REPRODUCTION
RESEARCH

Influence of tissue, age, and environmental quality on DNA methylation in *Alligator mississippiensis*

Benjamin B Parrott[1,2,*], John A Bowden[2,3,*], Satomi Kohno[1,2], Jessica A Cloy-McCoy[1,2], Matthew D Hale[1,2], Jacqueline T Bangma[1,2], Thomas R Rainwater[1,2,4], Phillip M Wilkinson[5], John R Kucklick[2,3] and Louis J Guillette Jr[1,2]

[1]*Department of Obstetrics and Gynecology, Medical University of South Carolina, Charleston, South Carolina, USA,* [2]*Hollings Marine Laboratory, 331 Fort Johnson Road, Charleston, South Carolina 29412, USA,* [3]*National Institute of Standards and Technology (NIST), Charleston, South Carolina, USA,* [4]*United States Fish and Wildlife Service, Charleston, South Carolina 29412, USA and* [5]*Tom Yawkey Wildlife Center, 407 Meeting Street, Georgetown, South Carolina 29440, USA*

Correspondence should be addressed to B B Parrott; Email: benbparrott@gmail.com

[*](B B Parrott and J A Bowden are co-first authors)

Abstract

Epigenetic modifications are key mediators of the interactions between the environment and an organism's genome. DNA methylation represents the best-studied epigenetic modification to date and is known to play key roles in regulating transcriptional activity and promoting chromosome stability. Our laboratory has previously demonstrated the utility of the American alligator (*Alligator mississippiensis*) as a sentinel species to investigate the persistent effects of environmental contaminant exposure on reproductive health. Here, we incorporate a liquid chromatography–tandem mass spectrometry method to directly measure the total (global) proportion of 5-methyl-2′-deoxycytidine (5mdC) in ovarian and whole blood DNA from alligators. Global DNA methylation in ovaries was significantly elevated in comparison with that of whole blood. However, DNA methylation appeared similar in juvenile alligators reared under controlled laboratory conditions but originating from three sites with dissimilar environmental qualities, indicating an absence of detectable site-of-origin effects on persistent levels of global 5mdC content. Analyses of tissues across individuals revealed a surprising lack of correlation between global methylation levels in blood and ovary. In addition, global DNA methylation in blood samples from juvenile alligators was elevated compared with those from adults, suggesting that age, as observed in mammals, may negatively influence global DNA methylation levels in alligators. To our knowledge, this is the first study examining global levels of DNA methylation in the American alligator and provides a reference point for future studies examining the interplay of epigenetics and environmental factors in a long-lived sentinel species.

Reproduction (2014) **147** 503–513

Introduction

Interactions between an organism's genome and the environment are thought to underlie the etiology of many complex disorders of the reproductive system. Epigenetic mechanisms have long been invoked as key mediators of these interactions, and more recent studies have begun to elucidate the molecular and biochemical nature of a broad array of epigenetic modifications (Packard 1901, Barker 1990). However, it remains unclear how combined exposures to ranges of environmental factors such as temperature, nutrition, and exposure to intricate mixtures of exogenous chemicals such as anthropogenic contaminants influence epigenetic programming. Here, we characterize tissue-specific global DNA methylation in a long-lived top

This paper forms part of a special issue of *Reproduction* on Endocrine Disrupters. This article was presented at the 7th Copenhagen Workshop on Endocrine Disrupters, 28–31 May 2013. The meeting was supported by the Danish Ministry of the Environment – Environmental Protection Agency as an activity under the Danish Centre on Endocrine Disrupters. Publication of this special issue has been supported by the Society for Reproduction and Fertility. The opinions or views expressed in this special issue are those of the authors, and do not necessarily reflect the opinions or recommendations of the Danish Ministry of the Environment – Environmental Protection Agency or the Society for Reproduction and Fertility. The Guest Editors for this special issue were Anna-Maria Andersson, Hanne Frederiksen, Niels Erik Skakkebæk, Rigshospitalet, Denmark, Kenneth M Grigor, Western General Hospital, Edinburgh, UK and Jorma Toppari, University of Turku, Finland.

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-13-0498
Online version via www.reproduction-online.org

predator, the American alligator (*Alligator mississippiensis*). Furthermore, we incorporate a model in which alligators originating from different environments are raised under controlled conditions to examine persistent effects of environmental quality and geographic location on global DNA methylation.

DNA methylation, consisting of the covalent addition of a methyl group to cytosine, represents the most fundamental and well-studied epigenetic modification to date. Cytosines directly preceding guanines (CpG dinucleotides) represent the predominant methylation sites in vertebrates. In mammals, the majority of DNA methylation patterning occurs during early development (Brandeis *et al*. 1993). However, the developmental dynamics of methylation patterning appear to be somewhat divergent across other vertebrates (Veenstra & Wolffe 2001, Stancheva *et al*. 2002, Potok *et al*. 2013). Once established, patterning is thought to retain a degree of temporal stability due to mitotic inheritance. Yet, studies investigating changes in DNA methylation patterning over short periods of time and in response to different stimuli have revealed a degree of context-dependent plasticity and variation (Woodfine *et al*. 2011, Barrès *et al*. 2012, Byun *et al*. 2012). For example, global hypomethylation of genomic DNA was observed in skeletal muscle in men and women shortly after exercise (Barrès *et al*. 2012). When taken together, these studies suggest that DNA methylation patterning remains stable over time but retains an underlying plasticity, allowing changes in response to specific cues.

Decreased levels of global genomic DNA methylation has been linked to aging, genomic instability, and malignant cancers (Gaudet *et al*. 2003, Rodriguez *et al*. 2006, Bollati *et al*. 2009, Hannum *et al*. 2013). In addition, environmental factors, such as exposure to air pollution and endocrine disrupting contaminants (EDCs), have also been shown to influence global levels of DNA methylation (Rusiecki *et al*. 2008, Baccarelli *et al*. 2009, Bromer *et al*. 2010). Yet, much of our current understanding of DNA methylation patterning in response to environmental cues is based on studies incorporating traditional laboratory models, cell culture, and associative epidemiological studies. Thus, a gap exists in our understanding of how long-term, chronic exposures to mixtures of contaminants affect DNA methylation patterning in real-world populations. The American alligator was chosen as a study model as this animal is long lived and has high site fidelity in the wild, and thus reflects ambient environmental conditions in its habitat. These characteristics provide an ideal model in which the aforementioned gap in our understanding can be addressed. Here, we report our findings utilizing the alligator as a sentinel to investigate persistent effects of environmental quality and geographic location on measures of genomic methylation levels.

Alligators living in Lake Apopka, Florida, undergo a combinatorial exposure to high levels of pesticides and agricultural contamination compared with those alligators living in a nearby, but relatively pristine lake, Lake Woodruff (Heinz *et al*. 1991, Guillette *et al*. 1999, Rauschenberger *et al*. 2007). A number of reproductive abnormalities, including altered gonadal development and decreased robustness of sexually dimorphic gene expression, have been reported in alligators originating from Lake Apopka (Milnes *et al*. 2005, 2008). Yet, understanding of the molecular mechanisms mediating the effects of poor environmental quality on reproductive health remain incomplete. Interestingly, our group has shown that many of these perturbations, including altered gonadal gene expression, persist in laboratory-reared juvenile alligators originating from field-collected eggs (Moore *et al*. 2010*a*, 2010*b*, 2011). Thus, the origins of these long-lasting impacts on reproductive health are likely to have roots in early development and may be mediated by epigenetic mechanisms. This study also incorporates animals derived from a different geographical location along the north central coast of South Carolina (Yawkey Wildlife Center). This population thrives near the northern boundary of the American alligator range and is exposed to more fluctuations in seasonal temperature and salinity compared with animals at Lake Woodruff or Lake Apopka.

