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

Braulio Ayala García[1]*, Alma L. Fuentes-Farías[2], Gabriel Gutiérrez Ospina[1]*.

Affiliation

[1]Systems Biology Laboratory, Departamento de Biología Celular y Fisiología, Instituto de Investigaciones Biomédicas. Universidad Nacional Autónoma de México, 04510, Ciudad de México, México.

[2]Animal Ecophysiology Laboratory, Departamento de Zoología, Instituto de Investigaciones Sobre Recursos Naturales, Universidad Michoacana de San Nicolás de Hidalgo,Morelia, Michoacán, Mexico.

Summary statement

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

Abstract

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 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 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 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 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 contexts, does not correlate with functional morphological trait variability.

*To whon correspondance should be adressed: braulioayala13@gmail.com +52 55 56 22 9195, gabo@biomedicas.unam.mx +52 55 56 22 9195

Introduction

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

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

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).

Methods

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 (2.5m^2). 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).

Assessing body condition

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

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:

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

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 length data from both male and female, mature and immature individuals.

Hematological parameters

Blood cell composition was evaluated in blood samples obtained from juvenile, male and female 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 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 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 lymphocytes, neutrophils, monocytes, basophils and eosinophils, following specific guidelines of amphibian hematology (Allender and Fry, 2008).

Morphometrics

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 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 bleacher for a few minutes to avoid damage. Digital photographies 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 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.

Estimating global DNA methylation (GDM)

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, Waltham MA, USA). DNA integrity was evaluated using 1% agarose gel electrophoresis. The percentage of 5-methylcitosine was estimated using an ELISA kit following manufacturer´s protocol (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 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 were incubated with 100 µl 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 µl of manufacturer's HRP 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 each sample was calculated after fitting logarithmic second-order regression on the manufacturer's standard curve absorbance values.

Statistical analysis and software

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) 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 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 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 count, packed cell volume and differential leukocyte number are presented as mean ± s.e.m. After testing equal variance, Shapiro-Wilk test was used to ensure normality of the data. To test differences 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 analysis and Sigma Plot 12 to create graphs.

Results

In this experiments, we tested the possible correlation between phenotypic variability and GDM by 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 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 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 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 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; $p<0.0001$).

Body condition, as estimated by the Scaled Mass Index, differed between LPu and SPt bullfrogs (Table 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 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, 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 males from SPt was 6.9-fold higher than that observed in specimens of both sexes raised in LPu (Table 2).

Global DNA methylation estimated in blood samples showed neither differences between sex or 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 correlation (not shown)

Discussion

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.

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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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Competing interests

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

Author contribution

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

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Data availability

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

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Figure legends

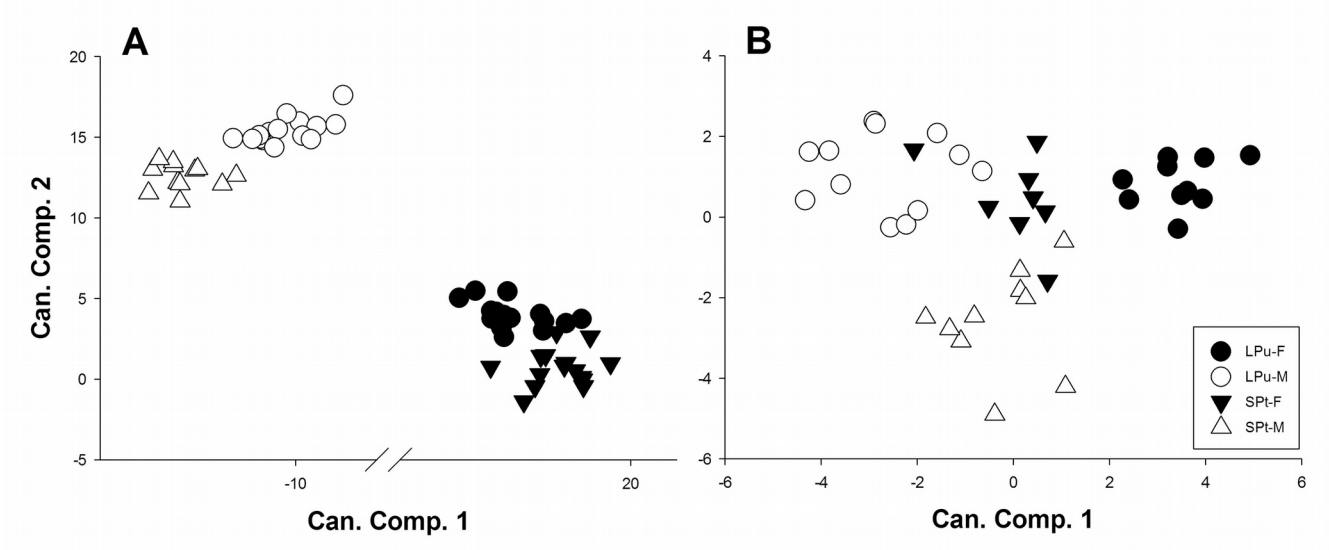

Figure 1. Linear and geometric morphometrics. Canonical variate analysis was performed on 7 lineal measurements for linear morphometrics (A), and on ten landmark extracted from ventral digital photos of the skull (B) of female and male bullfrogs. Canonical component (CP) 1 was plotted against CP2 for 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).

Tables

	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

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 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).

	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

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 post-hoc test on a One way-ANOVA.