

1 **Global DNA methylation, as estimated in blood samples, does not correlate with**
2 **variations of body condition, anatomical features and hematological parameters in**
3 **American bullfrogs (*Lithobates catesbeianus*) kept captive under distinct**
4 **environmental conditions.**

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14 **Summary statement**

Previous reports support that Global DNA methylation, as estimated in blood samples, correlate with
16 trait variability. Here we did not corroborate this assertion while testing in American bullfrogs that
displayed divergence on some phenotypic traits.

18

Abstract

20 Different levels of Global DNA Methylation (GDM) could have facilitated the emergence of new
species, without relying on gene mutations, through promoting ontogenetic phenotypic plasticity. If this
22 assertion was correct, one could expect individuals of the same species living under distinct
environmental conditions to be genetically similar, but having different GDM levels and being
24 phenotypically divergent. We tested this presumption by studying the relationship between variability
of functional morphological traits and GDM levels in American bullfrog (*Lithobates catesbeianus*), in
26 green houses located in two geographical sites. Our analyses revealed that body linear morphometry,
skull geometry, scaled mass index, packed cell volume and neutrophil counts differed significantly
28 among males and females within and between localities. GDM, nonetheless, was rather similar among
sex and locality groups. These results show that levels of GDM, at least under our experimental
30 contexts, does not correlate with functional morphological trait variability.

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36

38 **Introduction**

40 Shifts in DNA methylation are one of the epigenetic processes that are presumed to contribute to
41 generate non-mutational phenotype variability, within and across animal populations by coding
42 environmental information(Baerwald *et al.*, 2016). It is by modulating the degree of transcriptional
43 elongation(Rountree and Selker, 1997) and chromatin compaction/relaxation (Bogdanovic *et al.*, 2011),
44 that DNA methylation regulate patterns of gene expression and ontogeny (Dunican *et al.*, 2008).
45 Methylation takes place in the fifth carbon of cytosine residues (i.e., 5-methyl-cytosine), within gene
46 bodies, enhancers and promoter regions, in intergenic regions, among others (Stancheva *et al.*, 2002).
47 The chemical reaction involved in this process is catalyzed by a family of methyl-transferase
48 enzymes(Stancheva, Hensey and Meehan, 2001).

50 Different levels of Global DNA methylation (GDM) occur among vertebrate classes and
51 orders(Vanyushin *et al.*, 1973). Nowadays, it is presumed that shifts in global DNA methylation (GDM)
52 are important for understanding phenotype plasticity promoted by daily live interaction of the organism
53 with the environment(Zhu *et al.*, 2012), and hence, favor non-mutational phenotype variability during
54 vertebrate evolution(Ponger and Li, 2005). For instance, monotremes have the highest levels of
55 methylated DNA, followed by placentals and marsupials. In a similar vein, amphibian and fish DNA is
56 about twice as methylated as that of reptiles, birds and mammals(Jabbari *et al.*, 1997). Therefore, shifts
57 in GDM are thought to have contributed in driving the “cold to warm transition” during vertebrate
58 evolution(Varriale and Bernardi, 2006b). That environmental factors may shift GDM, is supported by
59 reports that show that fish living near the poles have higher values of GDM, as estimated in blood
60 samples, than those reported for fish living in temperate or tropical areas(Varriale and Bernardi, 2006a).
61 Since differences in GDM were reported to occur in fish species whose phylogenetic relatedness is
62 high, the results reported further support that interspecies GDM in part reflects differential
63 environmental exposure(Varriale, 2014). However, it remains unclear whether this might be true for
64 phenotype variability observed in individuals of a single species that are exposed to distinct
65 environments.

66

Domestication and artificial selection has traditional been a powerful tool to infer rules governing the evolution of species(Rivas Sanchez and Rivas Sanchez, 2015). Here, we turn to this strategy to evaluate whether trait variability, as analized through linear morphometry, skull geometric morphometry, body condition, whole white blood cell count, packed cell volume (PCV) and differential leukocyte counting, is correlated with GDM levels in bullfrogs subjected to distinct environments. Bullfrogs are an adequate experimental unit since their colonies display reduced genetic diversity(Bai *et al.*, 2012), but a great deal of phenotypic plasticity. In fact, this ability has made bullfrogs rather successful dwellers, They have literally colonized a variety of habitats throughout the globe (Govindarajulu, Price and Anholt, 2006).

