted on
88 population systems based on inter- taxon differences, especially when considering implications
89 for effective conservation and management of altered aquatic and wetland habitats (Joško and
90 Pabijan, 2020).

91 The aim of the present study was to (1) provide the necessary data for an overview of
92 the diet composition of the *P. esculentus* complex in Serbia in REL population systems; (2)
93 determine the dietary pattern and niche breadth for each taxon at three sites with different
94 habitat quality and anthropogenic pressure; (3) determine whether there are niche shifts in the
95 composition of the staple diet and/or dietary partitioning between three taxa in syntopic
96 population systems of green frogs.

97

98

MATERIALS AND METHODS

99

Study area

100 Frogs were collected in three consecutive years (2016 - 2018) at three sites in the South Banat
101 district, Serbia (Fig. 1). The sampling sites were selected according to the confirmed presence
102 of the REL system (Krizmanić and Ivanović, 2010) and represent typical habitats for water
103 frogs, but with different levels of preserved natural features and anthropogenic activities and
104 pressures: 1) Stevanove ravnice (STR) (44°49'57.8"N 21°18'33.1"E) is an alluvial plain of the
105 Danube within the special nature reserve "Deliblatska peščara" (Deliblato Sands). Here,
106 underground waters form semi-permanent pools along the sandy plains. During high water or
107 floods, these pools merge into larger water bodies that are connected to the Danube. The pools
108 are overgrown with helophytes, which form extensive reed belts. The area is traditionally used
109 as pastureland, but has retained its characteristic structure and composition of a mosaic
110 landscape characterised by an alternation of steppe and hygrophite vegetation depending on
111 the microtopography and water level. The frogs were collected in the wet meadows between
112 the ponds. 2) The Danube-Tisa-Danube Canal (DTD) (44°51'14.4"N 21°18'17.8"E) is an
113 artificial, man-made structure in which natural aquatic vegetation only grows in a very narrow
114 belt along the shoreline. Frogs have been collected along the canal in the macrophyte beds and
115 the canal embankment. The embankment is a popular picnic, recreation, camping and fishing
116 spot for locals and tourists with planned management activities. 3) Jaruga (JR) is a canal on the
117 edge of the outstanding natural landscape area "Karaš-Nera" near the village of Kusić
118 (44°52'30.8"N 21°28'16.0"E). It was built to regulate the flooding of the Nera River and to
119 ameliorate the surrounding agricultural land, but it has lost its main purpose and is no longer
120 used. The canal is surrounded on one side by a mosaic of agricultural fields and orchards and
121 on the other by floodplain vegetation and abandoned gravel pits. When the Nera is at low water,
122 which is the case for several months of the year, the canal has a low water flow. This allows the
123 formation of dense macrophyte beds, which consist mainly of underwater plants. Frogs were

124 collected within the canal where access was possible and in the floodplain meadows along the
125 canal.

126

127 *Data collection*

128 Frogs were sampled at night after the breeding season from May to October using dip nets, and
129 then transported to a field laboratory for further processing. Frogs were identified to taxon
130 according to Breka et al. (2020). Individuals with a snout-vent length < 55 mm (*P. ridibundus*)
131 or < 45 mm (*P. esculentus*, *P. lessonae*) were considered juveniles and not included in further
132 analyses (Mikulíček et al., 2014).

133 The prey samples were extracted from the stomach contents using the stomach flushing
134 method (Solé and Rödder, 2010), which allows to obtain residuals of the last foraging activity
135 without sacrificing the individual. Due to its ability to digest food very quickly, the stomach of
136 each frog was flushed immediately (within one hour) after capture to minimise the time between
137 frog capture and prey extraction. Stomach contents were stored individually and preserved in
138 vials containing 70% ethanol. Individual prey items were identified to the lowest taxonomic
139 level based on their digestive status. After stomach flushing, all frogs were released near their
140 capture site and checked to ensure they behaved normally.

141 The identification of prey taxa was carried out at the Institute of Zoology, University of
142 Belgrade - Faculty of Biology, Serbia, using published key references (Nilsson, 1996; Nartshuk,
143 2003; Horsák et al., 2013; Brock, 2017; Araneae, version 03.2022) and adopting the current
144 nomenclature (Fauna Europaea version 2.4, 2011). The length and width of intact prey were
145 measured using a digital calliper, while the lengths of incomplete prey were estimated using
146 known proportional ratios of body parts (Chinery, 1993). Prey volume was estimated from the
147 volume of a prolate spheroid (Quiroga et al., 2009). We considered stomach contents as a

148 random sample of food selected by an individual over an unspecified period of time prior to
149 capture.

150

151 *Data analysis*

152 All identified prey were categorised into 17 broader categories ("prey categories"). They were
153 assigned to categories defined by their ecomorphological characteristics to maximise clarity of
154 biological interpretation and variation in diet composition (Vignoli et al., 2009).