A promising strategy to measure global DNA methylation is to measure the proportion of 5-methyl-2′-deoxycytidine (5mdC) in relation to total 2′-deoxycytidine (dC) or 2′-deoxyguanosine (dG) directly using liquid chromatography–tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM) (Song *et al*. 2004, Kok *et al*. 2007, Ma *et al*. 2008, Quinlivan & Gregory 2008*a*, Liu *et al*. 2009, Le *et al*. 2011). Using direct measures that are independent of DNA sequence have become increasingly crucial to our understanding of global methylation as other assays, analyzing the methylation status of repetitive elements as a proxy for global methylation, have yielded inconsistent findings (Nelson *et al*. 2011). Here, we directly measure the relative genomic content of 5mdC employing dG as an internal calibrant. We examine global methylation levels in circulating blood cells and in ovarian tissue and find substantial differences between the two. Interestingly, we did not observe a correlation between global genomic methylation levels in blood and ovary across individuals, suggesting that methylation levels in the blood may not reflect those within the ovary. For female juvenile alligators originating from field-collected eggs but reared under identical conditions in the laboratory, no effect of site was observed. Finally, we compare levels of genomic methylation in juveniles with levels in wild-caught adults and find a dramatic decrease in global methylation in the latter, suggesting that age may be a crucial determinant of DNA methylation in alligators. This study provides the first data on the influence of tissue, site, and age on global DNA methylation in a

crocodilian and provides a foundation to further explore epigenetic mechanisms in long-lived sentinel species.

Materials and methods

Field collections and animal husbandry

All experiments performed in this study conformed to the guidelines set forth by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. All fieldwork and collections were performed under permits issued by the Florida Fish and Wildlife Commission, South Carolina Department of Natural Resources, and United States Fish and Wildlife Service. Adult alligators were captured at Lake Woodruff using a pole with a snare in April 2010 and a blood sample was immediately acquired and stored on ice in a heparinized vacutainer (BD Diagnostics, Franklin Lakes, NJ, USA) before centrifugation at 1500 ***g*** for 10 min at 4 °C. The pelleted cells were suspended in RNA later, placed on ice, and then stored at −20 °C.

For studies on juvenile alligators, eggs were collected shortly after oviposition from Lake Woodruff National Wildlife Refuge (FL), Lake Apopka (FL), and Yawkey Wildlife Center (SC) in June 2010 (as shown in Fig. 1). At least one egg from each clutch was necropsied shortly after collection and staged according to Ferguson (1985). For all clutches, embryos were collected before the stage at which temperature-dependent sex determination commences. Animals representing six clutches from Lake Apopka, ten clutches from Lake Woodruff, and ten clutches from Yawkey Wildlife Center were used in this study.

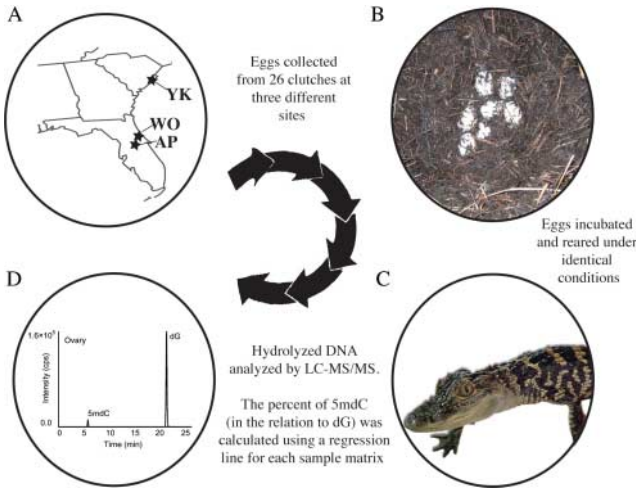

Figure 1 Experimental design. (A) Three Southeastern (USA) sites were selected for alligator egg collection: Yawkey Wildlife Refuge (YK), Lake Woodruff (WO), and Lake Apopka (AP). (B) Alligator eggs were collected from each site and incubated under identical conditions at female-producing temperature. (C) Alligator hatchlings were allowed to reach 1 kg in weight. DNA was isolated from ovaries and blood. (D) 5mdC and dG, from the isolated and hydrolyzed DNA, were examined using LC-MS/MS. A resultant chromatogram is shown for the LC-MS/MS analysis of DNA extract from an alligator ovary. For quantitation of percentage of 5mdC, calibration curves were prepared in triplicate (with each curve in triplicate analyzed twice) and were constructed by varying the amount of 5mdC while holding dG constant.

Eggs were placed in damp sphagnum moss and incubated in commercial incubators (Thermo Scientific, Waltham, MA, USA, Forma Environmental Chamber, model #3920) at the female-producing temperature of 30 °C until hatching. After hatching, each alligator was marked with a number monel tag attached to the webbing of the right rear foot and individuals were co-housed in flow-thru tanks (1000 l) at the Hollings Marine Laboratory. Water temperature was maintained at 32 °C. Animal health was checked daily and animals were fed a commercial crocodilian diet (Mazuri, Arden Hills, MN, USA) *ad libitum*. When each animal reached 1 kg in body mass, a blood sample was acquired immediately before killing and treated as described above for adult samples. There was no significant variation in the age of animals at the time of killing across the three sites. Animals were then necropsied and ovaries were placed in RNA later on ice before storing at −80 °C.

Nucleic acid extraction and DNA hydrolysis

Both RNA and DNA were simultaneously extracted from each tissue. RNA was extracted using the one-step acid phenol–guanidium thiocyanate–chloroform method described by Chomczynski & Sacchi (1987) with minor modifications. RNA was then further purified using the SV Total RNA Isolation System (Promega). Genomic DNA was isolated from the organic phase resulting from the RNA extraction by addition of back extraction buffer (4 M guanidine thiocyanate, 50 mmol/l sodium citrate, and 1 M Tris base). Samples were briefly mixed and centrifuged at 12 000 ***g*** for 30 min. The genomic DNA, contained in the resulting aqueous phase, was further purified by two rounds of phenol–chloroform extraction, followed by precipitation with ethanol. DNA and RNA were both assayed for quality and quantity using standard gel electrophoresis and a Nanodrop spectrophotometer. For subsequent methylation analysis, 1 µg of DNA was hydrolyzed to nucleosides as described by Quinlivan & Gregory (2008*b*).

Confirming sex by morphology and gene expression

All juvenile alligators included in this study were incubated at the female-producing temperature (30 °C) until hatching. Sex was initially assessed at the time of necropsy by the presence of oviduct. To further confirm the sex of the juveniles used in this study, gonadal aromatase mRNA expression levels were analyzed by quantitative PCR. Juvenile alligators show clear sexual dimorphic patterns in the expression of aromatase, with females displaying levels at least threefold higher than males (Kohno *et al.* 2008, Milnes *et al.* 2008). Both assessments (gross morphology and aromatase expression) classified all juvenile alligators included in this study as female.

