

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.**

Braulio Ayala García<sup>1\*</sup>, Alma L. Fuentes-Farías<sup>2</sup>, Gabriel Gutiérrez Ospina<sup>1\*</sup>.

6

**Affiliation**

8 <sup>1</sup>Systems Biology Laboratory, Departamento de Biología Celular y Fisiología, Instituto de  
Investigaciones Biomédicas. Universidad Nacional Autónoma de México, 04510, Ciudad de México,  
10 México.

<sup>2</sup>Animal Ecophysiology Laboratory, Departamento de Zoología, Instituto de Investigaciones Sobre  
12 Recursos Naturales, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, Mexico.

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.

32

\*To whom correspondance should be addressed: [braulioayala13@gmail.com](mailto:braulioayala13@gmail.com) +52 55 56 22 9195,

34 [gabo@biomedicas.unam.mx](mailto:gabo@biomedicas.unam.mx) +52 55 56 22 9195

36

## 38 **Introduction**

40 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  
42 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),  
44 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  
46 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  
48 enzymes(Stancheva, Hensey and Meehan, 2001).

50 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)  
52 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  
54 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  
56 about twice as methylated as that of reptiles, birds and mammals(Jabbari *et al.*, 1997). Therefore, shifts  
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  
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).  
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  
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  
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 (2.5m<sup>2</sup>). 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.

128

130

### ***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  $\mu$ 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  $\mu$ 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](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.

## 230 **Acknowledgements**

We thank Dr. Jesús Ramírez Santos and Dr. Margarita Gómez Chavarín for their technical support. We  
232 also thank Posgrado en Ciencias de la Producción y de la Salud Animal (UNAM), and the Consejo  
Nacional de Ciencia y Tecnología, through which this study was conducted.

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

## **Funding**

244 This study was funded with institutional resources if the Instituto de Investigaciones Biomédicas of the  
Universidad Nacional Autónoma de México. The first author was a PhD student granted by the Consejo  
246 Nacional de Ciencia de Ciencia y Tecnología (CONACYT).

## 248 **Data availability**

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

252

## **References**

254

**Allender, M. C. and Fry, M. M.** (2008) Amphibian hematology. *The veterinary clinics of North  
256 America. Exotic animal practice.* **11**(3), 463–80. doi: 10.1016/j.cvex.2008.03.006.

**Baerwald, M. R., Meek, M. H., Stephens, M. R., Nagarajan, R. P., Goodbla, A. M., Tomalty, K.  
258 M. H., Thorgaard, G. H., May, B. and Nichols, K. M.** (2016). Migration-related phenotypic  
divergence is associated with epigenetic modifications in rainbow trout, *Molecular Ecology.* **25**(8),  
260 1785–1800. doi: 10.1111/mec.13231.

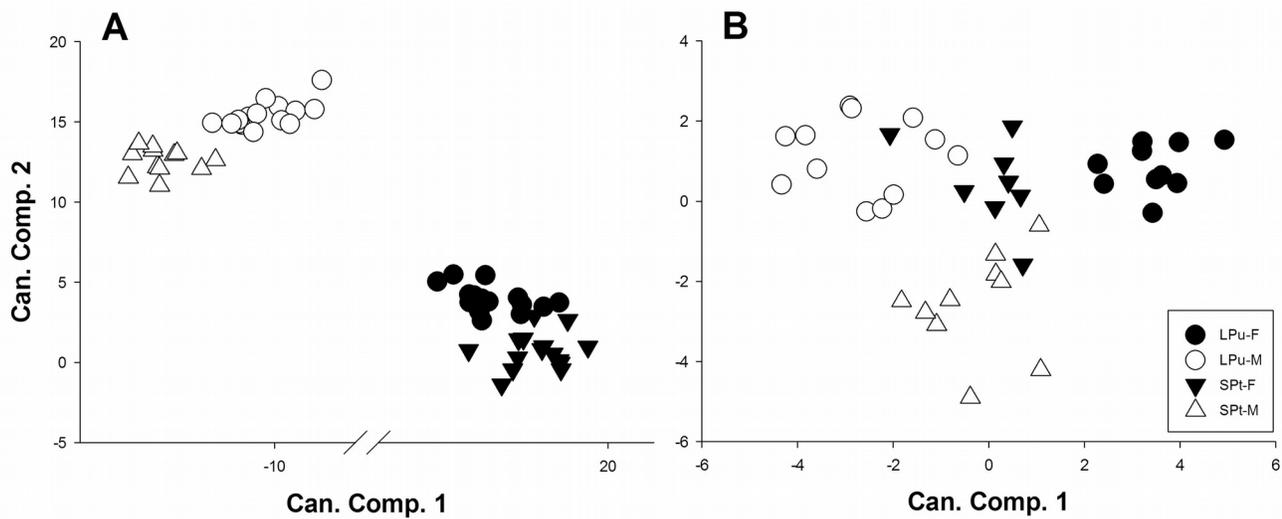
**Bai, C., Ke, Z., Consuegra, S., Liu, X. and Li, Y.** (2012). The role of founder effects on the genetic