155 To identify differences in diet composition between taxa and localities, we used a non-
156 parametric similarity analysis (ANOSIM using Euclidean distance with N=9999 permutations)
157 with pairwise comparisons based on a step-down sequential Bonferroni procedure. Variations
158 in diet were compared between taxa and localities using a repeated measure permutational
159 multivariate analysis of variance (PERMANOVA, with N=9999 permutations of the Euclidean
160 distance matrix). SIMPER analysis was used to identify the specific prey group contributing
161 most to the observed differences and the possible existence of niche shifts. All tests were
162 performed using the software programme PAST (Paleontological Statistics Software
163 Programme, Ver. 4.13 Hammer et al., 2001).

164 For further analysis, an explanatory matrix was created in which each frog was assigned
165 to a specific combination of locality and taxon (loc_tax): *STREsc*, *STRRid*, *STRLes*, *DTDEsc*,
166 *DTDRid*, *DTDLes*, *JRGEsc*, *JRGRid* and *JRGLes*.

167 The evenness and richness of prey categories were estimated using the Simpson index
168 (D') and Simpson dominance index (D), which are used in studies of niche differentiation as
169 indices of specialised measures of niche width and can provide insights into details of trophic
170 ecology (Krebs, 2014). All indices are given according to the notation in Hammer et al. (2001).
171 The analysis was performed in the diversity module of PAST, with the options "unbiased" and
172 "use ACE for S" selected. The ACE richness estimator corrects for the rare occurrence of food

173 items and, in our case, the effects of prey sample size (see: Gotelli and Colwell 2011, Hammer
174 et al., 2001). The standardised Levin's Index (B_A), as proposed by Hurlbert (1978), was used
175 to calculate niche width values for each loc_tax combination using Ecological Methodology
176 software, version 7.4 (Kenney and Krebs, 2003).

177 The available data were further analysed using multivariate statistical analysis. Following
178 the suggestion of Leps and Smilauer (2003), we chose linear redundancy analysis (RDA), a
179 multivariate direct gradient analysis (ter Braak and Prentice, 1988). It can be seen as a
180 constrained form of PCA in which the ordination of the variables of interest is constrained by
181 linear combinations of external explanatory variables. This allows us to assess how much of the
182 variation in the structure of a multivariate data set (e.g. species composition, in our case diet
183 composition) is explained (i.e. predicted) *a priori* by one or more independent external variables
184 (Legendre and Legendre, 1998). We chose this procedure because we opted for a method that
185 allows explicit prediction of food composition determination rather than exploratory post hoc
186 interpretation. The resulting RDA ordination plot is presented as a triplot with the following
187 symbology: 1) prey categories are shown as arrows (17 elements); 2) the three frog taxa and
188 three sampling sites are shown as symbols (six in total); and 3) additionally, the centroids of
189 the individual frogs sampled at each site are shown (nine in total). In this representation, the
190 distance between symbols approximates the average dissimilarity of prey composition as
191 measured by their Euclidean distances. These distances can be considered proportional to their
192 trophic overlap. The scaling of loadings and scores (prey categories and frog taxa, localities and
193 individual frogs in our case) focused on standardised prey category scores and a scaling that
194 preserves the relative distances between cases. The prey category arrows point in the direction
195 of the steepest increase in prey category abundance in the samples. The length of a diet category
196 arrow together with the angle with respect to an axis also indicates the relative contribution of
197 that category to the axes shown in the triplot as well as the dominance and contribution of each

198 diet category to the extracted gradients. The angle between the arrows indicates the approximate
199 correlation between the dietary categories: The approximate correlation is positive when the
200 angle is sharp and negative when the angle is more than 90 degrees. The projected orthogonal
201 distance of the loc_tax symbols on the arrows of the dietary categories indicates the relative
202 importance of this category in this sample. Ordination analysis was performed with CANOCO
203 5.15 (ter Braak and Smilauer, 2002). The significance of the two canonical axes was tested
204 using a permutation test. Additional post-hoc tests (t-test with XLSTAT, ver. 3.1, 2021) were
205 performed for the taxa and site assessment scores to confirm the trends observed by visual
206 interpretation of the resulting ordinations. For all analyses, $p < 0.05$ was set as the significance
207 level.

208

209

RESULTS

210 We captured 317 adult green frogs and obtained diet samples from 221 frogs. The breakdown
211 of samples by location and frog taxa is given in Table 1. Empty stomachs were present in 78
212 frogs (24.6%) and were excluded from further analysis. The taxon with the highest percentage
213 of empty stomachs was *P. ridibundus* (30%), while this percentage was slightly lower in the
214 other two taxa (18%). The site with the highest percentage of empty stomachs was STR (31%),
215 while DTD and JRG had a lower percentage of empty stomachs (18% and 14%, respectively).
216 Plant material (small leaves, seeds, lentils) was identified in 9 stomachs (3%) and was not
217 included in further analysis as we considered it as unintended prey items. A further 9 stomachs
218 (all *P. esc.*) contained unidentifiable prey items. In total, 1477 prey items were identified. The
219 frogs' diet consisted mainly of insects (88%), while the remaining 12% consisted of other small
220 invertebrates – spiders, crustaceans, molluscs (snails) and vertebrates. Insect larvae made up
221 20% of the total prey. In all three frog taxa, the most frequently observed prey groups were
222 typical ground-dwelling terrestrial invertebrates, e.g. ants, ground beetles, weevils, caterpillars

223 and spiders (50%), followed by aerial groups (e.g. midges, flying ants and leafhoppers, 36%)
224 and aquatic prey (6.5%).