Preparation of standards and calibration curves for instrument validation

Neat standards of 5mdC, dC, and dG monohydrate were purchased from Sigma and Santa Cruz Biotechnology respectively. The materials were weighed to the nearest 0.00001 g into 10 ml Falcon tubes using a Mettler Toldeo XS205 balance. Stock solutions were prepared in deionized

water (from a MilliQ Gradient A10, Millipore Corporation, Billerica, MA, USA) to final concentrations of 0.48917 mg/g (2.02 mmol/l) and 0.54870 mg/g (2.05 mmol/l) for 5mdC and dG respectively. A working solution for each 2′-deoxynucleoside was prepared to 20 μg/g (or ng/μl, at 82.8 and 74.8 μmol/l respectively) for the preparation of calibration curves. Two equimolar solutions of 5mdC and dG were prepared in hydrolysis buffer at concentrations of 5 and 50 μmol/l (each in triplicate) and were used to determine a relative response factor (RRF) to correct for variations in signal response resulting from differences in ionization efficiency. The equimolar solutions were used to tune the mass spectrometer.

Liquid chromatography–tandem mass spectrometry

2′-Deoxynucleoside standards and DNA extracts were analyzed in positive ion mode using an Applied Biosystems (AB) Sciex API 4000 triple quadrupole mass spectrometer with electrospray ionization using a TurboSpray Ion Source (Foster City, CA, USA). The instrument was operated in the MRM scan mode using ions that were the dominant protonated species, as well as the sodiated, dimer, and dimer-sodiated species ($[M+H]^+$, $[M+Na]^+$, $[2M+H]^+$, and $[2M+Na]^+$ respectively). The two dimer species were not detected for 5mdC. The following MRM transitions were monitored: 242.3→126.1 $[5mdC+H]^+$, 268.1→152.0 $[dG+H]^+$, 264.1→148.2 $[5mdC+Na]^+$, 290.2→174.2 $[dG+Na]^+$, 535.1→268.1 $[2\text{-}dG+H]^+$, and 556.6→290.4 $[2\text{-}dG+Na]^+$. Source (CAD 10, CUR 137 kPa, GS1 275 kPa, GS2 275 kPa, IS 5000 V, temperature 550 °C, and interface heater on) and compound-specific mass spectrometric parameters for each MRM transition were optimized. The compound-specific parameters (DP (V), EP (V), CE (eV), and CXP (V)) for each MRM transition were optimized to (25, 15, 20, and 10), (40, 10, 15, and 10), (25, 15, 20, and 10), (75, 15, 15, and 15), (75, 15, 15, and 15), and (75, 15, 15, and 15) for $[5mdC+H]^+$, $[dG+H]^+$, $[5mdC+Na]^+$, $[dG+Na]^+$, $[2\text{-}dG+H]^+$, and $[2\text{-}dG+Na]^+$ respectively. Nitrogen was used as the curtain gas. For each MRM transition, the dwell time was set to 200 ms.

The nucleosides were chromatographically separated using an Agilent 1100 LC (Palo Alto, CA, USA) equipped with a 2.1×150 mm Waters Atlantis dc18 column (5 μm i.d., Milford, MA, USA) and a gradient flow program consisting of solvent A (LC-grade water, BDH, West Chester, PA, USA) and solvent B (LC-grade methanol, Honeywell, Muskegon, MI, USA), with both solvents containing 0.1% formic acid (EMD, 98%, Darmstadt, Germany). For each injection, 5 μl was injected into a gradient flow rate set initially to 250 μl/min at 0% solvent A. The percentage of solvent A was increased to 20% from 0 to 10 min. At 10.01 min (to 17 min), the flow rate was changed to 220 μl/min and the percentage of solvent A was increased to 40%. At 17.01 min (to 22.0 min), the percentage of solvent A was decreased to 10%. At 22.01 min, the flow rate and percentage of solvent A were changed to 250 μl/min and 0% respectively, and these conditions were held until the end of the program (27 min). The column was heated to 35 °C using a column heater and the samples were kept at 8 °C in the autosampler tray until analysis. A sample chromatogram of 5mdC and dG separated in a DNA extract from ovary tissue is shown in Fig. 1.

Preparation of calibration curves for methylation measurements

For both the ovary and blood DNA samples, three separate calibration curves were included and analyzed in duplicate throughout a sample queue. Each calibration curve was prepared keeping dG constant at 10 μg/g (41.03 μmol/l). While dC was not used in our methods to determine methylation, it was necessary to include dC (stock solution at 10 μg/g or 37.43 mmol/l) for the purpose of preparing a calibration curve. At calibration levels from 82 nmol/l to 9.9 mmol/l (0.0, 0.1 (or 0.001 ng 5mdC/10 ng dG), 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, and 12% 5mdC), increasing amounts of 5mdC (mass) were added for each level while concomitantly decreasing amounts of dC (mass) were added, thus keeping the concentration of total dC=5mdC+dC constant across all calibration levels. The calibration levels were then designed to relate the percentage of 5mdC to the amount of dG. An overall calibration curve was then constructed from the sum of all the calibration curves analyzed, which was then used to determine the percent of 5mdC (to dG) present in the samples.

Handling of quality control and ovary/blood extracts

Post DNA hydrolysis, the samples (ovary and blood) were prepared in an autosampler vial and diluted to a total volume of 500 μl with hydrolysis buffer. The target DNA quantity per vial was 0.5 ng/μl, which resulted in ∼2.5 ng DNA/LC injection (employing 5 μl injections). A quality control (QC) DNA sample, extracted from visceral tissue from a juvenile alligator, was also included in this study to examine method performance and consistency. Since each matrix (for both ovary and blood samples) consisted of ∼300 sample injections per sample queue (which included ∼136 ovary and 104 blood DNA extract injections ($n=1$), along with 21 QCs, 80 calibration samples, and 50 blank injections), it was important to determine and evaluate method performance over the course of several days in regard to its feasibility as a population-based screening approach. For each sample type (ovary (O) and blood (B)), the QC material was included in triplicate (OQC1–3 and BQC1–3) and each sample in the triplicate was analyzed three times within a sample queue. The QC samples were processed in the same manner as the ovary and blood samples. The percentage of 5mdC (in relation to dG) for each individual replicate within the ovary and blood sample queue was 2.61±0.3% (or 0.0261±0.003 ng 5mdC/10 ng dG) for ovary OQC1–3 and 2.69±0.3% (or 0.0269±0.003 ng 5mdC/10 ng dG) for blood BQC1–3. The Relative Standard Deviation (RSD) for both sample queues combined for all the QC samples analyzed was 10%.

Sample and data processing prior to quantitation

Each ovary and blood DNA extract was examined ($n=1$) in a random order within a sample queue (two queues, one for each sample type). Each sample queue consisted of ∼300 injections, which included DNA extracts, QC samples (with replicates), calibration curve samples (in duplicate), and blanks. In post-sample analysis, the data were processed using Analyst v.1.5.2 (AB Sciex, Foster City, CA, USA).

