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Biogeographic patterns of color and size polymorphisms in the European tree frog (*Hyla arborea*)

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Abstract

Despite generally conserved phenotypes across radiations, Hylid frogs exhibit substantial intraspecific variation in size and coloration, especially the background color and lateral black and white stripe patterns (*linea marginalis*). We examined these traits in a phylogeographic context across 14 populations (276 adults) representative of the three genetic lineages reported in the widespread European tree frog *Hyla arborea*. Body size was measured and field photographs were examined to identify discrete color morphs and evaluate stripe intensity. Most of the polymorphism depended on populations, with no effect of genetic diversity and lineage, suggesting that size and color patterns in *H. arborea* likely result from phenotypic plasticity or micro-evolution at the local scale, potentially mediated by local selective pressures and drift. Frogs belonging to the southern Balkan lineage, however, did feature generally whiter stripes compared to the other lineages. Interestingly, our analyses suggested that the *linea marginalis* differ between sexes throughout the range: it was overall whiter in males and may thus provide a visual contrast to facilitate nocturnal detection by females, which were experimentally shown to prefer conspicuous mates. Finally, the body size of the frogs, which significantly differed between sexes and populations, did not follow any particular biogeographic pattern, running against Bergmann's rule. Our analyses provide a framework for future studies on the origin and maintenance of phenotypic variation in amphibians, especially to test the role of eco-geographical factors.

Keywords: amphibian, Balkans, coloration, *linea marginalis*, phylogeography.

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Résumé étendu

Introduction – La plupart des rainettes du paléarctique (genre *Hyla*) sont visuellement très semblables, malgré de fortes divergences génétiques. Pourtant, la taille et la coloration restent très variables entre les individus d'une même espèce. C'est notamment le cas de la couleur de fond, ainsi que de la ligne latérale (*linea marginalis*). Ces traits sont supposés être héritables chez les amphibiens, mais les facteurs régissant le maintien de leur variation restent méconnus. La teinte générale est censée optimiser le camouflage et la thermorégulation, tandis que la ligne latérale jouerait un rôle dans la détection nocturne et le choix des mâles par les femelles. Les conditions

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régionales et locales, ainsi que les différences génétiques entre populations, devraient donc affecter la distribution des morphotypes de coloration. De même, les facteurs éco-géographiques, et notamment les gradients climatiques, influencent théoriquement la taille des individus, qui peut varier du simple au double en fonction des populations. Pour mieux comprendre l'origine d'une telle variation phénotypique, nous avons examiné la taille et les motifs de couleur chez la rainette européenne (*Hyla arborea*) dans un contexte phylogéographique, sur la base de photographies prises sur 276 individus échantillonnés à travers l'aire de répartition (14 populations) au cours d'une campagne de terrain organisé en 2011, dans le cadre de nos recherches sur l'évolution de l'espèce. Par ailleurs, ces travaux ont montré qu'*Hyla arborea* comprenait trois lignées évolutives distinctes distribuées respectivement dans le sud de Balkans, le long de la côte adriatique, et le reste de l'Europe. Ces lignées sont-elles cryptiques, ou possèdent-elles des différences phénotypiques notables ?

MÉTHODOLOGIE – Dans cette étude, chaque individu a été mesuré (distance museau-cloaque), assigné à un morphe de couleur (vert clair, vert foncé et brun/gris) et a reçu des scores reflétant l'intensité des parties sombres et claires de la *linea marginalis*. Nous avons alors effectué des analyses statistiques basées sur les individus et sur les populations afin de tester si ces polymorphismes dépendaient du sexe, de la diversité génétique, ou de l'origine évolutive (lignée) et géographique (population, latitude, altitude) des rainettes.