225 The prey categories were as follows: Gastropoda (Gas.), Clitellata (Cli.), Arachnida
226 (Ara.), Myriapoda (Myr.), Malacostraca (Mal.), Ephemeroptera (Eph.), Odonata (Odo.),
227 Orthoptera (Ort.), Hemiptera (Hem.), Hymenoptera (Hym.), Coleoptera (Col.), Coleoptera
228 larvae(Col_1), Lepidoptera (Lep.), Lepidoptera larvae(Lep_1), Diptera (Dip.), Diptera
229 larvae(Dip_1) and Vertebrata (Ver.). The diet of all three frog taxa was dominated by insect
230 orders Hymenoptera (28%), Coleoptera (18%), Lepidoptera (17%) and Diptera (11%). Other
231 groups were represented with proportions below 10%. Rank-abundance curves of the 17
232 selected prey categories are shown in Fig. 2. Prey categories that were represented with a
233 frequency of more than 10% in the whole sample were considered as staple diet prey -
234 Hymenoptera, Coleoptera and Lepidoptera larvae. Those represented with a frequency of 5-
235 10% were considered common prey - Diptera, Arachnida, Hemiptera. Rare and accessory prey
236 groups were represented by the eleven remaining categories (less than 5%), e.g. snails,
237 springtails, annelids and centipedes, and vertebrates (small fish, froglets, smaller voles). In *P.*
238 *lessonae*, most prey items belonged to the dominant prey groups - Hymenoptera, Coleoptera
239 and Lepidoptera larvae, while rare food items were present in small proportions. In contrast to
240 *P. lessonae*, all 17 categories of prey were present in *P. ridibundus*. In addition to the
241 predominant prey categories, *P. ridibundus* also consumed prey from other diet categories, e.g.
242 Hemiptera, Gastropoda, Orthoptera, Odonata and Coleoptera larvae. The hybrid taxon also
243 consumed all 17 prey categories, and as the largest number of frogs analysed were identified as
244 *P. esculentus*, this influenced the pattern of the overall rank abundance curve. Rare prey
245 categories were present in the diet of the hybrid taxon, but in lower proportions than in *P.*
246 *ridibundus*.

247 The rank abundance curves for all three sites are shown in Fig. 3. The site STR was the
248 only one where all 17 prey categories were present. Compared to the rank abundance curves of
249 the total sample, there are some important differences: the most dominant prey categories were
250 different, while some prey categories that are considered staple diet were hardly present (e.g.
251 Lep_1. in the locality STR, < 2%). At the JRG and DTD sites, rare and accessory prey categories
252 were represented by less than 2%. We note that a slightly larger proportion of Orthoptera and
253 Lepidoptera were present in the DTD locality (11%) and Vertebrata in the STR locality (3%)
254 in relation to the total sample.

255 Overall, individuals from the *P. esculentus* complex consumed 6.75 ± 0.58 (mean \pm SE)
256 prey items with an average length of 10.39 ± 0.23 mm and a volume of 830.86 ± 62.76 mm³.
257 The largest average prey length and volume were found in *P. ridibundus* (12.24 ± 0.65 mm and
258 1515.58 ± 257.1 mm³, respectively) and the smallest in *P. lessonae* (9.07 ± 0.58 mm and 533.31
259 ± 91.62 mm³, respectively). However, *P. ridibundus* consumed fewer individual prey items
260 (4.97 ± 5.81), while *P. lessonae* consumed smaller prey items per stomach in greater numbers
261 (7.43 ± 11.14). This is consistent with the body size pattern of these species. The breakdown of
262 basic statistics and prey measurements in the total sample by taxon is shown in Table 2.

263 ANOSIM identified a weak but significant difference in diet between localities ($R =$
264 0.1 , $p < 0.01$), but not between taxa ($R = -0.03$, $p = 0.72$) (Fig. 4). Subsequent pairwise
265 comparisons showed that each locality differed significantly from all others (Table S1). In
266 addition, SIMPER analysis showed that the overall dissimilarity between localities was 85.74%
267 and 3 of the total 17 prey categories (Coleoptera, Hymenoptera and Lepidoptera larvae) yielded
268 more than 50.65% of cumulative dissimilarity (Table S2). PERMANOVA analysis supported
269 these results, finding a significant effect of locality ($F = 58.44$, $p < 0.01$) but not between taxa
270 ($F = 0.68$, $p = 0.65$) and the interaction between these two factors ($F = 0.94$, $p = 0.45$).