During MS and MS/MS tuning of 5mdC and dG standards, it was evident that in addition to the detection of a protonated ion, $[M+H]^+$, other forms of the 2′-deoxynucleosides were observed, including sodiated $[M+Na]^+$, dimer $[2M+H]^+$, and sodiated dimer $[2M+Na]^+$ species. The presence of additional forms of 2′-deoxynucleosides has been previously reported (Song *et al.* 2004, Ma *et al.* 2008). It has been shown in previous work from our laboratory that several of these additional species (sodiated and dimer) for specific 2′-deoxynucleosides (e.g. dC) can be present at levels above 10% of the protonated species (data not shown). Further, it has been previously reported by others that additional species can constitute anywhere from 2/7 to 1/3 of the approximate signal of additional species vs total signal response for the sum of all species (Song *et al.* 2004, Ma *et al.* 2008). To compensate for these potential contributions, all nucleoside forms for 5mdC and dG were tuned by mass spectrometer, incorporated into the LC-MS/MS method, and quantitated using the sum of the nucleoside area for all species. It should be noted that for the detection of 5mdC, only the sodiated adduct was detected, while all the adduct forms were represented for dG (and dC). Unlike other 2′-deoxynucleosides, the percent presence (%) of each additional species for 5mdC and dG (additional species area over $[M+H]^+$ species area) was negligible over the course of the ovary and blood sample batches and was recorded at 1.31 ± 1.69 and 1.08 ± 1.48 for $[5mdC+Na]^+$, 2.40 ± 0.13 and 2.61 ± 0.18 for $[dG+Na]^+$, 0.03 ± 0.02 and 0.03 ± 0.01 for $[2dG+H]^+$, and 0.01 ± 0.00 and 0.01 ± 0.00 for $[2\text{-}dG+Na]^+$ respectively.

Quantifying cytosine methylation

The peak areas for each 5mdC and dG species (including adducts and dimers) were examined and incorporated into a peak area ratio equation, $\Sigma 5mdC_{area}/(\Sigma dG_{area}\times 0.8971)$, where Σ is the sum of all the peak areas for the protonated, sodiated, and dimer species and 0.8971 is the experimentally determined RRF as described above. The RRF was determined by analyzing two equimolar solutions (5 and 50 μmol/l, each in triplicate) and comparing the relative peak areas of the sum of the 5mdC and dG peaks. The calibration curves were prepared in triplicate (with each curve in triplicate analyzed twice) and were constructed using the above peak area ratio calculation. Within the curve, only the amount (percentage, or mass fraction of ng 5mdc/10 ng dG) of 5mdC changed (0–12%, holding dG constant); thus, the percentage of 5mdC (in relation to dG) for each sample was calculated using the regression line derived from the sum of all the curves run for each sample matrix (ovary and blood). The calibration lines and R^2 values for the ovary and blood calibration curves were $y=0.0103x-0.0002$ (1.00) and $y=0.0127x+0.0003$ (1.00) respectively.

Statistical analyses

GraphPad Prism, version 6.0, was used to perform all statistical analyses. For all analyses, statistical significance was set at $P<0.05$. All global genomic methylation levels were calculated as ratios and were arcsin transformed before statistical analyses. A two-way ANOVA was used to test for differences in measures of global DNA methylation among sites and tissues. A one-way ANOVA was used to test for clutch effects on global DNA methylation. Unpaired *t*-tests were employed to compare differences in global genomic methylation levels from alligators of different ages. A one-way ANOVA was used to test for differences among sites and clutch effects.

Results

Site and tissue analysis of DNA methylation

DNA methylation in mammalian genomes is tissue specific (Maegawa *et al.* 2010, Herzog *et al.* 2013). Studies have linked both increased and decreased DNA methylation levels to chemical exposures in traditional laboratory models and humans (Dolinoy *et al.* 2007, Baccarelli *et al.* 2009, Zhu *et al.* 2012). To test whether tissue-specific and persistent site differences in genomic methylation are present in juvenile alligators, we analyzed the genomic content of cytosine methylation using LC-MS/MS. In alligators originating from each site, genomic methylation (reported as the proportion of methylated cytosines relative to the number of total CpGs, 5mdC/CpGs) was higher in ovaries in comparison with blood ($F_{2,234}=136.71$, $P<0.001$; Fig. 2A, Tables 1 and 2). However, both ovarian and blood methylation was similar among collection sites ($F_{2,234}=0.32$, $P=0.73$). These data indicate genomic methylation is higher in ovaries than in blood and thus tissue-specific DNA methylation patterns are present in juvenile alligators. However, persistent site-of-origin differences in genomic methylation were not observed.

DNA methylation among clutches

We next examined the potential of genetic contributions by examining variation among clutches. In late spring to early summer, female alligators lay a clutch of eggs that may be sired from one or multiple males (Lance *et al.* 2009). In an effort to determine whether a maternal effect on DNA methylation could be detected at the genome level, we compared variability of ovarian global genomic methylation among clutches (Fig. 2B). In all, DNA methylation was analyzed for individuals representing 26 clutches with an average of five eggs per clutch. A one-way ANOVA across all clutches revealed no significant variation among clutches ($F_{25,106}=0.82$, $P=0.71$).

Relationship between DNA methylation in blood and ovary

The collection of peripheral blood is the most universal means of obtaining a consistent minimally invasive biological DNA sample across a large number of organisms within a given population. However, in many cases, an understanding of how transcriptional and epigenetic endpoints measured in blood relate to

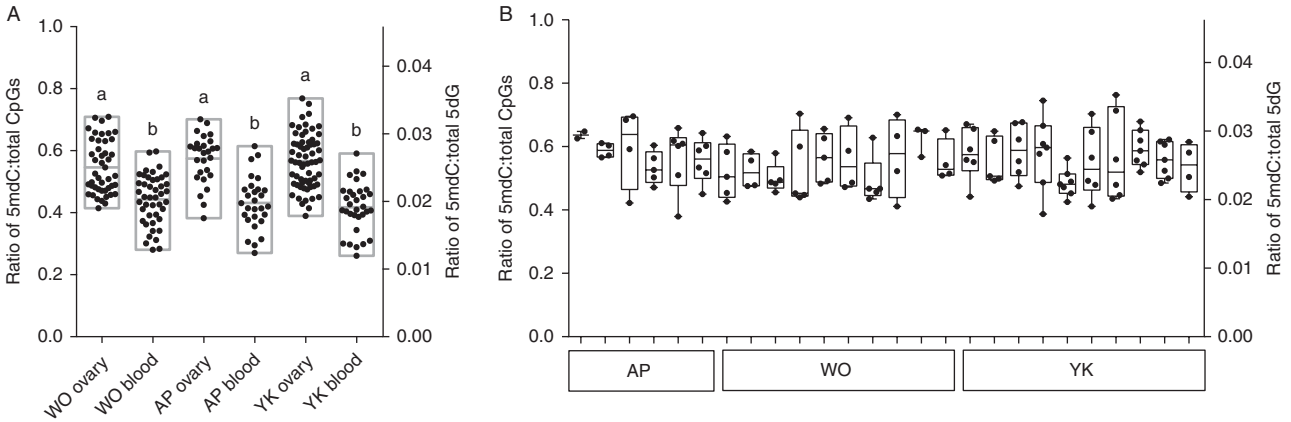

Figure 2 Comparison of global genomic methylation across tissues and sites. Different letters above groups indicate statistical significance for tissue comparisons. (A) Left *y*-axis indicates the proportion of methylated cytosines relative to CpGs dinucleotides in the genome, right *y*-axis indicates the proportion of methylated cytosines measured relative to guanosines. WO, Lake Woodruff; AP, Lake Apopka; and YK, Yawkey Wildlife Center. (B) Box-and-whisker plots of ovarian global genomic methylation across clutches. Left *y*-axis indicates the proportion of methylated cytosines relative to CpG dinucleotides in the genome, right *y*-axis indicates the proportion of methylated cytosines measured relative to guanosines. Whiskers indicate min. and max. No significant variation across clutches was observed.

other tissues is either inferred through speculation or is completely lacking. In an effort to determine whether levels of DNA methylation in blood correlate with those measured in the ovary, we performed a correlation analysis across individuals (Fig. 3). When DNA methylation levels in blood and ovary from all individuals were examined, a correlation between the two tissues was not observed ($n=101$, $P=0.94$, $r=0.008$). We reasoned that perhaps correlations might exist in animals from certain sites and not others, and if so, comparing all animals together may obstruct detection. However, correlations between DNA methylation levels in blood and ovary were also absent when analyzing individuals originating from each site (Fig. 3). These data suggest that levels of global DNA methylation in the blood are not linked to those measured in the ovary.

