

1 **Drivers of phenotypic variation along a Late Pleistocene range**  
2 **expansion route**

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8 **ABSTRACT**

9 Understanding how interindividual variation within populations drives the evolution of biodiversity patterns  
10 is a major challenge in ecology and evolutionary biology. By reshuffling species distribution in space and time,  
11 historical biogeographic processes have dramatically affected the structure of biodiversity. While the genetic  
12 legacy left by these historical processes within populations has been widely investigated, their effects on  
13 phenotypic diversity remain relatively unexplored. Here, we investigate whether dispersal-driven processes of  
14 historical biogeographic relevance, such as late Pleistocene range dynamics, have contributed to shape the  
15 geographic patterns of phenotypic trait variation. We focus on dispersal-related personality, morphological  
16 and performance traits in the Tyrrhenian tree frog, *Hyla sarda*, which underwent a northward range expansion  
17 from the Sardinia Island to the Corsica Island during the Last Glacial Maximum, when a temporary land-bridge  
18 connected these islands. We collected tree frogs from four geographic areas along the past expansion route,  
19 controlling for altitude, local habitat, demographic factors, and bioclimatic differences between geographic  
20 areas. Then, we scored variation in two personality traits, two performance traits, along with morphological  
21 traits likely involved in the dispersal process. Tree frogs from the northern area in Corsica were more prudent  
22 in a novel environment, they had significantly larger body sizes, longer limbs, wider heads, and displayed  
23 stronger take-off and adhesion performances compared to individuals from the source area in Sardinia. The  
24 results of our study suggest a non-random spatial sorting of the intraspecific variation in multiple phenotypic  
25 traits along the range expansion route. They also suggest that population differentiation in phenotypic traits  
26 associations might be a legacy of past biogeographic dynamics, identifying a potential driver of the current  
27 phenotypic architecture of animal populations.

28 **LAY SUMMARY**

29 Climatic oscillations of the Pleistocene have had dramatic impacts on species range dynamics and the structure  
30 of genetic diversity within them. However, did they also contribute to mould interindividual phenotypic  
31 variation? To help answer this question, in this study we examined spatial patterns of variation in personality,  
32 performance, and morphological traits along the Late Pleistocene range expansion route of the Tyrrhenian tree  
33 frog, *Hyla sarda*. We focused on these traits as they have been recently implicated in contemporary dispersal-  
34 driven processes, such as biological invasions. Our results show that individuals from newly established  
35 populations are shyer, take longer to make decisions, have a stronger tendency to jump when exploring new  
36 environments, are larger, have longer limbs and wider heads, and perform better in jumping and adhesion.  
37 Once other sources of variation are controlled, these spatial patterns of phenotypic variation appear as the  
38 result of non-random sorting processes occurring during the species' historical range expansion. Future studies  
39 should explore the genomic architecture of this phenotypic trait variation, to characterize further the intimate  
40 link between historical processes and the rising of intraspecific variation.

41 **SHORT TUNNING TITLE:** Phenotypic variation along a range expansion route

42 **KEYWORDS:** Pleistocene range expansion, dispersal, personality, locomotory performance, morphology,  
43 phenotypic variation, *Hyla sarda*

## 44 INTRODUCTION

45 The geography of interindividual variation within species has long fascinated ecologists and evolutionary  
46 biologists (Holt 2003; Des Roches et al., 2018; Raffard et al., 2019). With the advent and increasing availability  
47 of genetic and genomic resources, the study of this variation and the processes contributing to its shaping have  
48 boosted in the last decades (Hewitt 2000, 2004; Schmitt, 2007; Weiss & Ferrand, 2007; Fonseca et al., 2023).  
49 By analysing patterns of variation within organisms spanning the entire tree-of-life and all the continents,  
50 hundreds of phylogeographic and population genetic studies have identified the Pleistocene glacial–  
51 interglacial cycles as a major factor in this respect (Hewitt 2000, 2004; Schmitt, 2007; Weiss & Ferrand, 2007;  
52 Fonseca et al., 2023). These climatic cycles triggered periodic contractions and expansions of species' ranges  
53 and size of populations, leaving multiple imprints on their current genetic structure (Hewitt, 2004; Schmitt,  
54 2007; Weiss & Ferrand, 2007; Fonseca et al., 2023). While the link between these historical biogeographic  
55 processes and current spatial patterns of intraspecific genetic diversity has been widely investigated, the  
56 contribution of these past processes to the evolution of phenotypic variation within species is understudied  
57 (Simmons & Thomas, 2004; Cote et al., 2007; Phillips et al., 2010; Shine et al., 2011). Understanding the  
58 drivers of phenotypic diversity is essential to unravel the ecological and evolutionary processes that shape  
59 biodiversity and drive its evolution across space and time.

60 Recent studies have shown that inter-individual differences in dispersal ability can directly affect the  
61 success of colonisation into new areas and the establishment of new populations (Bowler & Benton, 2005;  
62 Cote et al., 2007; Clobert et al., 2009; Canestrelli et al., 2016a). The study of contemporary biogeographic  
63 processes (*e.g.* range variations in response to the ongoing climate change, and biological invasions) suggest  
64 that dispersers are a non-random sample of individuals characterised by specific phenotypic profiles (Fraser et  
65 al., 2001; Phillips et al., 2008; Cote et al., 2010; Canestrelli et al., 2016a; Louppe et al., 2017). Evolutionary  
66 and ecological processes experienced by any expanding population might drive the directional evolution of  
67 phenotypic traits at the range-edge of an expansion, particularly of those traits that directly influence dispersal  
68 (Pintor et al., 2008; Atwell et al., 2012; Brodin et al., 2013; Myles-Gonzalez et al., 2015; Gruber et al., 2017).  
69 Some personality traits are known to influence individual dispersal propensity (Cote et al., 2010; Canestrelli  
70 et al., 2016a). Both theoretical and experimental studies found that individuals from the range-edge of an  
71 expansion are bolder and more exploratory than those from the core population (Atwell et al., 2012; Myles-