271 The estimates of prey diversity and niche width for the nine loc_taxa groups are shown
272 in Table 3. Among the sites, the highest number of prey categories is documented at site STR
273 (S = 17), followed by DTD (S = 16), while the site with the lowest number of prey categories
274 is JRG (S = 15). In general, the lowest diversity of frog prey was found in frogs at site JRG,
275 which was accompanied by correspondingly higher values of dominance.

276 In terms of trophic niche width, the widest Levins-standardised niche width for all sites
277 was found in *P. ridibundus* ($B_A = 0.489$), while the narrowest niche width ($B_A = 0.186$) was
278 found in the second parental species *P. lessonae* at all three sites. The trophic niche width values
279 for the hybrid taxon *P. esculentus* were between the values of the parental species ($B_A = 0.365$).
280 The observed differences in the standardised Levin's measure of niche width between loc_tax
281 were not statistically significant.

282 The RDA triplot is shown in Fig. 5. The first RDA axis explains 4.16% of the variance
283 in Euclidean distance, and the proportion increases to 5.38% when the second axis is added.
284 About 50% of the unconstrained ordination is explained by the constrained axes, while the first
285 two axes of the unconstrained ordination were able to extract 17% of the variance in individual
286 frog diet composition. As observed, frog taxa and sites are arranged such that the first RDA
287 axis separates the JRG site from DTD and STR (two-tailed t-test of ordination values: $t_{219} =$
288 73.25 , $p < 0.01$). The second RDA axis separates two parental taxa, with *P. ridibundus* being
289 positive and *P. lessonae* being negative (two-tailed t-test of ordination scores: $t_{57} = -6.31$, $p <$
290 0.01). The hybrid taxon lies approximately in the middle of the ordination. Moreover, the
291 second axis separates the DTD locality from the other two localities (two-tailed t-test of
292 ordination values: $t_{219} = 26.37$, $p < 0.01$). The RDA with the two extracted gradients was
293 statistically significant ($F = 3.4$, $p < 0.01$).

294

295

DISCUSSION

296 The frogs of the *P. esculentus* complex were found to feed on a different invertebrates,
297 especially ground-dwelling arthropods and aerial insect groups. Aquatic prey made up only a
298 small proportion of the total prey, suggesting that the green frogs feed mainly on land or wait
299 terrestrial prey to come close to the water (“sit and wait” strategy). According to our data,
300 Hymenoptera, Coleoptera and Lepidoptera predominate in the diet of all three frog taxa of the
301 complex. A comparison of our results with the only published data on the diet of the green frog
302 in a REL system from Serbia, reported by Paunović et al. (2010), showed a similar dietary
303 spectrum, albeit with differences in the proportions of prey. In their study, the dominant prey
304 groups were Coleoptera, Hymenoptera and Gastropoda (each with a frequency of > 30%). In
305 our study Gastropoda were more abundant in the diet only in the STR locality (5%), a marshy
306 locality with similar characteristics to the locality in Paunović et al. (2010). Insect larvae were
307 less present in Paunović et al. (2010) (11%) than in our study (20%). In both studies, no
308 vertebrate prey was observed in *P. lessonae*, while in the other two species the proportion of
309 vertebrate prey was low (1 – 3%). Our results also agree with those of Karaica et al. (2016)
310 from REL population systems in the north-western part of Croatia, where the predominant prey
311 groups were Coleoptera, Diptera and Hymenoptera. In both studies, a higher proportion of
312 aquatic prey was observed in *P. ridibundus* than in the other two taxa. In contrast to our results,
313 no vertebrate prey was observed, and insect larvae were present in a much lower proportion
314 (1%). The diet composition of the frogs of the *P. esculentus* complex from Serbia was similar
315 to that in populations of *P. ridibundus* in Turkey (Çiçek and Mermer, 2006; Çiçek and Mermer,
316 2007), Bulgaria (Mollov, 2006; Mollov et al., 2010) and Russia (Ruchin and Ryzhov, 2002),
317 and to that in a mixed LE population system in Romania (Sas et al., 2007).

318 According to studies on the diet of green frogs, arthropods accounted for 90.1-97.3% of
319 the total prey in different population systems (Cogălniceanu et al., 2000; Ruchin and Ryzhov,
320 2002; Çiçek and Mermer, 2007; Rakojević et al., 2022), which is consistent with the results of