DNA methylation in blood and age

Decreased global DNA methylation levels are associated with aging and age-related cancers (Rodriguez *et al.* 2006, Bollati *et al.* 2009). Similar to humans, alligators are long-lived and undergo chronic exposure to a milieu of anthropogenic contaminants and thus represent an attractive model to study the effects of environmental quality on aging-related processes. To investigate whether the level of global DNA methylation within blood cells of alligators was decreased in adult alligators relative to juveniles, we measured DNA methylation from 16 adults captured on Lake Woodruff and compared the resulting levels to the juveniles reported in this study. Consistent with mammalian studies, juveniles originating from Lake Woodruff were observed to have elevated levels of global DNA methylation (44% CpG methylation, $n=44$) in comparison with their adult counterparts (Fig. 4; 36% CpG methylation, $n=16$, $T=4.07$, df$=58$, $P=0.0001$). Although alternative explanations are possible, taken at face value, these data suggest that age may influence global DNA methylation in these animals.

Discussion

The proposed strategy to characterize percent global methylation using the relative ratio of 5mdC:dG was designed to be simple, rapid, and inexpensive (no labeled standards), all being important components for providing a high-throughput platform for population-based screening. The most widely used approach for quantification of percent global methylation is to relate the peak area of 5mdC to the sum of the peak area for total dC (which is generally described as (5mdC)/(5mdC+dC)), either employing native or isotopically labeled 2′-deoxynucleosides for quantification by calibration curves (Friso *et al.* 2002, Kok *et al.* 2007, Ma *et al.* 2008, Quinlivan & Gregory 2008*a*, Liu *et al.* 2009, Le *et al.* 2011). However, recently, it was proposed to employ dG, instead of dC, to quantify percent methylation. This approach was based on the assumption that 5mdC+dC=dG (Parra *et al.* 2001, Song *et al.* 2004). While use of dC for quantitation has become the practiced approach, recent reports have indicated that dC can undergo multiple forms of modification, including 5mdC, 5-hydroxymethyl-2′-deoxycytidine,

Table 1 Descriptive Statistics comparing global methylation across sites. The uncertainty value is Standard Error from the Mean (SEM).

	CpG methylation mean % ±S.E.M. (*n*)		
Tissue	Apopka	Woodruff	Yawkey
Ovary	57±1.5 (27)	55±1.3 (45)	57±1.1 (64)
Blood	43±1.6 (28)	44±1.2 (44)	42±1.4 (32)

Table 2 Two-way ANOVA results comparing global methylation across tissues.

Source	df	*F*	*P*
Site	2	0.32	0.73
Tissue	1	136.71	<0.001
Interaction	2	1.93	0.15

5-formyl-2′-deoxycytidine, and 2′-deoxycytidine-5-carboxylic acid (Fu & He 2012). For monitoring subtle variations in percent global methylation, dG is more stable as an internal calibrant as it is less prone to modifications.

Currently, there are numerous methods for measuring global levels of genomic DNA methylation. In humans and traditional mammalian models, methylation levels of *LINE-1* and *Alu* satellite repeats utilizing pyrosequencing of PCR-amplified bisulfite-treated DNA are most commonly utilized as a proxy for global levels of genomic methylation (Yang *et al*. 2004). *LINE-1* and *Alu* repeats comprise a sizeable proportion of the genome and thus the methylation status of these repeats is likely to reflect that of the entire genome. However, some studies have failed to find a correlation between methylation levels of these elements and the methylation status of the genome in its entirety. Subsequently, calls for direct measures that are not biased in regard to sequence have recently been made for those studies investigating global methylation (Choi *et al*. 2009, Nelson *et al*. 2011). Another concern is that many epidemiological studies examining global genomic methylation have relied on DNA isolated from lymphocytes or buccal swabs as a surrogate for systemic genomic methylation. Yet, how global methylation in these tissues is correlated with other tissues is not well understood (Nelson *et al*. 2011).

By comparing our measures of global 5mdC content with the number of CpGs in the alligator genome, we were able to conclude that 55–57% of CpGs are methylated in ovarian tissues and 42–44% of CpGs are methylated in nucleated blood cells. These measures are lower than the 70–90% CpG methylation historically reported in human tissues (Ehrlich *et al*. 1982, Tucker 2001). Repetitive sequences are highly methylated in most vertebrates and are thought to comprise more than half of the human genome (Consortium 2001, de Koning *et al*. 2011). Interestingly, recent sequencing of the alligator genome revealed that repetitive elements only comprise an estimated 23% (St John *et al*. 2012), suggesting that the reduced global methylation in alligators relative to humans could be due to a decreased proportion of repetitive sequences. Further studies aimed at exploring methylation status at higher resolutions are needed to better understand these differences observed at the global level.

By examining global methylation in alligators raised under controlled conditions, but originating from sites with varying environmental qualities, we tested whether environmental quality during early development results in persistent alterations to global DNA methylation. Our inability to detect a site effect suggests that altered levels of global DNA methylation are not responsible for the persistent reproductive abnormalities previously described in juvenile alligators originating from Lake Apopka. However, whether alterations in methylation status are present at the locus or gene pathway level remain to be tested. Previous studies have documented rapid temporal fluctuations in levels of global DNA methylation in response to environmental cues (Baccarelli *et al*. 2009, Barrès *et al*. 2012). Thus, it is plausible that differences attributed to site might have been initially present but were subsequently lost while in captivity. Analyses examining the effects of site on wild-caught juvenile alligators are needed to determine whether site effects are present in wild populations.

Many human epidemiological studies to date have related global methylation, through both direct and indirect measures, to environmental exposures, the presence of cancer, and other diseases (Rusiecki *et al*. 2008, Choi *et al*. 2009, Terry *et al*. 2011). Most of these studies examine methylation levels in either white blood cells or cells obtained through buccal swabs, and it is unclear how these levels correlate with directly affected target tissues. In this study, we sought to examine tissue-specific differences in levels of global methylation in an environmental sentinel species and to investigate how these measures are related. We did not observe a correlation between global methylation levels in blood and ovary across individuals. Our findings suggest that measures of global methylation in the blood may not reflect relative methylation levels in other tissues. Studies examining global methylation across tissues in humans

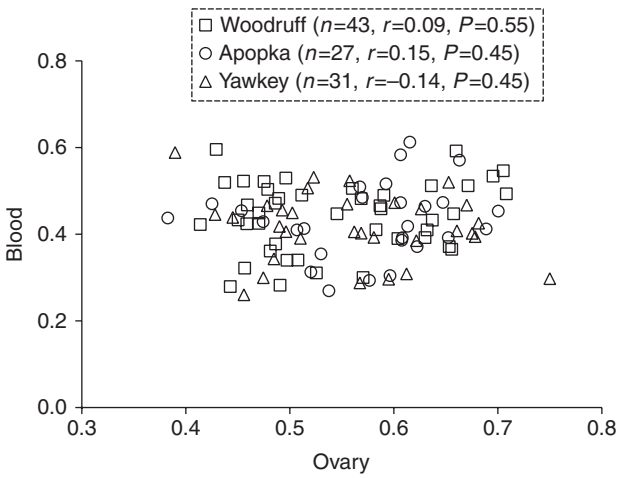

Figure 3 Scatter plot of levels of global genomic methylation in ovary and blood. Axes indicate the proportion of methylated cytosines relative to CpG dinucleotides in the genome. Individuals from different sites are indicated by symbols. Results from a correlation analysis are reported and did not reveal a correlation regardless of site.