76

78 **Methods**

80 ***Animals***

A total of sixty bullfrogs (*L. catesbeianus*; 1 year old; 15 males / 15 females per locality, n=15) were obtained from “La Purísima” (LPu) and “San Pedro Tlaltizapán” (SPt), two rustic greenhouses located at the State of Michoacán (19°52'11"N, 101°01'23"W) and at the Estado de México (19°20'11"N, 99°49'79"W), respectively. LPu (6033,465 ft) is about 6°C warmer than in SPt (8448,163 ft) through the year. In both sites male and female frogs were kept captive in groups of 100 individuals that co-inhabited large, cement-made tanks (2.5m²). Frogs were fed twice a day with commercial trout food provided by a local supplier (El Pedregal, Toluca, Estado de México; composition: 45% protein, 16% fat, 2.5% fiber, 12% ash, 12% humidity). Frogs were transferred to the laboratory in 200 liters plastic containers. Transportation took no more than six hours from frog capturing to freeing them in the laboratory enclosure. Frogs were kept under laboratory condition during 12 hours before sacrifice. All efforts were made to minimize stress; for instance, frogs were kept in a quiet and dark room and were not disturbed until sacrifice. Animal handling and experimentation followed federal guidelines recommended by the Mexican Official Norm on production, housing and handling of laboratory animals (NOM-62-ZOO-1999). All sections of this paper were made according to the “ARRIVE guidelines for reporting animal research” (Kilkenny *et al.*, 2010).

96

Assessing body condition

98 Body condition was estimated in LPu and SPt, male and female bullfrogs by using the Scaled Mass

100 Index(MacCracken and Stebbings, 2012). This condition index (CI), is better than the OLS residual method and other CI's because it correlates better with fat reserves and lean dry mass in reptiles and mammals (Peig, Green and Ame, 2009). The SMI was calculated using the following formula:

102
$$IME = Mi \left[\frac{Lo}{Li} \right] e^{bsma}$$

104 Where Mi and Li stand for individual mass and snout-vent length, Lo is the arithmetic mean of all the animals sampled, and “ $bsma$ ” is the coefficient of a standard major axis regression for all the mass and
106 length data from both male and female, mature and immature individuals.

108 ***Hematological parameters***

Blood cell composition was evaluated in blood samples obtained from juvenile, male and female
110 bullfrogs anesthetized with ice following their decapitation. Blood was collected in EDTA-treated tubes and stored at -4°C. Packed cell volume (PCV) was estimated after centrifuging 2ml of blood at 4,500
112 rpm for 15 minutes, and then measuring the volume of both serum and hematocrit. Leukocyte counts (WBC) were done in a hemocytometer after conventional Turk-solution treatment (for at least 5
114 minutes) of blood samples. Leukocyte differential counts were done on blood smears stained with Wright reagent for 5 minutes. A total of 100 cells in each smear were counted and classified as
116 lymphocytes, neutrophils, monocytes, basophils and eosinophils, following specific guidelines of amphibian hematology (Allender and Fry, 2008).

118

Morphometrics

120 Linear morphometric analysis was done on 7 lineal measurements taken with a precision Veneer caliper (0.01 cm error). Measures include snout-vent length, head width and length, timpanum and eye
122 diameter, leg size (femur plus tibio-fibula), feet (tarus plus metatarsus). For the geometric analysis, we used 10 skulls (n=10) that were previously cleaned with dissecting tools and using commercially
124 bleacher for a few minutes to avoid damage. Digital photographs from the ventral aspect of the skull were taken and used to place ten landmarks as suggested for the *Rana* genus (Fig. S1). The positioning
126 of the landmarks was done according to (Larson, 2002, 2005). TpsUtil and Tps2 (James Rohlf, Stony Brook University) software was used to generate landmark information from the digital images.