321 our study (92.4%). Most previous studies show that green frogs feed predominantly on
322 terrestrial prey (e.g. Çiçek and Mermer, 2007; Karaica et al., 2016; Rakojević et al., 2022),
323 which is consistent with our results (93.46%), with the largest proportion of aquatic prey found
324 in *P. ridibundus* (15.46%) and the smallest in *P. lessonae* (1.90%). However, according to
325 Ruchin and Ryzhov (2002), who based their results on a single taxon population (*P.*
326 *ridibundus*), the frogs consumed aquatic rather than terrestrial prey. This phenomenon is
327 generally not confirmed in other studies. Adult forms of invertebrates dominated the diet of
328 green frogs in our sample (79.83%), which is consistent with previous studies (e.g. Çiçek and
329 Mermer, 2007; Paunović et al., 2010). This suggests that members of the *P. esculentus* complex
330 generally seize more active prey, as adult invertebrates are generally more active than their
331 larvae. All three taxa had an almost equal proportion of larvae in their diet (about 20%),
332 although interestingly a high dominance of caterpillars (mainly moth larvae of the family
333 Geometridae) was recorded in the JRG locality (as much as 51.57% of the total prey, while in
334 other localities this percentage was much lower 10.44% in STR and only 3.9% in DTD). The
335 abundance of caterpillars in the diet of green frogs is probably not the result of their preference,
336 but the result of the abundance of caterpillars at the particular site, especially in samples
337 collected in late summer/early autumn. Although this result is not the subject of this article, it
338 could indicate a possible phenological effect specific to that site. However, this hypothesis
339 needs to be further confirmed.

340 Vertebrates were “on the menu” of *P. esculentus* and *P. ridibundus* at all three sites,
341 with the largest proportion found at STR. The most numerous vertebrate prey were newly-
342 metamorphosed frogs from the *P. esculentus* complex (besides small fish and voles). The STR
343 site with the highest percentage of cannibalism (3%) was also the site with the highest
344 percentage of empty stomachs. Intraspecific predation has been previously documented in
345 green frogs as a mechanism that enhances the survival of the individual under certain conditions

346 such as drought, lower food availability, etc. (Crump, 1992; Çiçek and Mermer, 2007). Since
347 in our study vertebrate prey was mainly present in autumn, the possibility of a phenological
348 effect of prey preference cannot be completely excluded. However, we consider it more likely
349 that an explanation for the observed phenomena is related to a relative prey deficit associated
350 with season and/or location.

351 The diet of all three green frog taxa is dominated by the same prey categories, which
352 means that there is no difference in the staple diet between the taxa. However, in the diet of the
353 parental species there is a difference in terms of rare and accessory (marginal) prey, especially
354 those that are present in the diet of *P. ridibundus*, while they are almost absent in the diet of *P.*
355 *lessonae*. In the diet of the hybrid taxon, most of these prey categories were present, but in
356 smaller or negligible proportions. These marginal prey categories are mostly represented by
357 larger prey such as Odonata, Gastropoda and Orthoptera and various species of vertebrates.
358 This could be due to the fact that *P. ridibundus* frogs are larger and can therefore consume
359 larger prey, whereas *P. lessonae* frogs fulfil their nutritional requirements with smaller prey,
360 but in larger numbers (Löw and Török, 1998). This could also indicate a tendency of smaller
361 green frog taxa to concentrate on fewer prey categories when foraging and would explain the
362 dominance of only three prey categories in the diet of *P. lessonae*. Each of the dominant prey
363 categories was most common in different locations. These differences in the dominance of prey
364 at all three sites can be explained by differences in the type and configuration of the habitats.
365 As the STR and JRG sites are floodplains under the strong influence of the surrounding rivers
366 (Danube and Nera), the presence of hydrophilic and aerial categories was to be expected. The
367 orchards surrounding the JRG site may also have had an influence on the high dominance of
368 moth larvae. At the DTD site, a deforested artificial embankment planted with clover,
369 influenced the dominance of epigeobiont species.

370 The RDA showed a high variance in the diet of the individual frogs. This is not an
371 unexpected result, as frogs are known to be opportunistic feeders, from which we conclude that
372 there should be a large heterogeneity in diet between individual frogs. The RDA revealed a
373 differentiation between the localities according to the dominance of certain prey categories. In
374 the JRG locality, for example, Lepidoptera larvae had the largest contribution, in the DTD
375 Coleoptera and Orthoptera stood out, while in the STR locality only Vertebrata and
376 Hymenoptera had relevant loadings. A very high dominance of a single prey category (e.g.
377 Lepidoptera larvae in JRG) indicates a lower diversity at this site, which is confirmed by the
378 values of the diversity indices. At STR, on the other hand, the arrows of the prey categories
379 were about the same length, which, together with the values of the diversity indices, indicates
380 higher prey diversity. The "swarm" of arrows pointing to a sampling site is an indicator of the
381 diversity of the food composition of that sample and consequently may indicate higher trophic
382 production/availability at that site. According to the results of the multivariate analyses, the frog
383 taxa are well separated both in ordination and at specific sites that differ in diet. For each
384 locality, the position of each frog score corresponds to the position of the respective taxon on
385 the second axis. The position of the centroids of the hybrid taxon was always close to the
386 locality score, whereas the centroids of the parental species were always above (*P. ridibundus*)
387 or below (*P. lessonae*) the locality score, reflecting their relative position on the secondary
388 gradient. We note that the ordination of taxa on the secondary gradient corresponds to the
389 ordination of the localities on the same gradient (*P. ridibundus* and the DTD locality have higher
390 positive scores on the second axis, *P. lessonae* and the STR and JRG have negative scores on
391 this axis). Furthermore, *P. lessonae* had the lowest values for prey diversity (as well as the
392 highest dominance of certain prey categories) and Levins' standardised niche breadth. We
393 conclude that *P. lessonae* has the narrowest trophic niche among the three taxa, which
394 contradicts previous studies in mixed population systems where the hybrid taxon had the