RÉSULTATS ET DISCUSSION – La taille, la couleur de base, ainsi que l'intensité et le contraste de la ligne latérale varient en fonction de la population échantillonnée, mais ne sont pas liés à la diversité génétique ni à l'origine géographique. Cela suggère que cette variation phénotypique dépend essentiellement de facteurs locaux (plasticité phénotypique, sélection, dérive). À noter cependant que les individus attribués à la lignée évolutive du sud des Balkans possèdent une ligne latérale généralement plus claire. Par ailleurs, les ratios noir/blanc de cette ligne semblent différer entre sexes : plus claire chez les mâles, elle pourrait ainsi augmenter le contraste visuel de la robe et faciliter le repérage par les femelles lors des accouplements nocturnes. Cette interprétation coïncide avec des expériences comportementales suggérant que les femelles rainettes choisiraient les partenaires les plus voyants. Concernant la taille, nos données confirment que les femelles sont significativement plus grandes que les mâles (45.4 mm *vs* 42.5 mm de moyennes). Cependant, l'absence de corrélation entre taille et altitude/latitude va à l'encontre de l'hypothèse répandue que les individus plus grands sont sélectionnés dans les environnements froids, afin d'optimiser la thermorégulation (règle de Bergmann). Notre étude fournit un cadre biogéographique potentiellement utile pour de futures études visant à comprendre l'origine et le maintien de la variation phénotypique chez la rainette verte.

Mots-clés : amphibiens, Balkans, coloration, ligne latérale, phylogéographie.

INTRODUCTION

Anuran amphibians are well-known for their great diversity of colorations and color motifs. While showing physiological and developmental plasticity (BRAUER 1991), these traits are often heritable (e. g. O' NEILL & BEARD 2010) and can be shaped by a multitude of genetic and evolutionary drivers (HOFFMANN & BLOUIN, 2000). Interestingly, the strong intraspecific variation of color phenotypes is often shared between distinct species. Hence, genetic divergence alone cannot explain the micro-evolution of color polymorphism among closely related lineages, which are mediated by other processes.

The same is true for body size, which may vary by several orders of magnitude depending on populations. For instance, the common toad *Bufo bufo* reaches enormous sizes in the Balkans (>20 cm for females) but is twice smaller in Central and Western Europe (DUFRESNES 2019). Understanding the origin of size variation has been a major focus in herpetological research (ADAMS & CHURCH 2008), but the genetic, environmental and developmental factors involved remain elusive. One prevailing idea is that body size is optimized to minimize heat loss in cooler climate (Bergmann's rule), and bigger individuals are selected in higher altitudes and latitudes (BERGMANN 1847, MAYR 1963), as widely seen in endotherms (ASHTON *et al.* 2000). Also suggested in ectotherms (RAY 1960), which regulate their temperature behaviorally, Bergmann's gradients are however not obvious in amphibians and reptiles (ASHTON 2002, ADAMS & CHURCH 2008) and the patterns greatly vary depending on taxonomic groups (ASHTON & FELDMAN 2003, OLALLA-TARRAGA & RODRIGUEZ 2007). Data from additional taxa are thus needed to get more comprehensive insights on the eco-geographic patterns ruling size variation, especially if the biogeographic history of species can be accounted for.

Complex phenotypic variation is characteristic of Western Palearctic hylids from the *Hyla arborea* complex. Despite a variety of background colors and skin patterns (dots, stripes), closely related species cannot be differentiated without genetic tools (e. g. *H. arborea*, *H. intermedia*, *H. molleri*, *H. orientalis, H. Perrini*), and are thus considered cryptic (DUFRESNES *et al.* 2018). In this group, the background color spans over a wide spectrum ranging from light green to dark green or brown/grey, which can be physiologically manipulated by individuals, in order to match their environment for camouflage (homochromy) or to improve thermoregulatory efficiency (VENCES *et al.* 2002, STEGEN *et al.* 2004, VITT & CALDWELL 2014). Moreover, tree frogs bear a highly variable *linea marginalis*, i. e. the black and white line running alongside of the body, which even allows to recognize individuals in capture-mark-recapture studies (e.g. PELLET *et al.* 2007). Last but not least, size is sexually-dimorphic but quite variable in tree frogs (3–6 cm, DUFRESNES 2019), and further seems to increase with latitude in some parts of the Western Palearctic (e. g. ÖZDEMIR *et al.* 2012).