- 262 structure of the invasive bullfrog (*Lithobates catesbeianus*) in China. *Biological Invasions*, 14(9),  
1785–1796. doi: 10.1007/s10530-012-0189-x.
- 264 **Bogdanovic, O., Long, S. W., van Heeringen, S. J., Brinkman, A. B., Gómez-Skarmeta, J. L.,**  
**Stunnenberg, H. G., Jones, P. L. and Veenstra, G. J. C.** (2011). Temporal uncoupling of the DNA  
266 methylome and transcriptional repression during embryogenesis. *Genome research*, **21**, 1313–1327.  
doi: 10.1101/gr.114843.110.
- 268 **Chang, H.-S., Anway, M. D., Rekow, S. S. and Skinner, M. K.** (2006). Transgenerational epigenetic  
imprinting of the male germline by endocrine disruptor exposure during gonadal sex determination.  
270 *Endocrinology*. **147**(12),5524–41. doi: 10.1210/en.2006-0987.
- Dunican, D. S., Ruzov, A., Hackett, J. a and Meehan, R. R.** (2008). xDnmt1 regulates transcriptional  
272 silencing in pre-MBT *Xenopus* embryos independently of its catalytic function. *Development*  
(Cambridge, England). **135**(7), 295–302. doi: 10.1242/dev.016402.
- 274 **Gama-Sosa, M. A., Midgett, R. M., Slagel, V. A., Githens, S., Kuo, K. C., Gehrke, C. W. and**  
**Ehrlich, M.** (1983). Tissue-specific differences in DNA methylation in various mammals. *BBA - Gene*  
276 *Structure and Expression*. **740**(2), 212–219. doi: 10.1016/0167-4781(83)90079-9.
- Govindarajulu, P., Price, W. M. S. and Anholt, B. R.** (2006). Introduced Bullfrogs (*Rana*  
278 *catesbeiana*) in Western Canada: Has Their Ecology Diverged?. *Journal of Herpetology*. **40**(2), 249–  
260. doi: 10.1670/68-05A.1.
- 280 **Jabbari, K., Cacciò, S., Pais De Barros, J. P., Desgrès, J. and Bernardi, G.** (1997). Evolutionary  
changes in CpG and methylation levels in the genome of vertebrates. *Gene*,. **205**(1–2), 109–118. doi:  
282 10.1016/S0378-1119(97)00475-7.
- Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M. and Altman, D. G.** (2010). Improving  
284 Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS*  
*Biology*. **8**(6), e1000412. doi: 10.1371/journal.pbio.1000412.
- 286 **Larson, P. M.** (2002). Chondrocranial development in larval *Rana sylvatica* (Anura: Ranidae):  
Morphometric analysis of cranial allometry and ontogenetic shape change. *Journal of Morphology*.  
288 **252**(2), 131–144. doi: 10.1002/jmor.1095.
- Larson, P. M.** (2005). Ontogeny, phylogeny, and morphology in anuran larvae: morphometric analysis  
290 of cranial development and evolution in *Rana* tadpoles (Anura: Ranidae). *Journal of morphology*.  
264(1), 34–52. doi: 10.1002/jmor.10313.
- 292 **Liebl, A. L., Schrey, A. W., Richards, C. L. and Martin, L. B.** (2013). Patterns of DNA Methylation  
Throughout a Range Expansion of an Introduced Songbird. *Integrative and comparative biology*.  
294 **53**(2),351-358. doi: 10.1093/icb/ict007.
- MacCracken, J. G. and Stebbings, J. L.** (2012). Test of a Body Condition Index with Amphibians.  
296 *Journal of Herpetology*. **46**(3), 346–350. doi: 10.1670/10-292.
- Miller, S. A., Dykes, D. D. and Polesky, H. F.** (1988). A simple salting out procedure for extracting