395 narrowest niche (Paunović et al., 2010; Karaica et al., 2016). Trophic diversity for the other two
396 taxa was approximately the same at all three sites, with the lowest values at the JRG site and
397 the highest at the STR site. The positions of their centroids on the RDA plot support this result.
398 The direct gradient analysis presented in this study provides further insight into these patterns.
399 Since the gradient extraction in RDA is hierarchical, we can interpret the two gradients as
400 follows: 1) the first, dominant gradient, predicting site position, is an environmental gradient
401 related to habitat quality, defining the differential trophic availability of potential food
402 components specific to a site through differences in secondary production; 2) the second,
403 subdominant gradient predicting species position is a taxon-specific gradient separating
404 parental species from hybrids. It probably follows the characteristics of their life form and
405 differences, especially between parental species - different details of foraging strategy, size-
406 related prey acquisition, etc.

407 The success of the "sit-and-wait" predation strategy depends, among other conditions
408 (high mobility of prey and low energy requirements of the predator), on a fairly high prey
409 density (Moore and Biewener, 2015). A high proportion of certain taxa in the diet could
410 therefore also be due to the fact that green frogs, as "sit-and-wait" predators, compensate for
411 the rarity or low specific mass of the prey taken by the number of individuals taken (Löv and
412 Török, 1998). This would also suggest that prey items are acquired in proportion to their
413 dominance in the immediate environment. We hypothesise that differences in the prey
414 composition of all three taxa in the study area are influenced by differences in the local
415 availability of prey groups, possibly resulting from differences in habitats and their secondary
416 production. A review of the diet composition of the *P. esculentus* complex across its range
417 revealed that some dominant taxonomic prey groups were consistent, but other prey categories
418 varied in abundance. This suggests that the diet of frogs of the *P. esculentus* complex is
419 determined by prey availability and habitat characteristics rather than active selection.

420 Consequently, niche shifts among localities and variations in prey composition are to be
421 expected, as green frogs from the *P. esculentus* complex can be characterised as non-selective
422 predators that show a generalist feeding habit and consume prey according to their body size
423 and other morphological features such as the mouth gap (Mollov, 2008).

424 In summary, the diet of all three taxa of the *P. esculentus* complex is diverse but
425 regionally specific. There is no difference in the staple diet between the taxa of the complex,
426 but in the less frequent and rare prey categories, especially in the parental species *P. ridibundus*
427 and *P. lessonae*. Understanding feeding habits and trophic ecology is of particular importance
428 for syntopic and marginal populations of the complex and for understanding the details of co-
429 occurrence in human-modified landscapes. This is particularly important for *P. lessonae*, one
430 of the parental species, as the southern Banat represents its southern geographical boundary.
431 Although *P. lessonae* is classified as a Least Concern Species (LC) by the International Union
432 for Conservation of Nature and populations are declining worldwide (Kuzmin et al., 2009), it
433 can be considered threatened at the regional level as it is exposed to constant and increasing
434 environmental threats throughout its range, which increase the risk of local extinction (Sjögren-
435 Gulve, 1994; Vukov et al., 2015).

436

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SUPPLEMENTARY MATERIAL

447 Supplementary material associated with this article can be found at <[http://www-](http://www-9.unipv.it/webshi/appendix/index.html)

448 [9.unipv.it/webshi/appendix/index.html](http://www-9.unipv.it/webshi/appendix/index.html)> manuscript number 13529

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accepted manuscript

601

TABLES

602 **Table 1.** Breakdown of the total number of frogs caught by taxon and location (abbreviations

603 are given in the text)

Locality/Taxon	<i>P. rid</i>	<i>P. les</i>	<i>P. esc</i>	Total
STR	46	11	129	186
DTD	11	9	53	73
JRG	6	9	43	58
Total	63	29	225	317

604

605 **Table 2.** Basic statistics of green frogs and their stomach contents by taxon

	<i>P. rid</i>	<i>P. les</i>	<i>P. esc</i>
	(<i>n</i> = 63)	(<i>n</i> = 29)	(<i>n</i> = 225)
Average body size of frogs (mm)	75.8 ± 1.32	69.06 ± 1.48	72.62 ± 0.71
Average number of consumed prey items per stomach	4.97 ± 5.81	7.43 ± 11.14	6.95 ± 8.70
Average length of the consumed prey items (mm)	12.24 ± 0.65	9.07 ± 0.58	10.26 ± 0.26
Average volume of the consumed prey items (mm ³)	1515.58 ± 257.1	533.31 ± 91.62	757.27 ± 68.1