The evolutionary causes of color polymorphism in *Hyla* have received some attention. Green and brown amphibian pigmentations only differ by their type of chromatophores (HOFFMANN & BLOUIN, 2000). Different color morphs may thus frequently arise (DUFRESNES *et al.* 2011, NIKOLIĆ *et al.* 2016), and be mediated by predatory selection (favoring a color matching the environment) or adaptation to solar irradiation. Moreover, experiments on *H. arborea* suggested that flank stripes enhanced male conspicuousness and facilitated detection and localization by females during nocturnal breeding (GOMEZ *et al.* 2009). Yet, such selection should be sex-antagonistic, i. e. favoring conspicuous colors in males due to sexual selection, but dull colors in females to escape predation (conspicuous individuals being easier to hunt). Neutral forces like drift should also play a significant role for all types of heritable polymorphism. At the regional level, tree frog metapopulations experience frequent extinction and recolonization events, and thus strong drift. For instance, Gomez *et al.* (2011) suggested that local processes (selection and drift) were likely the main driver of background color variation in French *Hyla arborea*, among which this trait was accordingly not associated to the genetic distances between populations. At the continental level, amphibian populations experienced contrasted evolutionary dynamics during the Quaternary, with recent post-glacial recolonization in northern ranges, but long-term refugial persistence of separate lineages in southern ranges, where populations maintained higher levels of diversity (Schmitt 2007). In the same way they affected the distribution of genetic diversity, these processes could thus have also affected the distribution of phenotypic variation.

Here, we investigated size and color polymorphism in the European tree frog *H. arborea*, in respect to its genetic diversity and evolutionary history. The phylogeography of *H. arborea* has been well documented (Dufresnes *et al.* 2013), and provides a nice framework to assess phenotypic variation. The species diversified in the Balkan Peninsula during the previous Quaternary glacials, forming three young evolutionary lineages present along the Adriatic coast (~200'000 years of divergence, i.e. two ice ages), the southern Balkans, and Central Europe (~100'000 years of divergence, i.e. one ice age). The latter lineage recolonized Northern and Western Europe after the last glacial maximum, losing substantial amounts of the refugial diversity in the process (Dufresnes *et al.* 2013). Did these lineages independently evolved private phenotypic variation during their recent evolutionary history, and what factors maintain their color and size polymorphisms?

To address these questions, we measured hundreds of individuals across the range of *H. arborea*, and qualitatively assessed their background coloration and the intensity of the black and white lines of the *linea marginalis*. Through individual and population-based comparisons, we specifically tested whether these traits depend on i) the evolutionary lineages documented and the genetic diversity of populations, as expected if genetic divergence and expansion-associated drift are the main drivers; ii) the sex of individuals, as expected under sex-antagonistic selection; or iii) the populations only, as expected if phenotypic variation depends on local conditions.

METHODS

Data collection

Adult tree frogs were collected throughout the Balkans during spring 2011 as part of our fieldwork expedition to study the phylogeography of *H. arborea*, with a particular focus on sex chromosome evolution (Dufresnes *et al.* 2013, 2014). This agenda channeled our sampling towards large population samples of both sexes (ideally n > 10 males and 10 females), adequate for the present study. Another population from Western France (Normandy, NOR) sampled in spring 2013 complemented the dataset. Individuals were photographed from the side so that both the background color and the *linea marginalis* were visible. In total, 14 populations were included (n = 276 individuals, 176 males and 100 females), covering all three genetic lineages: S-Balkans (CRE, CGR, TES1-4, SSR), Adriatic (DAL, KRK, IST) and Central/NW-Europe (ZAG, VOJ, NEU, NOR). See Table 1 for the geographic coordinates of localities and their sample sizes. Genetic diversity indices (observed heterozygosity H_o and allelic richness A_r) were available from Dufresnes *et al.* (2013) for all but three of these populations (CGR, SSR, NOR). Body size (Snout-Vent Length, SVL) was measured for a large subset of individuals (n = 194) with a 1 mm ruler.

Indices of background color and lateral stripes

Background coloration is highly variable in tree frogs, especially because of their physiological plasticity. However, three classes of coloration can be differentiated in *H. arborea* (see also Nikolić *et al.* 2016) : a typical light green ("green"), a darker, olive-like green ("olive"), and a brown/grey color ("brown") (figure 1). Frogs may harbor lighter and darker tints for each base color, but still fall within these three discrete categories (figure 1).