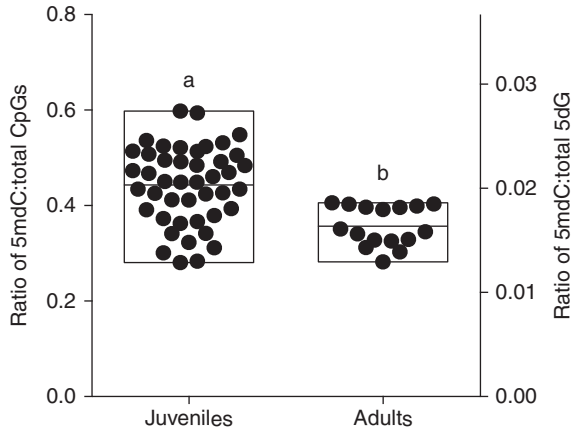

Figure 4 Comparison of global genomic methylation in blood samples from juvenile and adult animals from Lake Woodruff (FL). Left *y*-axis indicates the proportion of methylated cytosines relative to CpG dinucleotides in the genome; right *y*-axis indicates the proportion of methylated cytosines measured relative to guanosines. Measures of global genomic methylation are lower in adults (0.37±0.011 5mdC/CpGs) compared with juveniles (0.46±0.013 5mdC/CpGs). Statistical significance is indicated by different letters.

have reported similar findings, with correlations between tissues being rare (van Bemmel *et al.* 2012). These findings along with those presented here raise further questions regarding the influence or lack thereof of metabolic factors, such as one-carbon metabolism, on systemic global methylation levels across individuals. Collectively, these studies point toward the use of global methylation levels in circulating blood cells as a potential biomarker or as a means to understanding exposure histories, but at this point, conclusions regarding the methylation status of other tissues are premature.

We found that average global methylation levels were substantially higher in juvenile alligators originating from Lake Woodruff compared with adults from the same lake. However, there did appear to be some juvenile alligators with levels of DNA methylation that were similar to adults. It is well established that aging is associated with decreased levels of global methylation in peripheral blood cells in mammals (Fuke *et al.* 2004, Bollati *et al.* 2009). Yet, to our knowledge, this association has not been demonstrated in non-mammalian vertebrates. While other influences, such as captivity, cannot be ruled out, given our current knowledge of the relationship between aging and global methylation, it is probable that the differences observed between juvenile and adult alligators in this study are due to age. Further studies focused on measuring global methylation levels in wild alligator populations across a range of ages are needed to better understand the relationship between DNA methylation and aging. Furthermore, studies comparing the relationships between DNA methylation and aging across sites with differing environmental factors, such as varying degrees of contamination, could potentially reveal the influence of environmental factors on the aging process.

DNA methylation plays a critical role in mediating interactions between the genome and the environment. Numerous studies in humans and traditional laboratory models have demonstrated a sensitivity of global methylation levels to environmental factors. Measures of global methylation remain a cost-effective strategy for assessing epigenetic status and potential environmental impacts across a population. Further studies aimed at determining environmental impacts in wild populations of alligators and other sentinel species should aid in our understanding of how DNA methylation in blood cells, and also in other tissues, is affected by long-term combinatorial exposures.

Discussion from the meeting

Laura Vandenberg (Medford, USA): You have seen environmental effects on animals at every age you have studied, and the same individuals continue to have these defects on repeated sampling. Is that because the animals continue to live in the same contaminated areas, or is it an organizational effect due to an early hit which persists during life?

L J Guillette (Charleston, USA): Neonatal exposure and continued exposure are both important, and it is difficult to separate the two components. Alligators have problems when they hatch. We have kept some in captivity with a clean environment, and the problems persist. However, same age animals in the wild with continued contaminated exposure for 1–3 years have greater severity of the same problems. Therefore, birth defects are augmented by continued environmental exposure, possibly due to exactly the same mechanisms. Early studies on endocrinology, before the concept of endocrine disrupting chemicals (EDCs) had been considered, showed that developing embryos exposed to abnormal hormones had more dramatic effects when stimulated again with hormones in later life, whether steroid or non-steroid hormone. The embryonic hit is pump priming for future exposure.

Åke Bergman (Stockholm, Sweden): The environment around the NASA space center must have other chemical pollutants in addition to flame retardants and metals, particularly fluorinated compounds such as PFOS and polyfluorinated hexosulphonates. These are present around airports in Sweden. Have you examined them?

L J Guillette: Rocket fuel contains perchlorate, 2½ tons of which are projected from the nozzles of the rocket at 9000 °C at every launch. This produces a cloud of HCl and water with a pH of 0.5. There are thyroid defects around NASA and we were asked to investigate perchlorate levels: however, none was present. All the perchlorate is complete vaporized, and the highly acid cloud at extreme temperatures oxidizes and vaporizes metal compounds in the vicinity. The biggest

environmental problem around rocket sites is the presence of many weird metals and metal compounds. We are also looking at flame retardants and fluorinated compounds.

Sander van den Driesche (Edinburgh, UK): You demonstrated DNA methylation in the SOX9 promoter gene and indicated a difference in expression between males and females. Given that gender determination is temperature dependent, is there a difference in methylation of SOX9 at different temperatures?

L J Guillette: We incubated embryos at a temperature which would induce male differentiation by sex reversal, and added an estrogen. This resulted in a male pattern of methylation although the confounding factor of an added steroid hormone has to be considered in case the estrogen was having an effect. There was no difference in the methylation enzymes between males and females which is to be expected because other genes are being methylated. This indicates that SOX9 methylation is not controlled by temperature but is regulated by other upstream events. The trigger for environmental sex determination is not clear but it is upstream of SOX9. Aromatase is not the trigger for ovarian differentiation but the determining factor can be overridden by an estrogen signal.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology; nor does it imply that the materials or equipment identified are necessarily the best for the purpose.

Funding

This article is based on work presented at the 7th Copenhagen Workshop on Endocrine Disrupters, which was supported by the Danish Ministry of the Environment – Environmental Protection Agency. Publication of this special issue was supported by the Society for Reproduction and Fertility. This work was funded in part by the South Carolina Center of Economic Exellence Center for Marine Genomics and an exploratory research grant to L J Guillette from NIST.

Acknowledgements

The authors would like to acknowledge employees of the Florida Fish and Wildlife Conservation Commission for logistical support. This study would not be possible without the continued support of Allan Woodward, Arnold Brunell, and Patrick Delaney in regard to fieldwork and permitting. They would also like to thank Lance Koch, the refuge manager at Lake Woodruff, for his assistance in this project. In addition, they would also like to acknowledge Jamie Dozier of the South Carolina Department of Natural Resources for providing access and logistical support at the Yawkey Wildlife Center. Finally, they would like to acknowledge an anonymous reviewer and Lori Cruze of the Medical University of South Carolina for their guidance and suggestions regarding statistical analyses.