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Estimating global DNA methylation (GDM)

132 DNA was isolated from blood samples using the propanol extraction protocol (Miller, Dykes and
Polesky, 1988). DNA concentration and purity was estimated using a Nano-drop 1000 (Thermo-Fisher,
134 Waltham MA, USA). DNA integrity was evaluated using 1% agarose gel electrophoresis. The
percentage of 5-methylcytosine was estimated using an ELISA kit following manufacturer's protocol
136 (Zymo D5326, Irving CA, USA). Briefly, 100ng of double stranded DNA were denatured for 5 minutes
in a thermocycler (Axygen Union City CA, USA). Then, DNA samples were adsorbed each to the
138 walls of wells of 96-well plates. Each assays was conducted by duplicate. After blocking, anti-5-mC
primary monoclonal antibody (1:2,000) and HRP secondary antibody (1:1,000) were mixed, and wells
140 were incubated with 100 μ l of this solution for 1 hour at 37°C (information about animals used to raise.
Following several washes, HRP activity was revealed by adding 100 μ l of manufacturer's HRP
142 developer during 10 to 60 minutes at room temperature. Absorbance was estimated at 480 nm with the
aid of an ELISA plaque reader (Biotek, Winooski VT, USA). The percentage of DNA methylation in
144 each sample was calculated after fitting logarithmic second-order regression on the manufacturer's
standard curve absorbance values.

146

Statistical analysis and software

148 Morphological data from both groups were compared by using a multivariate analysis conducted
through canonical discriminant tests (CDA). We used JMP statistical software (SAS, version 10)
150 and MorphoJ 2.0 (Obtained from Klingenberg's lab official
site:http://www.flywings.org.uk/morphoj_page.htm). This method was used instead of principal
152 component analysis (PCA) because it maximizes the degree to which pre-defined groups can be
distinguished. To find any difference between experimental groups, CV1 and CV2 were used to
154 perform Two-way ANOVA (R 3.1.1) on linear morphometrics; for geometric analysis, Mahalanobis
procruster distance was calculated (MDA. MorphoJ 2.0). Body condition, whole white blood cell
156 count, packed cell volume and differential leukocyte number are presented as mean \pm s.e.m. After
testing equal variance, Shapiro-Wilk test was used to ensure normality of the data. To test differences
158 between groups from body condition, hematology and GDM variables, we used a One-way ANOVA
followed by a Tuckey post-hoc test, with an alpha level of < 0.05 . R 3.1.1 was used for statistical
160 analysis and Sigma Plot 12 to create graphs.

162

164 **Results**

In this experiments, we tested the possible correlation between phenotypic variability and GDM by
166 using bullfrogs from two localities, a species that display a great deal of environmentally driven
phenotypic plasticity in the absence of genetic mutations. Both female and male bullfrogs were larger
168 in SPt than in LPu. The overall morphometry between females from both sites was similar, whereas
differences could be seen in their male counterparts (Fig. 1A). Differences between female and male
170 frogs from LPu were greater than in SPt, thus sex and locality exert a combined effect. (2-ANOVA;
 $p=0.0004$). This results suggests that sexual dimorphism is present at both sites but is more noticeable
172 in LPu. On the other hand, skull geometry behaved differently in both populations (Fig. 1B). In LPu,
there was a sharp difference between female and male skull shape ($p<0.0001$). This was not observed
174 in SPt (MDA; $p=0.1$). Lastly, even though no differences in skull shape were documented between
females of both localities (MDA; $p=0.2$), males did differ significantly (MDA; $p<0.0001$).

176

Body condition, as estimated by the Scaled Mass Index, differed between LPu and SPt bullfrogs (Table
178 1). Both male and female frogs from SPt had a higher body condition than LPu ones (ANOVA,
 $p<0.0001$). As shown in Table 1, PCV levels were higher in both male and female frogs from LPu than
180 SPt animals (ANOVA; $p<0.0001$). WBC counts on the contrary, did not differ between among groups
(ANOVA; $p=0.96$). Overall, neutrophil number was greater in LPu than in SPt. In the former locality,
182 nonetheless, neutrophil number was higher in male than in female frogs (ANOVA; $p<0.05$. Table 2). In
SPt, monocyte number was 3.4-fold higher in males than in females. In addition, monocyte number of
184 males from SPt was 6.9-fold higher than that observed in specimens of both sexes raised in LPu (Table
2).