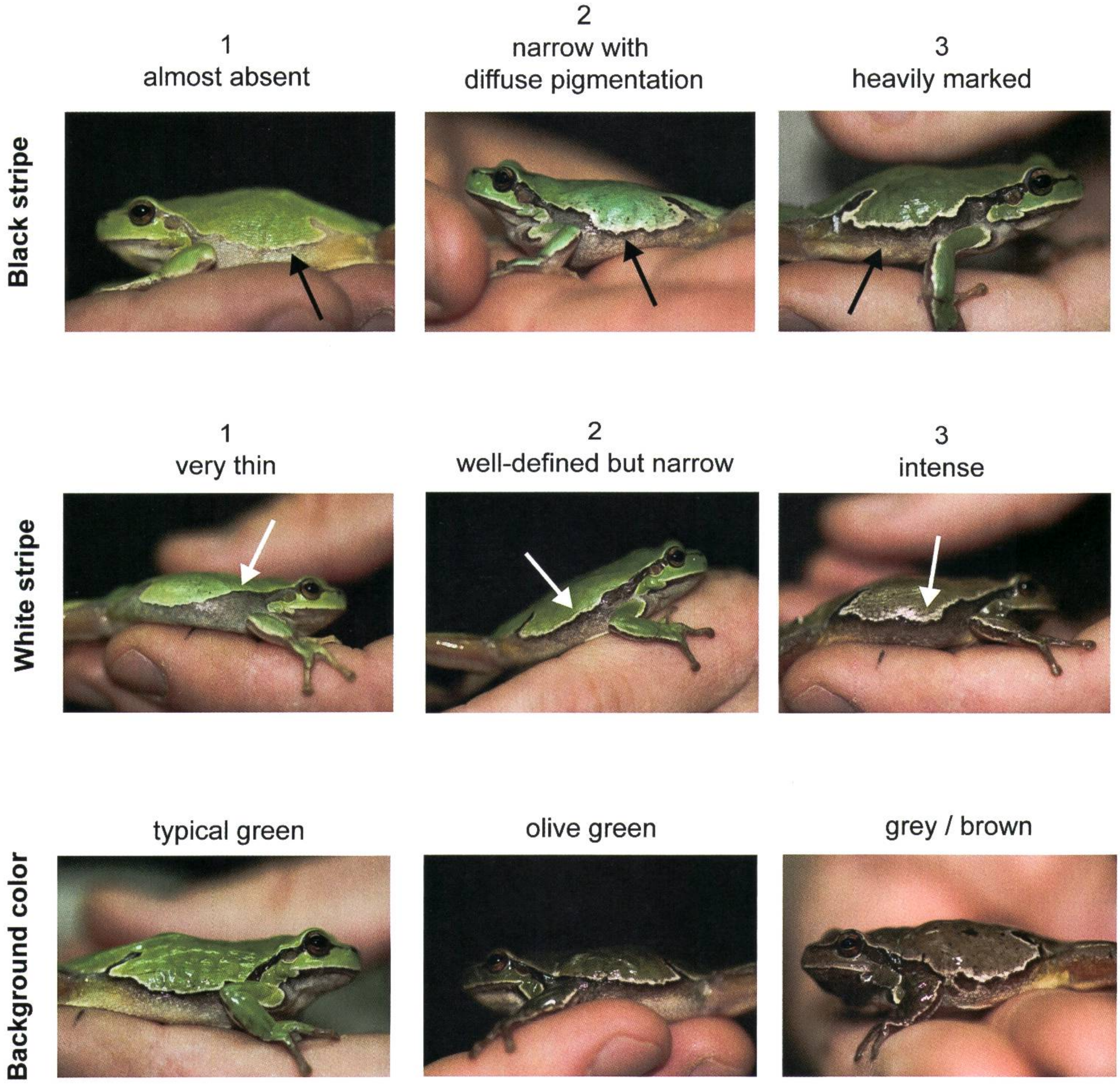

Figure 1. Coloration indices scored from the photographs : the black and white components of the *linea marginalis*, and the three distinct color morphs. These indices were independently scored by each author for every individual. Photos credit : HCFT-2011.

The white and black lines composing the *linea marginalis* greatly vary in shape and intensity. The complex patterns together with the heterogeneity of our data (photographs under different conditions) preclude any formal quantification. Instead, we classified the intensity of the white and black stripes separately, each under three categories: "1" stripe almost absent; "2" well-defined stripe but narrow (with diffuse melanism for the black stripe); "3" heavily marked / intense stripe. figure 1 exemplifies these categories for both lines.