References

Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua AA, Suh HH, Zanobetti A, Sparrow D, Vokonas PS & Schwartz J 2009 Rapid DNA methylation changes after exposure to traffic particles. *American Journal of Respiratory and Critical Care Medicine* **179** 572–578. (doi:10.1164/rccm.200807-1097OC)

Barker DJ 1990 The fetal and infant origins of adult disease. *BMJ* **301** 1111. (doi:10.1136/bmj.301.6761.1111)

Barrès R, Yan J, Egan B, Treebak Jonas T, Rasmussen M, Fritz T, Caidahl K, Krook A, O'Gorman Donal J & Zierath Juleen R 2012 Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metabolism* **15** 405–411. (doi:10.1016/j.cmet.2012.01.001)

van Bemmel D, Lenz P, Liao LM, Baris D, Sternberg LR, Warner A, Johnson A, Jones M, Kida M, Schwenn M *et al.* 2012 Correlation of LINE-1 methylation levels in patient-matched buffy coat, serum, buccal cell, and bladder tumor tissue DNA samples. *Cancer Epidemiology, Biomarkers & Prevention* **21** 1143–1148. (doi:10.1158/1055-9965.EPI-11-1030)

Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, Sparrow D, Vokonas P & Baccarelli A 2009 Decline in genomic DNA methylation through aging in a cohort of elderly subjects. *Mechanisms of Ageing and Development* **130** 234–239. (doi:10.1016/j.mad.2008.12.003)

Brandeis M, Ariel M & Cedar H 1993 Dynamics of DNA methylation during development. *BioEssays* **15** 709–713. (doi:10.1002/bies.950151103)

Bromer JG, Zhou Y, Taylor MB, Doherty L & Taylor HS 2010 Bisphenol-A exposure *in utero* leads to epigenetic alterations in the developmental programming of uterine estrogen response. *FASEB Journal* **24** 2273–2280. (doi:10.1096/fj.09-140533)

Byun HM, Nordio F, Coull BA, Tarantini L, Hou L, Bonzini M, Apostoli P, Bertazzi PA & Baccarelli A 2012 Temporal stability of epigenetic markers: sequence characteristics and predictors of short-term DNA methylation variations. *PLoS ONE* **7** e39220. (doi:10.1371/journal.pone.0039220)

Choi JY, James SR, Link PA, McCann SE, Hong CC, Davis W, Nesline MK, Ambrosone CB & Karpf AR 2009 Association between global DNA hypomethylation in leukocytes and risk of breast cancer. *Carcinogenesis* **30** 1889–1897. (doi:10.1093/carcin/bgp143)

Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159. (doi:10.1016/0003-2697(87)90021-2)

Consortium TGS 2001 Initial sequencing and analysis of the human genome. *Nature* **409** 860–921. (doi:10.1038/35057062)

Dolinoy DC, Huang D & Jirtle RL 2007 Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *PNAS* **104** 13056–13061. (doi:10.1073/pnas.0703739104)

Ehrlich M, Gama-Sosa MA, Huang L, Midgett RM, Kuo KC, McCune RA & Gehrke CW 1982 Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells. *Nucleic Acids Research* **10** 2709–2721. (doi:10.1093/nar/10.8.2709)

Ferguson MWJ 1985 Reproductive biology and embryology of the crocodilians; in Gans C, Maderson P, Billet F (eds): Biology of the Reptilia, Vol 14, pp 329–491 (Wiley and Sons, New York).

Friso S, Choi S-W, Dolnikowski GG & Selhub JA 2002 A method to assess genomic DNA methylation using high-performance liquid chromatography/electrospray ionization mass spectrometry. *Analytical Chemistry* **74** 4526–4531. (doi:10.1021/ac020050h)

Fu Y & He C 2012 Nucleic acid modifications with epigenetic significance. *Current Opinion in Chemical Biology* **16** 516–524. (doi:10.1016/j.cbpa.2012.10.002)

Fuke C, Shimabukuro M, Petronis A, Sugimoto J, Oda T, Miura K, Miyazaki T, Ogura C, Okazaki Y & Jinno Y 2004 Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study. *Annals of Human Genetics* **68** 196–204. (doi:10.1046/j.1529-8817.2004.00081.x)

Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H & Jaenisch R 2003 Induction of tumors in mice by genomic hypomethylation. *Science* **300** 489–492. (doi:10.1126/science.1083558)

Guillette LJ Jr, Woodward AR, Crain DA, Pickford DB, Rooney AA & Percival HF 1999 Plasma steroid concentrations and male phallus size in juvenile alligators from seven Florida (USA) lakes. *General and Comparative Endocrinology* **116** 356–372. (doi:10.1006/gcen.1999.7375)

Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, Klotzle B, Bibikova M, Fan JB, Gao Y *et al.* 2013 Genome-wide methylation profiles reveal quantitative views of human aging rates. *Molecular Cell* **49** 359–367. (doi:10.1016/j.molcel.2012.10.016)

Heinz GH, Percival HF & Jennings ML 1991 Contaminants in American alligator eggs from lakes Apopka, Griffin and Okeechobee, Florida. *Environmental Monitoring and Assessment* **16** 277–285. (doi:10.1007/BF00397615)

Herzog E, Galvez J, Roks A, Stolk L, Verbiest M, Eilers P, Cornelissen J, Steegers E & Steegers-Theunissen R 2013 Tissue-specific DNA methylation profiles in newborns. *Clinical Epigenetics* **5** 8. (doi:10.1186/1868-7083-5-8)

Kohno S, Bermudez DS, Katsu Y, Iguchi T & Guillette LJ Jr 2008 Gene expression patterns in juvenile American alligators (*Alligator mississippiensis*) exposed to environmental contaminants. *Aquatic Toxicology* **88** 95–101. (doi:10.1016/j.aquatox.2008.03.006)

Kok RM, Smith DE, Barto R, Spijkerman AM, Teerlink T, Gellekink HJ, Jakobs C & Smulders YM 2007 Global DNA methylation measured by liquid chromatography–tandem mass spectrometry: analytical technique, reference values and determinants in healthy subjects. *Clinical Chemistry and Laboratory Medicine* **45** 903–911. (doi:10.1515/CCLM.2007.137)

de Koning AJ, Gu W, Castoe TA, Batzer MA & Pollock DD 2011 Repetitive elements may comprise over two-thirds of the human genome. *PLoS Genetics* **7** e1002384. (doi:10.1371/journal.pgen.1002384)

Lance SL, Tuberville TD, Dueck L, Holz-Schietinger C, Trosclair PL III, Elsey RM & Glenn TC 2009 Multiyear multiple paternity and mate fidelity in the American alligator, *Alligator mississippiensis*. *Molecular Ecology* **18** 4508–4520. (doi:10.1111/j.1365-294X.2009.04373.x)

Le T, Kim K-P, Fan G & Faull KF 2011 A sensitive mass spectrometry method for simultaneous quantification of DNA methylation and hydroxymethylation levels in biological samples. *Analytical Biochemistry* **412** 203–209. (doi:10.1016/j.ab.2011.01.026)

Liu Z, Wu J, Xie Z, Liu S, Fan-Havard P, Huang THM, Plass C, Marcucci G & Chan KK 2009 Quantification of regional DNA methylation by liquid chromatography/tandem mass spectrometry. *Analytical Biochemistry* **391** 106–113. (doi:10.1016/j.ab.2009.05.012)