606

607 **Table 3.** Summary of frogs' diet diversity indices and niche breadth

Loc_tax	STRRid	DTDRid	JRGRid	STRLes	DTDLes	JRGLes	STREsc	DTDEsc	JRGEsc
Number of prey categories (S)	16	11	9	7	11	8	17	14	15
Prey abundance in sample (n)	99	43	52	73	40	48	537	277	308
Simpson Index (D')	0.86	0.83	0.69	0.40	0.76	0.64	0.81	0.80	0.71
Simpson's dominance index (D)	0.15	0.17	0.31	0.60	0.24	0.36	0.19	0.20	0.29
Levins' standardized measure of trophic niche breadth (B_A)	0.3	0.37	0.14	0.04	0.2	0.11	0.28	0.25	0.15

608

609

FIGURE LEGENDS

610 **Fig. 1.** Map of the South Banat district (dotted border) in northern Serbia showing three sites

611 where green frogs (*P. esculentus* complex) were sampled: STR (orange circle), DTD (pink

612 square) and JRG (green triangle).

613 **Fig. 2.** Rank abundance curve for 17 selected prey categories (abbreviations are given in the

614 text) for three taxa of green frogs. The order of the food categories for the individual species

615 corresponds to their order in the total sample.

616 **Fig. 3.** Rank abundance curve for three localities (abbreviations are given in the text).

617 **Fig. 4.** Box whisker plot of the ANOSIM analysis comparing the diet of frogs at three

618 different localities. Boxes indicates values from 25th (bottom) to 75th (top) percentile;

619 horizontal black line indicates the median.

620 **Fig. 5.** Redundancy analysis (RDA) triplot of standardised taxon/locality scores derived from

621 correlation matrices of 17 prey categories analysed (abbreviations as in text). The scaling

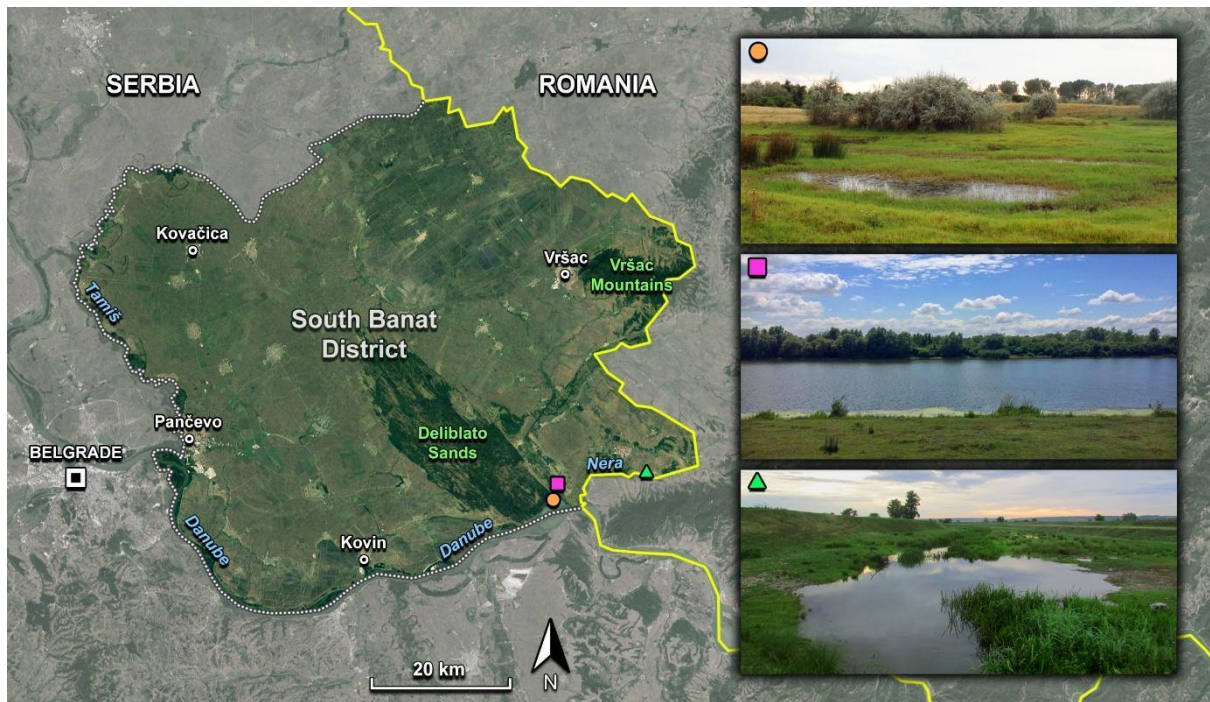
622 preserves the distances between the loc_tax combinations, the angles between the vectors

623 indicate the correlation, the length of the arrows the dominance.