Because these scorings are partly subjective, even more so when it comes to intermediate individuals, the five of us independently assigned a background color (COL: "green", "olive", "brown"), a black stripe score (BS: "1"; "2", "3") and a white stripe score (WS: "1", "2", "3") to each individual. We then computed our average scores for each variable. For each individual, mean BS and WS scores were further combined to build up two additional variables: the black / white ratio (BWR = BS / WS) and the total stripe intensity (TSI = BS + WS).

Statistical analyses

All analyses were conducted in R (R Core Team, 2016). A first set of analyses was performed at the individual level, in order to test for sex and population effects. The effect of population was initially checked by analyses of variance (ANOVAs) for the size (SVL) and stripe variables (BS, WS, BWR and TSI), and by a χ^2 test for the background coloration (which consists of the proportions of each color morphs). Then, we predicted each variable by sex (male or female) and phylogeographic lineage (S-Europe, Adriatic, or Central/N-Europe) by generalized linear mixed models (GLMMs), accounting for populations as a random factor. A backward selection procedure was applied, and significance was assessed by 1'000 bootstrap replicates (Faraway 2006).

A second set of analyses was conducted at the population level, where we assessed whether the average score and variability in stripe and color depend on the genetic diversity of populations. To this end, for each population, we computed the variance of each four stripe variables (BS, WS, BWR and TSI), and calculated an index of color diversity based on the relative frequencies of each color morph, as $C_d = \frac{n}{n-1}(1 - \sum_i x_i^2)$, where n is the sample size of the population and x_i the frequency of morph i. We fitted linear regression models to predict indices by the observed heterozygosity H_o, and the allelic richness A_r, available from Dufresnes *et al.* (2013). In addition, we also performed linear regressions to predict body size (SVL) by genetic diversity, latitude, and altitude. Only male sizes were considered, given that SVL showed sex-specific differences (see Results) and females were not captured in all populations (Table 1).

RESULTS

Background color

The proportion of the three background color morphs significantly differed between populations ($\chi^2 = 59.2$, $P < 0.001$, d.f. = 20, figure 2, 3), but were neither predicted by sex ($P = 0.75$), lineages ($P = 0.87$), nor by their interaction ($P = 0.71$) in the GLMM. At the population level, the diversity of color morphs C_d was not related to H_o nor A_r (Table 2).

Lateral stripes

All four stripe variables significantly varied among localities (ANOVA' $F = 3.1\text{-}8.1$, $P < 0.001$ in all cases). According to the GLMMs, the black / white ratio (BWR) was further affected by

Table 1. Geographic origin of populations, number of frogs examined for the color polymorphism (detailed per sex) and average body size (SVL in mm, sample sizes in parenthesis).

Code	Latitude	Longitude	Sample size	SVL (♂)	SVL (♀)
CRE	35.1897	25.4557	51 (25 ♂ - 26 ♀)	42.8 (25)	45.6 (25)
CGR	38.4426	21.1251	2 (1 ♂ - 1 ♀)	-	-
SSR	42.3288	21.8964	2 (1 ♂ - 1 ♀)	-	-
TES1	40.7220	22.6671	15 (12 ♂ - 3 ♀)	42.5 (12)	52.3 (3)
TES2	41.1816	22.7611	21 (21 ♂)	44.0 (21)	-
TES3	40.8200	22.8490	21 (21 ♂)	44.8 (21)	-
TES4	40.5454	22.5949	20 (21 ♂)	37.9 (20)	-
DAL	43.9405	15.5221	4 (2 ♂ - 2 ♀)	44.5 (2)	45.0 (2)
KRK	45.1704	14.6229	23 (12 ♂ - 11 ♀)	40.5 (12)	43.5 (11)
IST	45.3405	13.8101	36 (18 ♂ - 18 ♀)	49.3 (6)	50.7 (6)
ZAG	45.5435	15.5729	22 (11 ♂ - 11 ♀)	42.2 (11)	44.2 (11)
VOJ	44.7422	20.1382	38 (18 ♂ - 20 ♀)	36.7 (3)	38.0 (3)
NEU	47.9261	16.8634	8 (4 ♂ - 4 ♀)	-	-
NOR	49.3318	-0.6280	13 (9 ♂ - 4 ♀)	-	-

sex (being white-biased in males) and by lineage (being white-biased in S-Europe compared to the two other regions) (Table 2, figure 2D, 3). Accordingly, the white stripe score (WS) differed by lineage, while the black stripe (BS) did not (Table 2). The total stripe intensity (TSI) appeared irrespective of sex and lineage (Table 2, figure 2C). At the population level, the genetic diversity (H_o and A_r) was not related to the the variance of TSI, BWR, BS and WS, nor their population average (Table 2).