Ma H, Zhang W, Hu J, Yu Z, Chen Y, Luo Q, Shi X, Zhang Y, Song R & Zhou Z 2008 Analysis of global DNA methylation levels in human blood using high-performance liquid chromatography/tandem electrospray ionization mass spectrometry. *European Journal of Mass Spectrometry* **15** 555–561. (doi:10.1255/ejms.1007)

Maegawa S, Hinkal G, Kim HS, Shen L, Zhang L, Zhang J, Zhang N, Liang S, Donehower LA & Issa JP 2010 Widespread and tissue specific age-related DNA methylation changes in mice. *Genome Research* **20** 332–340. (doi:10.1101/gr.096826.109)

Milnes MR, Bermudez DS, Bryan TA, Gunderson MP & Guillette LJ Jr 2005 Altered neonatal development and endocrine function in *Alligator mississippiensis* associated with a contaminated environment. *Biology of Reproduction* **73** 1004–1010. (doi:10.1095/biolreprod.105.041012)

Milnes MR, Bryan TA, Katsu Y, Kohno S, Moore BC, Iguchi T & Guillette LJ Jr 2008 Increased posthatching mortality and loss of sexually dimorphic gene expression in alligators (*Alligator mississippiensis*) from a contaminated environment. *Biology of Reproduction* **78** 932–938. (doi:10.1095/biolreprod.107.064915)

Moore BC, Kohno S, Cook RW, Alvers AL, Hamlin HJ, Woodruff TK & Guillette LJ 2010*a* Altered sex hormone concentrations and gonadal mRNA expression levels of activin signaling factors in hatchling alligators from a contaminated Florida lake. *Journal of Experimental Zoology. Part A, Ecological Genetics and Physiology* **313** 218–230. (doi:10.1002/jez.595)

Moore BC, Milnes MR, Kohno S, Katsu Y, Iguchi T & Guillette LJ Jr 2010*b* Influences of sex, incubation temperature, and environmental quality on gonadal estrogen and androgen receptor messenger RNA expression in juvenile American alligators (*Alligator mississippiensis*). *Biology of Reproduction* **82** 194–201. (doi:10.1095/biolreprod.109.077305)

Moore BC, Milnes MR, Kohno S, Katsu Y, Iguchi T, Woodruff TK & Guillette LJ 2011 Altered gonadal expression of TGF-β superfamily signaling factors in environmental contaminant-exposed juvenile alligators. *Journal of Steroid Biochemistry and Molecular Biology* **127** 58–63. (doi:10.1016/j.jsbmb.2011.01.004)

Nelson HH, Marsit CJ & Kelsey KT 2011 Global methylation in exposure biology and translational medical science. *Environmental Health Perspectives* **119** 1528–1533. (doi:10.1289/ehp.1103423)

Packard AS 1901 In *Lamarck, the Founder of Evolution: His Life and Work with Translations of His Writing on Organic Evolution*. New York: Longmans, Green, and Co. (http://dx.doi.org/10.5962/bhl.title.12263).

Parra R, Pastor MT, Perez-Paya E & Amo-Marco JB 2001 Effect of *in vitro* shoot multiplication and somatic embryogenesis on 5-methylcytosine content in DNA of *Myrtus communis L*. *Plant Growth Regulation* **33** 131–136. (doi:10.1023/A:1017571911028)

Potok ME, Nix DA, Parnell TJ & Cairns BR 2013 Reprogramming the maternal zebrafish genome after fertilization to match the paternal methylation pattern. *Cell* **153** 759–772. (doi:10.1016/j.cell.2013.04.030)

Quinlivan EP & Gregory JF 2008*a* DNA methylation determination by liquid chromatography–tandem mass spectrometry using novel biosynthetic [U-^{15}N]deoxycytidine and [U-15N]methyldeoxycytidine internal standards. *Nucleic Acids Research* **36** e119. (doi:10.1093/nar/gkn534)

Quinlivan EP & Gregory JF III 2008*b* DNA digestion to deoxyribonucleoside: a simplified one-step procedure. *Analytical Biochemistry* **373** 383–385. (doi:10.1016/j.ab.2007.09.031)

Rauschenberger RH, Wiebe JJ, Sepulveda MS, Scarborough JE & Gross TS 2007 Parental exposure to pesticides and poor clutch viability in American alligators. *Environmental Science & Technology* **41** 5559–5563. (doi:10.1021/es0628194)

Rodriguez J, Frigola J, Vendrell E, Risques RA, Fraga MF, Morales C, Moreno V, Esteller M, Capella G, Ribas M *et al.* 2006 Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. *Cancer Research* **66** 8462–9468. (doi:10.1158/0008-5472.CAN-06-0293)

Rusiecki JA, Baccarelli A, Bollati V, Tarantini L, Moore LE & Bonefeld-Jorgensen EC 2008 Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. *Environmental Health Perspectives* **116** 1547–1552. (doi:10.1289/ehp.11338)

Song L, James SR, Kazim L & Karpf AR 2004 Specific method for the determination of genomic DNA methylation by liquid chromatography–electrospray ionization tandem mass spectrometry. *Analytical Chemistry* **77** 504–510. (doi:10.1021/ac0489420)

Stancheva I, El-Maarri O, Walter J, Niveleau A & Meehan RR 2002 DNA methylation at promoter regions regulates the timing of gene activation in *Xenopus laevis* embryos. *Developmental Biology* **243** 155–165. (doi:10.1006/dbio.2001.0560)

St John JA, Braun EL, Isberg SR, Miles LG, Chong AY, Gongora J, Dalzell P, Moran C, Bed'hom B, Abzhanov A *et al.* 2012 Sequencing three crocodilian genomes to illuminate the evolution of archosaurs and amniotes. *Genome Biology* **13** 415. (doi:10.1186/gb-2012-13-1-415)

Terry MB, Delgado-Cruzata L, Vin-Raviv N, Wu HC & Santella RM 2011 DNA methylation in white blood cells: association with risk factors in epidemiologic studies. *Epigenetics* **6** 828–837. (doi:10.4161/epi.6.7.16500)

Tucker KL 2001 Methylated cytosine and the brain: a new base for neuroscience. *Neuron* **30** 649–652. (doi:10.1016/S0896-6273(01)00325-7)

Veenstra GJ & Wolffe AP 2001 Constitutive genomic methylation during embryonic development of *Xenopus*. *Biochimica et Biophysica Acta* **1521** 39–44. (doi:10.1016/S0167-4781(01)00280-9)

Woodfine K, Huddleston JE & Murrell A 2011 Quantitative analysis of DNA methylation at all human imprinted regions reveals preservation of epigenetic stability in adult somatic tissue. *Epigenetics & Chromatin* **4** 1. (doi:10.1186/1756-8935-4-1)

Yang AS, Estecio MRH, Doshi K, Kondo Y, Tajara EH & Issa J-PJ 2004 A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Research* **32** e38. (doi:10.1093/nar/gnh032)

Zhu ZZ, Hou L, Bollati V, Tarantini L, Marinelli B, Cantone L, Yang AS, Vokonas P, Lissowska J, Fustinoni S *et al.* 2012 Predictors of global methylation levels in blood DNA of healthy subjects: a combined analysis. *International Journal of Epidemiology* **41** 126–139. (doi:10.1093/ije/dyq154)

Received 2 October 2013
First decision 4 November 2013
Revised manuscript received 13 December 2013
Accepted 7 January 2014