624

625

Fig. 1

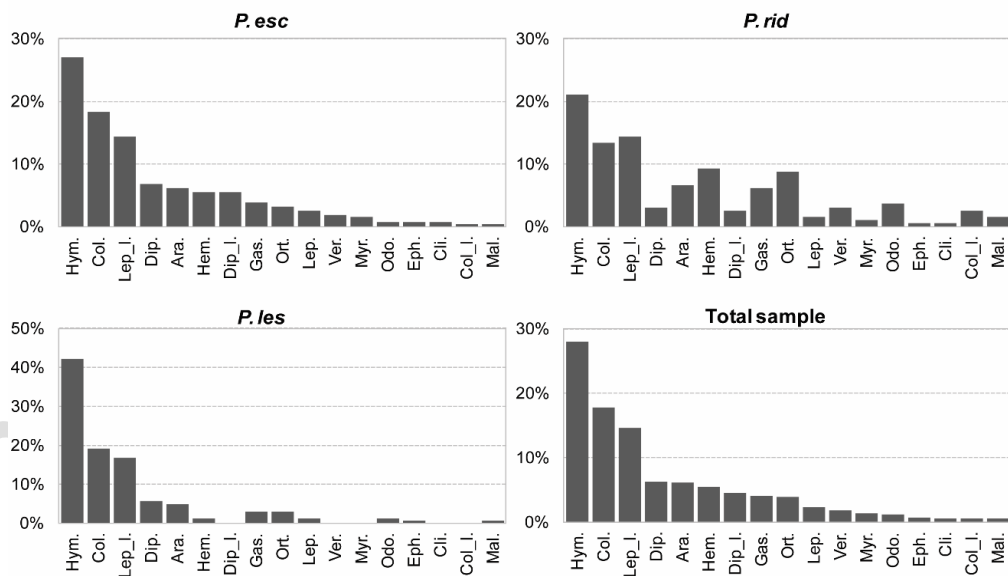


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Fig. 2

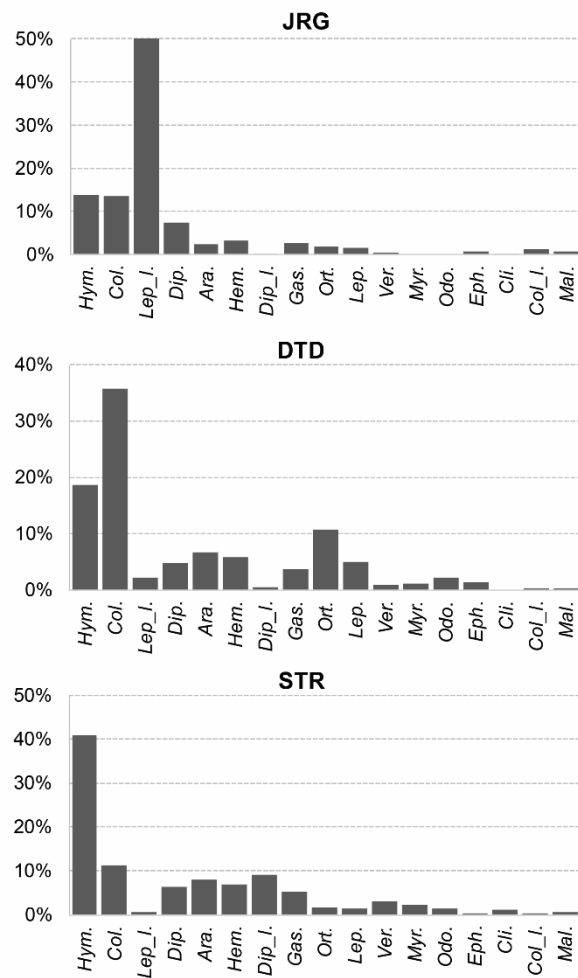


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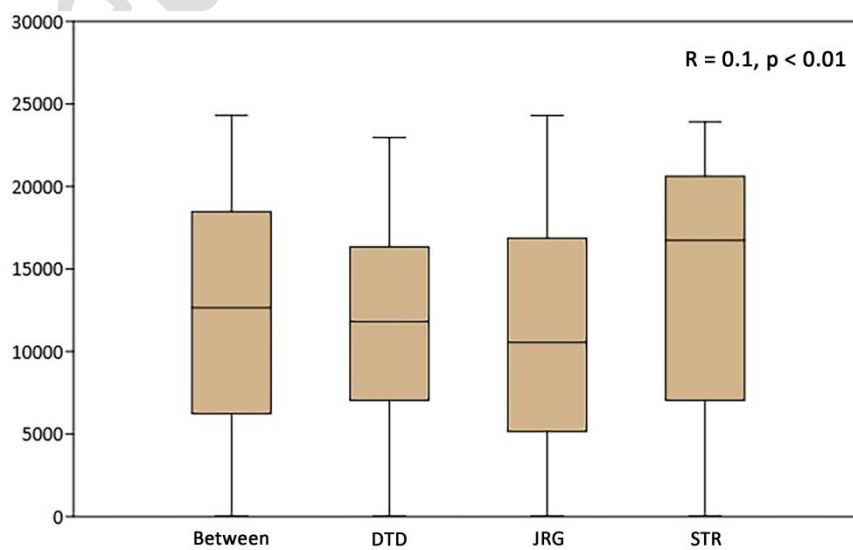
632 **Fig. 3**



633

634

635 **Fig. 4**



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