Body size

Snout-Vent Length (SVL) significantly differed among populations (ANOVA' $F = 13.9$, $P < 0.001$). Sex was a significant predictor in the GLMM, but not lineage, nor their interaction (Table 2, figure 2B). Average male SVL was not related to the the H_o , A_r (Table 2), the latitude ($P = 0.62$) or the altitude ($P = 0.99$) of populations. The average sizes for each population are reported in Table 1. Over the entire range, female SVL averaged 45.4 mm and male SVL averaged 42.5 mm.

DISCUSSION

We present a range-wide phylogeographic analysis of the variation at three phenotypic traits in *Hyla arborea*: the overall background coloration, the color pattern of the lateral stripe *linea marginalis*, and body size. Most of this polymorphism depended on localities, but none of it was associated to genetic diversity and differentiation. This result is consistent with a similar analysis among French populations (GOMEZ *et al.* 2011), and suggests that micro-evolution and/or phenotypic plasticity at the local level are the main drivers of color patterns in *H. arborea*, e. g. as a response to local selective pressures, environmental conditions, or rapid demographic fluctuations resulting from meta-population dynamics.

Table 2. P-values associated with each quantitative variable in the statistical tests (individual-based: GLMMs; population-based: linear regression models). SVL: Snout-Vent Length (tested for males only); TSI: Total Stripe Intensity; BWR: Black / White Ratio; BS: Black Stripe; WS: White Stripe; C_d: Color Diversity; H_o: Observed Heterozygosity; A_r: Allelic Richness (scaled to five individuals). H_o and A_r originate from Dufresnes *et al.* (2013). Significant P-values are highlighted in bold.

Individual-based (GLMM)	**Sex**	**Lineage**	**interaction**
SVL	**<0.001**	0.20	0.25
TSI	0.17	0.33	0.68
BWR	**0.015**	**0.005**	0.40
BS	0.82	0.11	0.28
WS	**0.003**	**0.02**	0.97

Population-based (LM)	**H_o**	**A_r**
average SVL (♂)	0.90	0.85
C_d	0.78	0.80
average TSI	0.89	0.82
variance of TSI	0.98	0.98
average BWR	0.06	0.05
variance of BWR	0.44	0.50
average BS	0.27	0.28
variance of BS	0.69	0.57
average WS	0.11	0.08
variance of WS	0.97	0.95

Yet, some aspects of the results may still reflect forces acting at a more global scale. A previous study (Gomez *et al.* 2009) suggested that some visual features were selected by females, namely the color of the vocal sac (as a cue of male quality) and the lateral stripe (enhancing nocturnal detection of mates). In an experimental setup, females accordingly preferred conspicuous males (Gomez *et al.* 2009). Here the *linea marginalis* was significantly whiter in males than females, which would fit this latter hypothesis (figure 2). While the overall intensity of the stripe appears unimportant, the white component may enhance contrast with the dark line and the background color. Most olive or brown individuals were males (figure 2A), and their whiter lateral stripes would thus provide a particularly striking picture on these dark backgrounds.

Furthermore, the *linea marginalis* appears significantly whiter in the southern phylogeographic lineage compared to the Adriatic and Central European ones (figure 2D, 3). This specificity may be associated to the genetic divergence of southern populations, and/or to environmental factors linked to their Mediterranean habitats. It is worth noting however, that the more intense solar radiation in southern compared to northwestern ranges should rather trigger a selective response in the opposite direction, i.e. towards melanism. Experimental UV-B exposure is known to cause skin darkening in *H. arborea* (Langhelle *et al.* 1999). Yet, tree frogs can also respond physiologically by adjusting their overall tint, which can be rapid (especially under stress, Vitt & Caldwell 2014), and so the brightness of the lateral stripe