186

Global DNA methylation estimated in blood samples showed neither differences between sex or
188 locality (Table 1). Moreover, there was no correlation between this variable with any of the others. A
multivariate analysis of the phenotypic variation in response of GDM did not any significant
190 correlation (not shown)

192 **Discussion**

194 Shifts in GDM are thought to contribute in generating phenotype variability during evolutionary
processes (Jabbari *et al.*, 1997; Varriale and Bernardi, 2006b). Even though significant differences in

196 GDM seem to associate with trait variability when comparing animal classes (Jabbari *et al.*, 1997), this
presumption may not be valid for explaining trait variability among individuals belonging to the same
198 species. Accordingly, here we showed that bullfrogs raised in two localities differ significantly in body
linear morphometry, skull geometry (Fig. 1), scaled mass index, packed cell volume and neutrophil
200 counts (Tables 1 - 2). GDM, however, was similar between all groups, and not correlated with other
variables (Table 1). Hence GDM, under current experimental context, does not correlate with trait
202 variability.

204

The fact that GDM did not correlate with any phenotypic variable is at odds with previous studies. For
206 instance, fish species belonging to the same family are known to have different GDM levels depending
on the temperature they are exposed to. This indeed is the case for *Symphodus tinca*, a Mediterranean
208 fish that exhibits the highest levels of GDM among the members of Labridae family (Varriale and
Bernardi, 2006a). Moreover, expecting that GDM levels could differ between bullfrogs from SPt and
210 LPu was a reasonable presumption since even different organ within single individuals may have
different GDM levels, as shown in rats, mice and monkeys (Gama-Sosa *et al.*, 1983). Although we do
212 not currently have an explanation for these counterintuitive results, it is fair to say that we cannot rule
out fully that global methylation is unrelated with trait variability since differential methylation may
214 occur in distinct genes throughout the genome for individuals coming from one locality or the other.
Accordingly, ELISA GDM analysis may not be the correct tool for approaching inter-individual
216 variation within single species. It may be more instructive to analyze global patterns of DNA
methylation as shown for fish (Baerwald *et al.*, 2016), birds (Liebl *et al.*, 2013), reptiles (Venegas *et al.*,
218 2016) and mammals (Chang *et al.*, 2006).

220 Recent evidence suggests that GDM may participate in the process of generating non-mutational
phenotypic variability in natural conditions (Varriale, 2014). Bullfrogs living out of their native range
222 exhibit a highly variable phenotype that could be attributed to the effect of being exposed to distinct
environmental niches (Govindarajulu, Price and Anholt, 2006). In this work, we found no correlation
224 between morphological nor physiological traits with levels of GDM. However, our results show that
intraspecific sex variability is also present in bullfrogs even under relatively controlled conditions
226 (Figs. 1A-B), thus suggesting that sexual dimorphism in bullfrogs can be influenced by environmental
factors. Future studies must establish whether global patterns of DNA methylation can explain these
228 interesting findings.

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234

Competing interests

236 The authors declare no competing financial interests regarding the elaboration of the study and the
preparation of the manuscript.

238

Author contribution

240 First and last author contributed equally to the presented study. Second author contributed with
preparation of the manuscript and data analysis.