72 Gonzalez et al., 2015; Canestrelli et al., 2016a; Gruber et al., 2017). These traits are predicted to influence all  
73 dispersal stages (*i.e.*, departure, transience, and settlement; Dingemanse et al., 2003; Cote and Clobert,  
74 2007; Cote et al., 2010; Canestrelli et al., 2016a), resulting in a rapid evolution of divergent personalities along  
75 an expansion route. Locomotory performance is also a key factor for expansion dynamics. During a range  
76 expansion, individuals may be spatially sorted based on their locomotor abilities (Phillips, et al., 2010; Shine  
77 et al., 2011). Evidence suggests that this sorting often drives rapid evolution in locomotory traits (Travis &  
78 Dytham, 2002; Phillips et al., 2010; Shine et al., 2011), leading to morphological characteristics that enhance  
79 dispersal rates compared to individuals in long-invaded areas (Phillips et al., 2006; Phillips et al., 2008;  
80 Llewellyn et al., 2010; Louppe et al., 2017). For example, in the cane toad (*Rhinella marina*), a fast dispersal  
81 rate with straighter paths and longer legs has been documented at the edge of the invaded Australian range  
82 (Phillips et al., 2008; Alford et al., 2009; Llewellyn et al., 2010). When seen from a historical perspective,  
83 these studies of contemporary biogeographic processes indicate that the existing knowledge gap regarding the  
84 phenotypic legacy of past biogeographic events within species likely represents the ‘great missing’ piece in  
85 our understanding of the structure of biodiversity and its evolution over time.

86 In this study we aim to fill in this gap, by exploring how dispersal-driven processes may have shaped  
87 the contemporary geographic patterns of intraspecific variation in personality, performance, and  
88 morphological traits along the past expansion route of the Tyrrhenian tree frog *Hyla sarda* (Bisconti et al.,  
89 2011a, Spadavecchia et al., 2021). Previous phylogeographic and population genetic investigations on this  
90 amphibian species have addressed its Late Pleistocene range dynamics and historical demography (Bisconti et  
91 al., 2011a; Spadavecchia et al., 2021). During the last Glacial Maximum, when the sea levels were about 120m  
92 lower than at present, a temporary land-bridge connected the islands of Sardinia and Corsica (Van Andel &  
93 Shackleton, 1982; Shackleton et al., 1984), and this event allowed a northward range expansion of the  
94 Tyrrhenian tree frog, from Sardinia to Corsica (Bisconti et al., 2011a; Spadavecchia et al., 2021). After the last  
95 Glacial Maximum, when the sea levels rose, the newly established populations in Corsica were separated from  
96 their source populations in Sardinia, thus impeding the subsequent flow of individuals from the rear into the  
97 newly colonised areas. Recent studies investigating spatial patterns of variation along the historical expansion  
98 route in this species, showed a clear differentiation in rates of physiological ageing, different telomere

99 dynamics, and differences in anti-predator strategies (Liparoto et al., 2020; Canestrelli et al., 2021;  
100 Spadavecchia et al., 2023), among ancestral and derived populations.

101 This study tested the hypothesis that historical biogeographic events of dispersal contribute to shape  
102 patterns of phenotypic diversity. We expect that Sardinia and Corsica treefrogs would differ in dispersal-related  
103 phenotypic traits, such as personality, morphology, and locomotor performance. We also tested the hypothesis  
104 that this legacy of past events could be detected in the geographic pattern of variation of these dispersal-related  
105 phenotypic traits. Specifically, if dispersers are a non-random sample of individuals, we predict that, compared  
106 to Sardinia tree frogs, Corsica tree frogs should be bolder, and have longer limbs and better jumping  
107 performance because these traits would enhance dispersal capability

## 108 **METHODS**

### 109 **Sampling and housing**

110 Sample collection of tree frogs was carried out along a latitudinal transect from the ancestral area of the glacial  
111 refuge in central-eastern Sardinia to the northern part of the island of Corsica (Figure 1 and Table 1). A total  
112 of 93 tree frogs were collected in spring 2018 from four areas (Figure 1; Table 1). Individuals were captured  
113 with hand nets during the first hours of the night, after acoustic and visual localization and then transported to  
114 the housing facilities at Department of Ecological and Biological Sciences, Tuscia University. Housing  
115 conditions are described in detail in Bisconti et al., 2023.

116 The sampling design was purposefully planned to reduce possible confounding effects linked to local  
117 habitat, kinship or inbreeding between individuals: a) two areas per island were sampled, and two breeding  
118 sites for each area, collecting individuals from different ponds within each site (see Table 1 and Figure 1); b)  
119 all the sampling areas were located on the coasts on the eastern side of species distribution, to avoid both  
120 current and past bioclimatic differences among sampling sites, as shown by previous species distribution model  
121 (Bisconti et al., 2011a); c) tree frogs were collected from ponds exclusively located in coastal areas to avoid  
122 any effect linked to environmental conditions correlated to the altitude or the distance from the coast; d) finally,  
123 to avoid any effect due to demographic bottlenecks usually associated with colonisation processes through  
124 jump dispersal (Frankham, 1998) rather than through spatial diffusion processes, other islands inhabited by *H.*  
125 *sarda* were intentionally excluded from this study (i.e. Elba and Capraia islands; see Bisconti et al., 2011a,b;  
126 Bisconti et al., 2023).

127 **Ethical note**

128 All the procedures were performed under the approval of the Institute for Environmental Protection and  
129 Research 'ISPRA' (protocol #5944), Ministry of Environment 'MATTEM' (protocol #8275), Regione Sardegna  
130 (#12144) and Prefecture of Corsica (#2A20180206002 and #2B20180206001).

131 Permission to temporarily house amphibians was granted by the Local Health and Veterinary Centre,  
132 with license code 050VT427. All handling procedures were approved by the Ethical Committee of the Tuscia  
133 University for the use of live animals. During captivity the animals were monitored daily. No adverse effects  
134 on the overall health of tree frogs were observed during the procedures. The animals were released in the  
135 original sampling locations at the end of the experimentation.