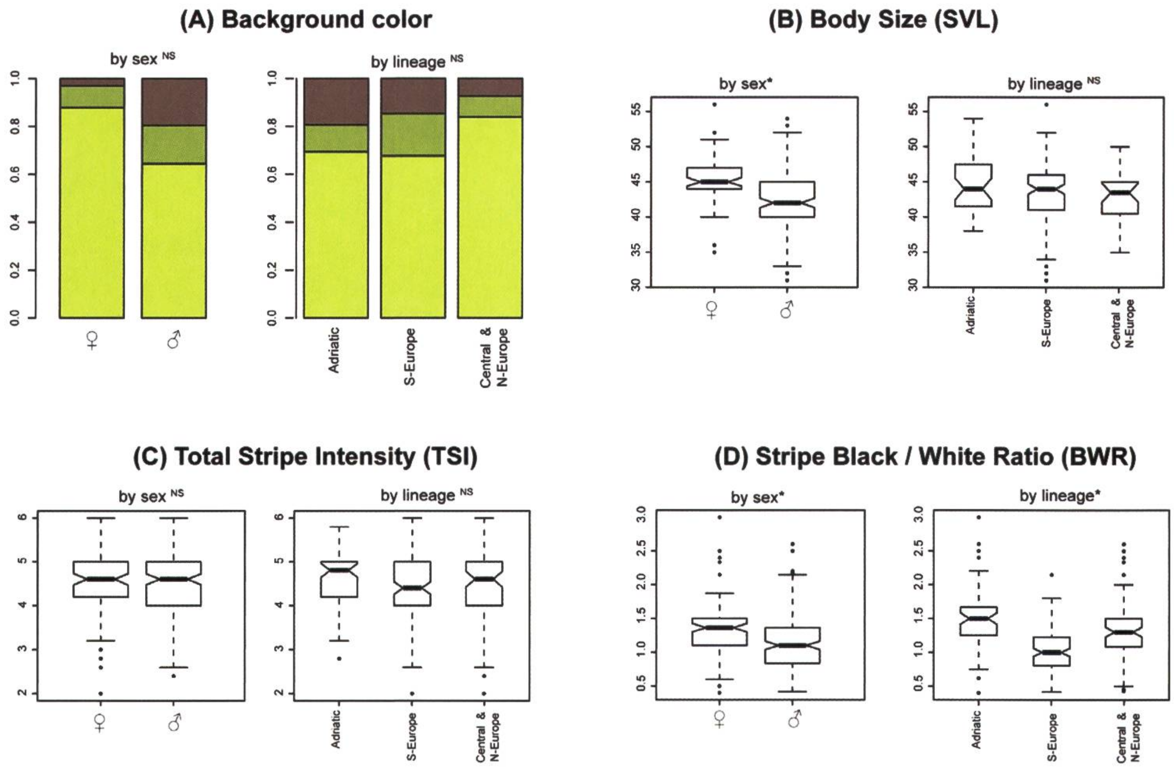

Figure 2. Variation in the proportion of color morphs (A), body size (B), total stripe intensity (C) and black / white ratio (D), in respect to sex and phylogeographic lineage. *: significant differences ; ^{NS}: no significant difference. See Table 2 and the Results section for details.

may have little influence on thermoregulation. Therefore, while the proximate mechanisms underlying the coloration traits investigated here remain elusive, the documented variation may serve as a basis to test specific hypotheses in future experimental setups.

Finally, our analyses confirmed that female tree frogs were overall larger than males throughout the range of *H. arborea* (figure 2), a known sexual dimorphism of European hylids (Nöllert & Nöllert 2003, Dufresnes 2019). Contrarily to previous work from Anatolia (*H. orientalis*, Özdemir *et al.* 2012), we did not find a relationship between size and latitude (nor altitude). This result agrees with most studies on amphibians, which similarly provide little support for Bergmann's rule (Adams & Church 2008). The strong population effect rather reflects local conditions in constraining body size, such as food availability, seasonal activity or the age structure of populations. Amphibians grow until sexual maturity, which age was shown to depend on local conditions (Cvetković *et al.* 2008). Fine-scaled demographic and ecological data will be required to appreciate the complex eco-geographical factors driving phenotypic variation in *H. arborea*.