242

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248 **Data availability**

250 The data used for the analysis of this study can be found in Dryad: (after submission

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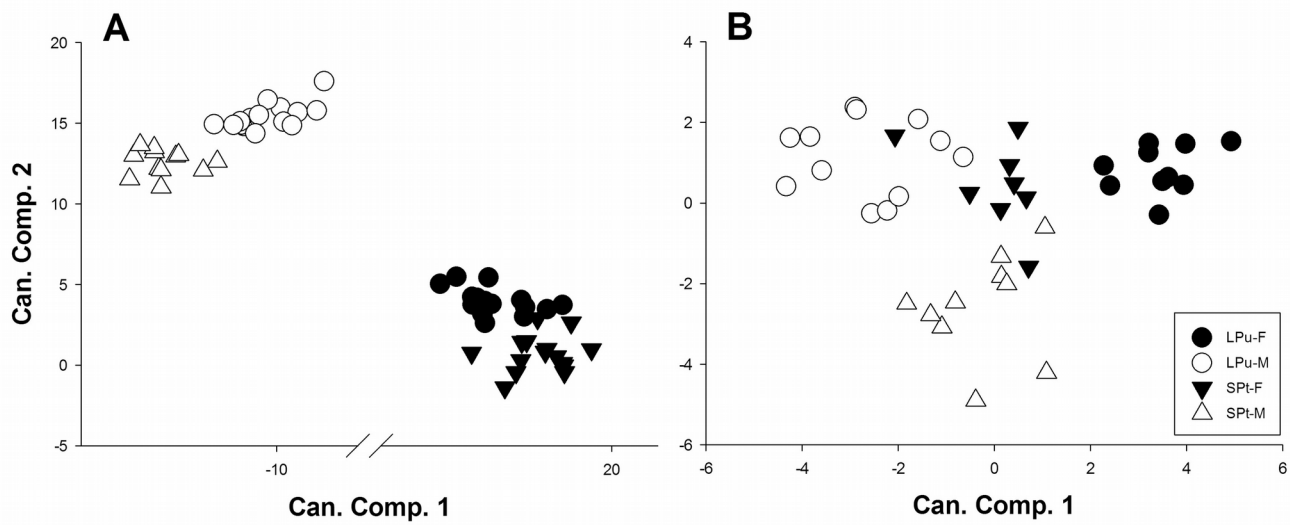
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Figure legends

334



336 **Figure 1. Linear and geometric morphometrics.** Canonical variate analysis was performed on 7
 338 lineal measurements for linear morphometrics (A), and on ten landmark extracted from ventral digital
 340 photos of the skull (B) of female and male bullfrogs. Canonical component (CP) 1 was plotted against
 CP2 for every frog. We used 30 frogs (15 female and 15 male, n=15) for linear morphometrics and 20
 skulls (10 female, and 10 male, n=10) from each of the two localities. Black circles correspond to LPu
 females, open circles to LPu males, black triangles to SPt females and open triangles to males (n = 15).

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Tables

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	SMI		PCV (%)		WBC (# cells)		5-mC (%)	
LPu-F	268.5±5.48	a	34.6±0.88	a	3584.6±239	a	5.24±0.31	a
LPu-M	268.9±10.47	a	35.9±0.91	a	3746.6±176	a	4.86±0.25	a
SPt-F	335.9±7.44	b	27.2±1.39	b	3797.7±355	a	5.15±0.24	a
SPt-M	317.1±6.51	b	29.5±1.21	b	3731.8±324	a	5.56±0.25	a

Tuckey-PH α 0.05

354

356 **Table 1. Physiological parameters and global content of 5-mC.** Scaled mass index, packed cell
volume, whole blood cell count and the percentage of 5-mC were measured in 15 male and 15 femal
358 frogs from two localities (LPu and Spt, n= 15). Mean and s.e.m. are reported. Letters indicate statistical
significance after Tuckey post-hoc test on a One way-ANOVA. For PCV, WBC and 5-mC, blood
samples were used (for sample processing see materials and methods).

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362

	Neutrophils		Lymphocytes		Monocytes		Basophils		Eosinophils	
LPu-F	1154.27±169.9	ab	1997.16±225.1	a	36.61±8.63	a	230.55±51.1	a	125.83±26.16	a
LPu-M	1464.53±128.1	b	1791.14±103.1	a	31.07±7.6	a	233.17±49	a	197.92±49	a
SPt-F	730.84±63.5	a	2093.4±236.3	a	68.69±19.8	a	413.32±75.9	a	191±29.4	a
SPt-M	933.61±120	a	1927.84±189.1	a	233.65±40.8	b	349.13±53	a	236.27±50	a

Tuckey-PH α 0.05

366 **Table 2. Differential leukocyte count.** The five principal cell types in blood samples were measured in
15 male and 15 femal frogs from two localities using a conventional Wright staining technique (LPu
and SPt, n=15). Mean and s.e.m. are reported, and letters indicate statistical significance after Tuckey
368 post-hoc test on a One way-ANOVA.

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