136 **Behavioural tests**

137 To address geographic variation in individual behaviour of *H. sarda*, the study was focused on two personality  
138 traits that are commonly associated with dispersal propensity: exploration attitude in a novel environment and  
139 boldness (Canestrelli et al., 2016a). Exploratory activity is considered crucial in promoting dispersal, as  
140 individuals with a greater propensity toward exploration are expected to crowd at the expanding range edge  
141 (Dingemanse et al., 2003; Cote et al., 2010). Exploration activity was investigated by measuring the latency to  
142 exploring a novel environment, and the time spent in exploration. Propensity to disperse was also assessed, by  
143 investigating boldness, that is, an individual's propensity to take risks (Réale et al., 2007; Canestrelli et al.,  
144 2016a). Bolder individuals are expected to be more willing to accept the intrinsic risks of dispersal (Stamps,  
145 1985; Bonte et al., 2012), and thus likely to disperse earlier than shyer conspecific. To assess these personality  
146 traits, two distinct behavioural tests were performed, as described below, and each test was repeated after 10  
147 days to measure temporal consistency. All the tests were performed in the housing room, to avoid any changes  
148 in humidity and temperature that could affect the analysed behavioural features (Duellman & Trueb, 1994).

149 *Exploration in a novel environment*

150 Considering the arboreal lifestyle of *H. sarda*, exploration tests were carried out in a cylindrical arena allowing  
151 both vertical and horizontal movements. The arena was enriched with plants (*Epipremnum aureum*) and an  
152 oakwood that allows individuals to hide, eliminating the possibility that the measured behaviours reflected  
153 abnormal fear or anxiety rather than a spontaneous exploratory behaviour (see Bisconti et al., 2023). This test  
154 has been described in detail Bisconti et al., (2023). Briefly: after a 5-minute acclimatation period, individuals

155 were left to explore the arena for 10 minutes, and the individual exploration behaviour was recorded using an  
156 HD video camera (Nova Germany model DVR AHD-7908). The following variables were extract using Boris  
157 5.1.3 (Friard & Gamba, 2016): 1) latency to explore (s); 2) duration of activity (expressed as percentage of  
158 duration of test); 3) duration of time spent on the arena ground floor; 4) jumping activity, expressed as  
159 percentage of time in activity (moving) spent jumping.

#### 160 *Boldness*

161 This test was performed to assess the individual behaviour across the boldness-shyness behavioural axis. The  
162 latency to exit from a shelter was considered as a proxy of the individual propensity to take risk. A HD video  
163 camera (Nova Germany model DVR AHD-7908) was used to record a rectangular arena where all animals  
164 were caged into a cylindrical dark shelter for a 6-minute acclimatation period and then left free to exit (Bisconti  
165 et al., 2023). The software Boris 5.1.3 was used to measure the time it took for an individual to exit from the  
166 shelter.

#### 167 **Locomotory performance tests**

168 To investigate the geographic variation in locomotory performances of *H. sarda*, we assessed two traits that  
169 are expected to play a crucial role in an arboreal tree frog dispersal: jumping force, and adhesion force. The  
170 jumping force was measured at take-off (Marsh & John-Alder 1994; Nauwelaerts & Aerts 2006 and references  
171 therein). The adhesion force was also considered a proxy of dispersal abilities because it plays a main role  
172 during the landing phase (Duellman & Trueb, 1994; Bisconti et al., 2023; Emerson & Diehl 1980; Federle et  
173 al. 2006; Smith et al. 2006; Bijma et al., 2016). The jumping force was quantified by means of jumping tests,  
174 while the adhesion force was quantified by a stickiness tests, as described below. Before each test, all the  
175 individuals were hydrated for 1 minute, and then weighted using a scale Acculab model (ATILON ATL-224-  
176 I).

#### 177 *Jumping test*

178 Jumping tests were carried out in a rectangular arena, videotaped with a video camera (Panasonic DMC-  
179 FZ300) placed laterally to the setup. Each tree frog was fitted with an accelerometer data logger (xy-4 units,  
180 Technosmart, Rome, 9.15 x 15 x 4 mm, 1 gr weight including battery) set to record triaxial acceleration (0 - 4  
181 g) at 100 Hz, and the tree frog was induced to jump by stimulating its caudal region (Mitchell & Bergmann,  
182 2016). Five jumps were collected for each individuals (see Bisconti et al., 2023 for a detailed description of

183 this test). The dynamic body acceleration for each dimension was extracted from the downloaded data (x, y,  
184 z; Wilson et al., 2006; Shepard et al., 2008). The logger's x axis recorded sway, the y axis recorded surge, and  
185 the z axis recorded heave (Halsey et al., 2008). Framework 4 software were used (version 2.5) to add these  
186 values and get the Vector sum of Dynamic Body Acceleration [ $VeDBA = (Ax^2 + Ay^2 + Az^2)$ ]. Five VeDBA  
187 values for each individual were quantified, one for each jump, and the highest value of VeDBA was retained  
188 as the individual maximum jumping force at take-off [ $VeDBA \times mass$ ] and considered for downstream  
189 analyses.

#### 190 *Stickiness test*

191 A smooth plastic rotating wheel with a moderate and constant angular speed (3 revolutions per minute) was  
192 used to test the maximum adhesiveness force. Five falls for each individual were recorded using Nova  
193 Germany model DVR AHD-7908. The software Tracker (version 4.11.0) was used to extract the angle of fall  
194 (radian) for each of the five falls, and the largest value was selected for the subsequent analyses. The maximum  
195 adhesion force was calculated as [ $\cos(\alpha) \times mass \times g$ ] using Barnes et al. (2006)'s standard protocol (see Bisconti  
196 et al., 2023, for further details).

#### 197 **Morphological traits**

198 Anurans with an arboreal lifestyle are characterised by morphological adaptations to better perform during  
199 jumps and landings (Kamada et al., 2017; Bijma et al., 2016). Those morphological traits that typically play a  
200 direct role in these two phases of locomotion were investigated, that is, body size and limb lengths (Phillips et  
201 al., 2006). However, data on another morphological trait that is intrinsically linked to jumping performance in  
202 tree frogs were also collected: the head width. Indeed, there is evidence that the head width plays a crucial role  
203 improving the ability of tree frogs in performing jumps, especially by maintaining the posture during the  
204 jumping phase, and during the landing phase by hitting the substrate first (Reilly et al., 2016; Kamada et al.,  
205 2017).