- 298 DNA from human nucleated cells. *Nucleic Acids Research*. **16**(3), 1215–1215. doi:  
10.1093/nar/16.3.1215.
- 300 **Peig, J., Green, A. J. and Ame, C.** (2009). New perspectives for estimating body condition from  
mass / length data : the scaled mass index as an alternative method. **118**(12), 1883–1891. doi:  
302 10.1111/j.1600-0706.2009.17643.x.
- Ponger, L. and Li, W.-H.** (2005). Evolutionary diversification of DNA methyltransferases in  
304 eukaryotic genomes. *Molecular biology and evolution*. **22**(4), 1119–28. doi: 10.1093/molbev/msi098.
- Rivas Sanchez, D. F. and Rivas Sanchez, D. F.** (2015) ‘Identifying potential for fisheries-induced  
306 evolution on behavioral traits of a Skagerrak cod (*Gadus morhua*) population. Master thesis, University  
of Oslo, 2015. Available at: <https://www.duo.uio.no/handle/10852/46128> (Accessed: 13 June 2017).
- 308 **Rountree, M. R. and Selker, E. U.** (1997). DNA methylation inhibits elongation but not initiation of  
transcription in *Neurospora crassa*. *Genes & development*. **11**(18), 2383–95.
- 310 **Stancheva, I., El-Maarri, O., Walter, J., Niveleau, A. and Meehan, R. R.** (2002) .DNA methylation  
at promoter regions regulates the timing of gene activation in *Xenopus laevis* embryos. *Developmental*  
312 *biology*. **243**(1), 155–65. doi: 10.1006/dbio.2001.0560.
- Stancheva, I., Hensey, C. and Meehan, R. R.** (2001) .Loss of the maintenance methyltransferase,  
314 xDnmt1, induces apoptosis in *Xenopus* embryos. **20**(8), 1963–1973. doi: [10.1093/emboj/20.8.1963](https://doi.org/10.1093/emboj/20.8.1963)
- Vanyushin, B. F., Mazin, A. L., Vasilyev, V. K. and Belozersky, A. N.** (1973) .The content of 5-  
316 methylcytosine in animal DNA: The species and tissue specificity. *BBA Section Nucleic Acids And*  
*Protein Synthesis*. **299**(3), 397–403. doi: 10.1016/0005-2787(73)90264-5.
- 318 **Varriale, A.** (2014). DNA Methylation, Epigenetics, and Evolution in Vertebrates: Facts and  
Challenges. *International journal of evolutionary biology*. **2014**, 1–7. doi: 10.1155/2014/475981.
- 320 **Varriale, A. and Bernardi, G.** (2006a). DNA methylation and body temperature in fishes. *Gene*. **385**,  
111–121. doi: 10.1016/j.gene.2006.05.031.
- 322 **Varriale, A. and Bernardi, G.** (2006b). DNA methylation in reptiles. *Gene*. **385**, 122–127. doi:  
10.1016/j.gene.2006.05.034.
- 324 **Venegas, D., Marmolejo-Valencia, A., Valdes-Quezada, C., Govenzensky, T., Recillas-Targa, F.**  
**and Merchant-Larios, H.** (2016). Dimorphic DNA methylation during temperature-dependent sex  
326 determination in the sea turtle *Lepidochelys olivacea*. *General and Comparative Endocrinology*. **236**,  
35–41. doi: 10.1016/j.ygcen.2016.06.026.
- 328 **Zhu, Z. Z., Hou, L., Bollati, V., Tarantini, L., Marinelli, B., Cantone, L., Yang, A. S., Vokonas, P.,**  
**Lissowska, J., Fustinoni, S., Pesatori, A. C., Bonzini, M., Apostoli, P., Costa, G., Bertazzi, P. A.,**  
330 **Chow, W. H., Schwartz, J. and Baccarelli, A.** (2012). Predictors of global methylation levels in blood  
DNA of healthy subjects: A combined analysis. *International Journal of Epidemiology*. **41**(1), 126–139.  
332 doi: 10.1093/ije/dyq154.

## 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).

342

344

346

## Tables

348

350

352

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

Tuckey-PH  $\alpha$  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).

360

362

	Neutrophils	Lymphocytes	Monocytes	Basophils	Eosinophils
<b>LPu-F</b>	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
<b>LPu-M</b>	1464.53±128.1 b	1791.14±103.1 a	31.07±7.6 a	233.17±49 a	197.92±49 a
<b>SPt-F</b>	730.84±63.5 a	2093.4±236.3 a	68.69±19.8 a	413.32±75.9 a	191±29.4 a
<b>SPt-M</b>	933.61±120 a	1927.84±189.1 a	233.65±40.8 b	349.13±53 a	236.27±50 a

Tuckey-PH  $\alpha$  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.

370

372

374

376

378