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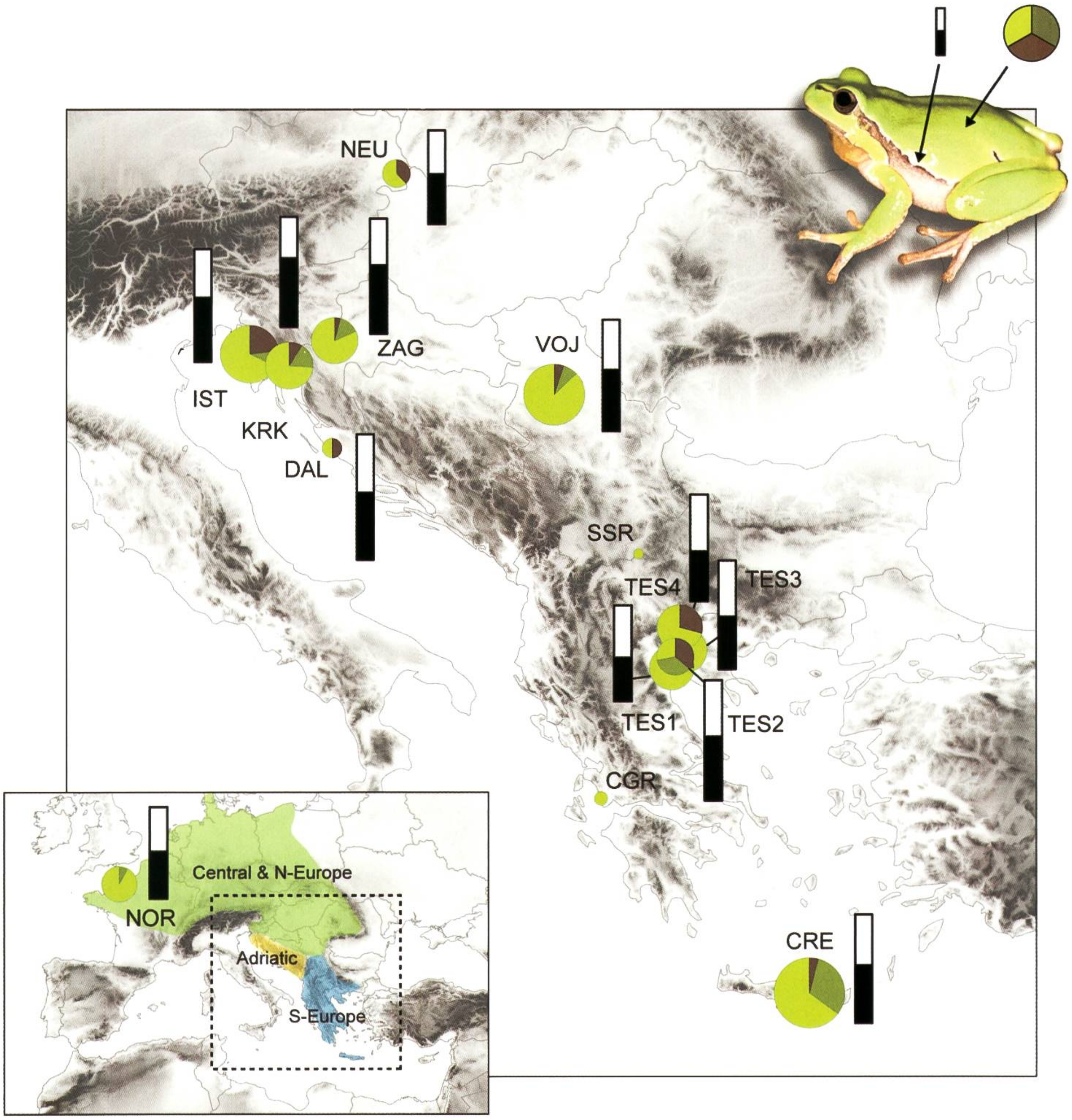

Figure 3. Geographical pattern of the proportion of color morphs (pie charts), and the black and white variation of the *linea marginalis* (barplots). Pie sizes are proportional to sample size. The bottom left frame shows the distribution of the three phylogeographic lineages, according to Dufresnes *et al.* (2013). Photo credit: C. Dufresnes.

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