206 Measures of the following morphological traits were collected: (1) snout–vent length (SVL; mm); (2)  
207 head width (mm); (3) forelimb length (mm); (4) hindlimb length (mm). To obtain standardised measures, the  
208 tree frogs were anaesthetised by a two-minute submersion in a solution of MS-222 (0.05% m/v), placed in the  
209 prone position on 1x1 cm grid paper, parallel to the plane of a camera lens, and photographed using a Panasonic  
210 LUMIX (DMC-FZ300) digital camera. All photographs were taken with the same camera settings and under



211 the same lighting conditions. After the photographs, each individual was placed in a humid box until it fully  
212 recovered, and then reintroduced into its own fauna box. The measures were then extracted by the photographs  
213 and analysed by using ImageJ 1.52 (National Institutes of Health, USA).

#### 214 **Data analysis**

215 All statistical analyses were performed using R software version 4.3.1 (R Core Team 2023). To evaluate the  
216 repeatability of the behavioural traits, we run Generalized Linear Mixed effect (GLMM) based repeatability  
217 models using the “rpt” function of the *rptR* package (Schielzeth & Nakagawa, 2011; Stoffel et al., 2017),  
218 setting as Poisson distribution datatype; duration of activity and duration of time spent on the arena floor level  
219 were treated as proportion datatype. We entered each behavioural trait as a dependent variable and individual  
220 as a random factor. We considered the behavioural variables as personality traits when their repeatability value  
221  $R$  is  $> 0.2$ , and the lower bound of the CI was  $> 0.0$  (Brodin et al., 2013; Kelleher et al., 2017; Kelleher et al.,  
222 2018 and references therein). Preliminary models showed that entering sampling location within each island  
223 as a random factor did not improve the fit of the model (i.e. the AIC value was not reduced beyond 2); thus, it  
224 was not subsequently considered. Also, running the models with or without females did not change at all both  
225 the model fit and the results, and thus we retained females for all the subsequent analyses.

226 We tested the hypothesis of a phenotypic change across the past expansion route of *Hyla sarda* from  
227 Sardinia to Corsica. First, we assessed the differences in personality, performance, and morphological traits  
228 between the two islands by running Generalized Linear Models (GLMs; “lm” function in the basic package  
229 *stats*) entering each variable singly as dependent variables, and island as fixed factor. We also run the analyses  
230 including sampling location within each island as a random factor in generalized linear mixed-effect models  
231 (GLMMs; “lmer” function in the *lme4* package); AIC values comparison was applied to select the best-fit  
232 modelling approach. For repeatable personality traits, we used the mean between the two repetitions; the  
233 variables were square-root (frequency of jumping events) or log10 (all the others) transformed to meet the  
234 assumption of residuals normality. To exclude the effect of the body size on the morphological traits, for each  
235 measured trait we extracted the residuals of the regression with the SVL. Normality and homoscedasticity of  
236 model residuals were evaluated to ascertain the goodness of model fit. For the selected best-fit models, we  
237 contrasted the estimated marginal means and standard errors from the models as computed using the *emmeans*  
238 *r* package.

239 Then, we tested the association of phenotypic variation with the distance from the estimated area of  
240 origin of the range expansion from Sardinia to Corsica as estimated in Spadavecchia et al. (2021). We set a  
241 distinct linear regression model between each behavioural, performance and morphological variable and the  
242 distance from the area of origin, measured in km using the Google Earth (Google Inc.) “ruler” function.

## 243 **RESULTS**

244 The repeatability coefficients of the behavioural traits with the respective CI and p-value of are reported in  
245 Table 2. Three out of the five investigated traits resulted significantly repeatable: boldness ( $R = 0.40$ ), the  
246 frequency of jumping events during the arena exploration ( $R = 0.35$ ), and the latency to explore a novel  
247 environment ( $R = 0.31$ ). Conversely, activity and duration of time spent on the arena floor were not  
248 significantly repeatable and thus they were excluded from subsequent analyses.

249 We found substantial differences between Sardinia and Corsica populations for all the analysed traits.  
250 The AIC model comparison (Supplementary Table 1) showed that entering sampling location within each  
251 island as a random factor did not improve the fit of the model (*i.e.* the AIC value was not reduced) for all the  
252 traits but two (performance traits), supporting the general independence of the pattern found from local  
253 sampling site conditions.

254 We found significant differences in personality traits between Sardinia and Corsica individuals (Table  
255 3), with Corsica individuals showing a shyer profile and higher jumping frequencies. The latency to exit from  
256 the shelter and the latency to explore were longer for Corsica individuals than those showed by Sardinia  
257 individuals ( $p = 0.032$  and  $p < 0.001$ , respectively). The jumping frequency during exploration was  
258 significantly higher in Corsica than in Sardinia ( $p = 0.001$ ). The estimated marginal means and standard errors  
259 for personality differences between Sardinia-Corsica are shown in Figure 2A-2C.

260 Sardinia and Corsica populations differed also in jumping force at the take-off and in stickiness, with  
261 tree frogs from Corsica showing higher performance values than individuals from Sardinia (Table 3 and Figure  
262 2). Entering sampling location within each island as a random factor improved the model fit for both the  
263 performance traits (lowest AIC; see Supplementary Table 1). However, whereas between-island differences in  
264 stickiness were significant both in GLM and GLMM ( $p < 0.001$ ), jumping force at the take-off resulted  
265 significant in GLM ( $p = 0.001$ ) but not significant when entering sampling location as a random factor ( $p =$

266 0.094). The estimated marginal means and standard errors for performance differences between Sardinia-  
267 Corsica are shown in Figure 2D-2E.

268 All morphological traits showed significant differences between Sardinia and Corsica populations  
269 (Table 3 and Figure 2F-2I). Corsica tree frogs were larger in body size ( $p < 0.001$ ), with longer forelimbs ( $p =$   
270 0.044) and hindlimbs ( $p = 0.026$ ), and had wider head size ( $p = 0.011$ ).

271 Finally, we found significant associations between phenotypic variation and the distance from the  
272 estimated area of origin of the range expansion from Sardinia to Corsica (see Spadavecchia et al., 2021) for all  
273 the analysed traits, except for hind limb length and head width (Figure 3).

## 274 **DISCUSSION**

275 We found substantial and significant differences between *H. sarda* individuals from the two islands. Corsica  
276 tree frogs showed a shyer behavioural profile, a longer decision-making time, a significant attitude to jump  
277 during exploration, and a more efficient dispersal-related performance and different morphological traits pared  
278 to Sardinian tree frogs.

279 The spatial patterns of phenotypic variation observed could be explained by several scenarios.  
280 Variation in bioclimatic conditions between the two islands could contribute to shape the differences found.  
281 Climatic conditions do, indeed, affect habitat features, that in turn, could differentially influence dispersal and  
282 the ability to avoid predators, especially for an arboreal species (Martin et al. 2005). However, previous studies  
283 showed comparable bioclimatic conditions between the coastal areas considered in this study (Bisconti et al.,  
284 2011; see section 2.1), which makes a contribution of climatic variation highly improbable in explaining the  
285 observed patterns of phenotypic differentiation (Bisconti et al., 2011a). An alternative explanation for these  
286 patterns might stem from differences in the ecological community between the two islands. In particular,  
287 differences in the predatory environment encountered within the two islands could contribute much to promote  
288 local adaptations and mould phenotypic variation (Kang et al. 2017; Gavriilidi et al., 2022). Corsica and  
289 Sardinia are continental islands that share their geological origin and most of the Pleistocene history (Blondel  
290 et al., 2010) and, consequently, the ecological communities of coastal environments largely overlap between  
291 these islands (Blondel et al., 2010). There are not noticeable differences between islands in the presence of  
292 organisms that are known to predate the Tyrrhenian tree frog (mostly birds and snakes; Lanza et al., 2007).  
293 Lastly, in principle, some genetic divergence between Sardinia and Corsica populations might contribute to

294 explaining the observed pattern of phenotypic differences (Lipshutz, et al., 2017; Stamp et al., 2020). However,  
295 previous phylogeographic and population genetic structure analyses showed the absence of any remarkable  
296 differences in the levels of genetic diversity, and the lack of a spatial genetic structure between Sardinia and  
297 Corsica populations, disproving this potential contribution to the observed pattern of phenotypic variation  
298 (Bisconti et al., 2011a,b).

299 In the absence of clear influences from environmental or genetic factors on the geographic patterns of  
300 phenotypic variation, our results provide support to the hypothesis that the formation of these geographic  
301 patterns has been promoted by a non-random spatial sorting of individuals by dispersal propensity/ability,  
302 during the past range expansion phase along the south-north axis (Figure 2). In the following sections, we  
303 will discuss the putative implications of our findings for the evolution of each phenotypic trait, during the  
304 range expansion phase from Sardinia to the Corsica Island.

305 The shy behavioural profile shown by the Corsica individuals appears counterintuitive, as dispersers  
306 at the range edge have frequently been described as bolder and more exploratory than those living in long-  
307 established areas (*e.g.* Pintor et al., 2008; Liebl & Martin, 2012; Atwell et al., 2012; Myles-Gonzalez et al.,  
308 2015; Canestrelli et al., 2016a; Gruber et al., 2017). However, recent empirical studies showed that a shy and  
309 more prudent behavioural profile is an efficient alternative strategy to navigate in a novel environment, where  
310 the density of conspecific individuals is lower than in core populations (Gavriilidi et al., 2022; Mazza &  
311 Eccard, 2023; Eccard et al., 2023). On the contrary, a shy profile in expanding populations could enable a  
312 more careful assessment of the surrounding environment, thereby reducing risks and ultimately increasing the  
313 chances of survival (Groothuis & Carere 2005; Réale et al. 2010; Mazza & Eccard, 2023). By requiring less  
314 energy investment in exploration, this behavioural profile could favour metabolic resource allocation to other  
315 critical functions pivotal for a successful colonisation, such as foraging, growth, and reproduction (Verbeek et  
316 al., 1994; Deerenberg et al., 1998; Cockrem, 2007; Coppens et al., 2010). Besides, the higher propensity of  
317 Corsica tree frogs to jump during their overall activity duration, compared to individuals from the source  
318 island, may have facilitated a cost-benefit optimization for the dispersers. Despite the energetic costs associated  
319 with a jumping locomotory mode, such strategy could enable individuals to cover longer distances with a  
320 single jump, interspersed with pauses for energy recovery and for predatory and environmental context  
321 assessment, before exploring again. The higher propensity to jump shown by tree frogs from Corsica is

322 reflected in their better locomotory performance than those from Sardinia. As key traits in determining species  
323 dispersal ability, locomotory performance traits play a direct role in dispersal processes and, consequently, in  
324 the overall success of expansion (Phillips et al., 2006; Phillips et al., 2008; Llewellyn et al., 2010; Louppe et  
325 al., 2017). The performance trait analysis revealed that Corsica tree frogs exhibit greater jumping and adhesion  
326 forces than those from Sardinia. In such an arboreal species, both traits could significantly enhance dispersal  
327 ability, playing a crucial role during both the initial jumping phase and the subsequent landing phase. This  
328 higher jumping performance may contribute to covering longer distances with each jump while minimizing  
329 the risk of missing the target during landing (see material and methods; Duellman and Trueb, 1994; Kosmala  
330 et al., 2017). More performant profiles shown by Corsica individuals during dispersal are in line with findings  
331 from several other studies exploring locomotory performance traits in contemporary expanding populations  
332 (Phillips et al., 2008; Alford et al., 2009; Llewellyn et al., 2010). Recent studies on invasive species have  
333 investigated the evolution of locomotory performance traits at the range-edge of expanding populations,  
334 revealing a significant improvement in the locomotor abilities of dispersers, thus suggesting that, during an  
335 expansion, individuals may be spatially sorted based on their dispersal-related performance ability (Phillips et  
336 al., 2008, Alford et al., 2009; Llewellyn et al., 2010; Shine et al., 2011; Louppe et al., 2017).

337 Morphological analyses showed that Corsica tree frogs have a larger body size, longer limbs, and a  
338 wider head width than those from Sardinia. All these features imply a better performance of these individuals  
339 during the dispersal and the colonisation of new environments. A larger body size would confer several  
340 advantages to individuals during an expansion. Studies on amphibians showed that larger individuals have a  
341 better locomotory performance both on land and in water (Cabrera-Guzman et al., 2013). Moreover, a larger  
342 size plays an important role in other essential functions particularly crucial for an amphibian during  
343 colonisation, such as avoiding dehydration (Kosmala et al., 2020), allowing to be more performant in capturing  
344 prey (Cabrera-Guzman et al., 2013) and also increasing the reproductive success, whereby the larger male  
345 would be more successful both in intraspecific competition and during the amplexus (Bowcock et al., 2013;  
346 Herrel et al., 2012; Clarke et al., 2019). Similarly, longer limbs improve locomotion, and, thus, the dispersal  
347 ability (Phillips et al., 2006; Clarke et al., 2019). Longer legs allow a greater propulsive force in jumping  
348 (Kamada et al., 2017; Bijma et al., 2016). For example, in the invasive *Rhinella marina*, individuals at the edge  
349 of the Australian invasion front had longer legs, a trait significantly enhancing invasion success (Phillips et al.,

350 2006). In the context of jumping amphibians like tree frogs, longer forelimbs could also contribute to  
351 improving landings, while dampening the energy of jumps upon touchdown (Bijma et al., 2016). Lastly,  
352 colonising individuals with a wider head would likely adapt better to landings after jumps. The increased  
353 propulsion resulting from longer legs might lead to more forceful landings and given that the head is the first  
354 part of the body to arrive on a surface, a larger head width could confer better management of shock absorption  
355 during collisions.

## 356 **CONCLUSION**

357 This study provides novel evidence that historical range expansion could have played a role in explaining the  
358 geographic patterns of intraspecific phenotypic variation in natural populations. Rapid, substantial, and  
359 directional evolution in dispersal-enhancing traits had been already documented in populations currently  
360 undergoing range expansion (Phillips et al., 2006; Liebl and Martin, 2012; Gruber et al., 2017; Kosmala et al.,  
361 2017). Although studies of ongoing invasion provide an excellent opportunity to investigate the causes and  
362 consequences of dispersal dynamics, they do not enable the investigation of their long-term legacies, in an  
363 evolutionary perspective (Canestrelli et al., 2016a, b). The selective advantages associated with adaptive,  
364 dispersal-enhancing, traits during an expansion phase may be transient, as suggested by several empirical  
365 evidence (Travis & Dytham, 2002; Brown et al., 2007; Phillips et al., 2008; Brown et al., 2014). Recently  
366 established populations may indeed restore variation (phenotypic and genotypic) through a slower flow of  
367 individuals from the rear or may evolve novel trait states in response to demographic density increases in newly  
368 colonised areas (Cobben et al., 2015; Canestrelli et al., 2016a; Perkins et al., 2016). To date, we are not aware  
369 of studies addressing if the predicted directional changes in dispersal-enhancing traits persist in populations  
370 long after the expansion stops, and the demographic conditions promoting spatial sorting processes vanish  
371 (Cobben et al., 2015; Canestrelli et al., 2016a; Perkins et al., 2016). In this frame, the expansion history of the  
372 Tyrrhenian tree frogs could offer the opportunity to learn about the long-term legacy of non-neutral processes  
373 at play during a range expansion. Finally, to investigate the genomic underpinnings of the phenotypic evolution  
374 promoted by historical range expansions appears the next mandatory step to reach a more complete  
375 understanding of the eco-evolutionary processes moulding spatial and temporal patterns of variation of  
376 biological diversity (see Libro et al., 2022).

377 **Author contributions**

378 Daniele Canestrelli, Claudio Carere, and Roberta Bisconti designed research; Daniele Canestrelli, and Roberta  
379 Bisconti performed research; Andrea Chiocchio, David Costantini and Claudio Carere analyzed data, Roberta  
380 Bisconti, Andrea Chiocchio, and Daniele Canestrelli wrote the paper with inputs from the other authors.

381

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393

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395

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646 **FIGURES AND TABLES**647 **Table 1.** Geographic coordinates and sample size of the sites sampled to study phenotypic variation within  
648 *Hyla sarda* populations.

649

Island	Sampled site	Latitude	Longitude	N
<b>Corsica</b>	Aleria	42.1132	9.5221	14
	San Giuliano	42.2677	9.5184	10
	Etagn de Canettu	41.4456	9.2035	10
	T10	41.4603	9.2170	13
<b>Sardinia</b>	Stazzo Pulcheddu	41.1639	9.3620	8
	Porto Pollo	41.1844	9.3303	15
	Cala Ginepro	40.4481	9.7920	14
	Siniscola	40.5814	9.7691	9

650

651 **Table 2.** Summary of GLMM-based repeatability (R) estimates from multiplicative model. Parametric  
652 bootstrapping (number of iterations = 1,000) was used to calculate the CI interval and the likelihood ratio test  
653 to estimate the P-value of the repeatability distribution. Significant traits are shown in bold.

654

655

Variable	Estimate	95 % CI	P-value
Latency to explore	0.31	0.09-0.50	<b>0.002</b>
Activity	0.04	0.00-0.10	0.051
Time on floor	0.07	0.00-0.21	0.183
Jumping events	0.35	0.14-0.53	<b>0.001</b>
Latency to exit	0.40	0.20-0.55	<b>&lt;0.001</b>

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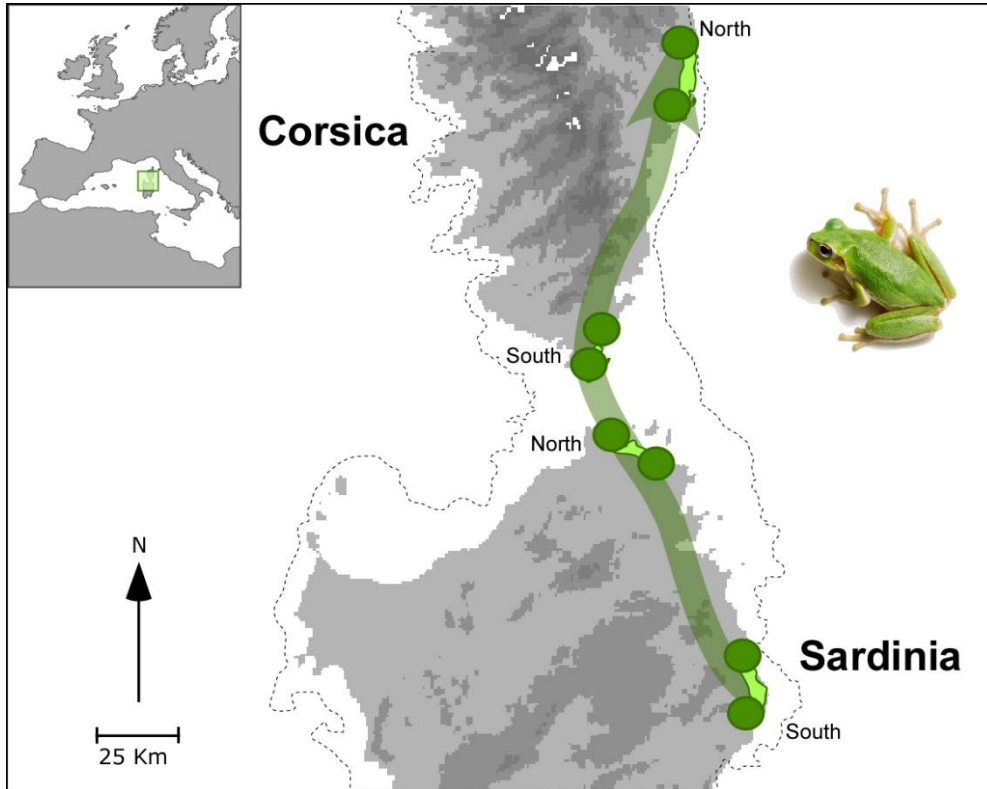
663 **Table 3.** Linear models showing differences between Corsica and Sardinia Island populations in behavioural,  
664 performance, morphological traits. Coefficient estimates ( $\pm SE$ ) of these models are shown with both  
665 behavioural and performance traits as dependent variable and island as fixed factor. Significant contrasts are  
666 shown in bold.  
667

Phenotypic trait	Dependent variable	Reference	Level	Coefficient	SE	t-Value	P
<b>Behaviour</b>	Latency to explore	C	S	-0.526	0.131	- 4.028	< <b>0.001</b>
	Jumping events	C	S	-1.256	0.358	-3.504	<b>0.001</b>
	Latency to exit	C	S	-0.396	0.181	-2.185	<b>0.032</b>
<b>Performance</b>	Jumping	C	S	-3.390	0.987	-3.433	<b>0.001</b>
	Stickiness	C	S	-7.731	1.969	-3.926	< <b>0.001</b>
<b>Morphology</b>	SVL	S	C	-0.238	0.062	-3.844	< <b>0.001</b>
	Forelimb	S	C	-0.062	0.030	-2.059	<b>0.044</b>
	Hindlimb	S	C	-0.100	0.044	-2.288	<b>0.026</b>
	Head width	S	C	-0.032	0.0124	-2.595	<b>0.011</b>

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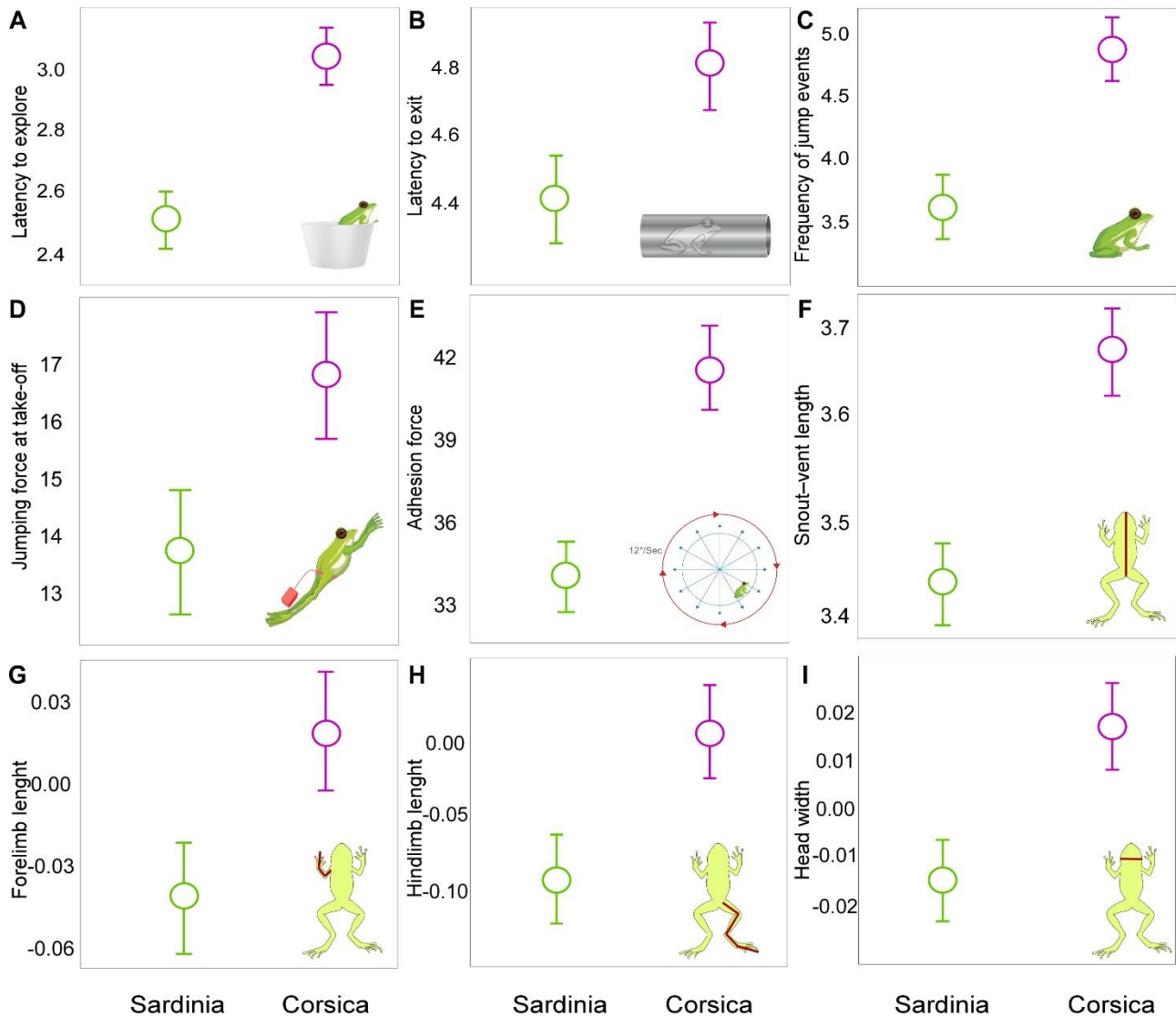
670 **Figure 1.** Geographical distribution of the eight sampled populations of the Tyrrhenian tree frog (*Hyla sarda*).  
671 The dashed line indicates the approximate coastline location during the Last Glacial Maximum (Thiede, 1978).  
672



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675 **Figure 2.** Estimated marginal means and standard errors from linear models are shown for each island for all  
 676 phenotypic traits studied in the Tyrrhenian tree frog (*Hyla sarda*). Personality traits include A) latency to  
 677 explore (in seconds), B) latency to exit (in seconds), and C) frequency of jump events (%). Performance traits  
 678 include D) jumping force at take-off (in Newton) and E) adhesion force (in Newton). Morphological traits  
 679 include F) snout-vent length (SVL), G) forelimb length, H) hindlimb length, and I) head width (all expressed  
 680 in millimetres). Morphological measurements are indicated by red lines within each panel.



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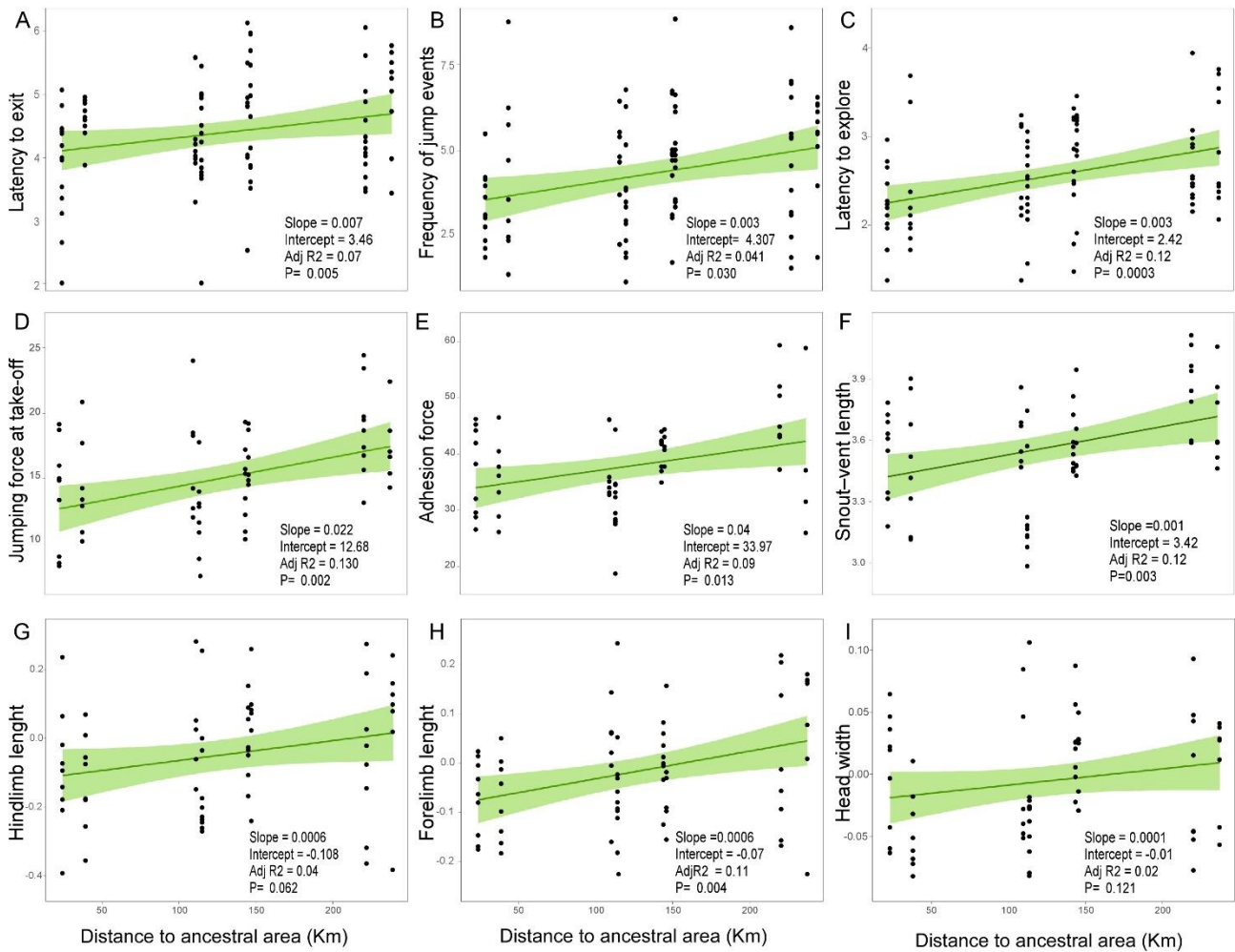
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687 **Figure 3.** Phenotypic trait variation along the inferred historical range expansion route of the Tyrrhenian tree  
 688 frog (*Hyla sarda*). Results are based on linear regression models examining the relationship between distance  
 689 from the area of origin (in km) and each behavioural, performance, and morphological trait analysed in this  
 690 study.  
 691



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