RESEARCH ARTICLE

Low Incubation Temperature Induces DNA Hypomethylation in Lizard Brains

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ABSTRACT

Developmental stress can have organizational effects on suites of physiological, morphological, and behavioral characteristics. In lizards, incubation temperature is perhaps the most significant environmental variable affecting embryonic development. Wall lizards (*Podarcis muralis*) recently introduced by humans from Italy to England experience stressfully cool incubation conditions, which we here show reduce growth and increase the incidence of scale malformations. Using a methylation-sensitive AFLP protocol optimized for vertebrates, we demonstrate that this low incubation temperature also causes hypomethylation of DNA in brain tissue. A consistent pattern across methylation-susceptible AFLP loci suggests that hypomethylation is a general response and not limited to certain CpG sites. The functional consequences of hypomethylation are unknown, but it could contribute to genome stability and regulation of gene expression. Further studies of the effects of incubation temperature on DNA methylation in ectotherm vertebrates may reveal mechanisms that explain why the embryonic thermal environment often has physiological and behavioral consequences for offspring. *J. Exp. Zool. 00:1–6, 2016.* © 2016 Wiley Periodicals, Inc.

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INTRODUCTION

Novel environmental conditions can induce developmental stress and may cause a wide range of morphological, physiological, and behavioral modifications. For example, in ectotherms, exposure to temperatures at the extremes of a species' tolerance has been shown to affect offspring size and shape as well as their physiology, behavior, and cognition (Deeming, 2004; Booth, 2006; Amiel et al., 2014). These physiological and behavioral effects can be long lasting (e.g., Flores et al., '94; Trnik et al., 2011) and may even span several generations (Warner et al., 2013). This raises the possibility that temperature affects epigenetic systems that regulate gene expression, a commonly hypothesized route to "developmental programming" in mammals (Gapp et al., 2014; Fernandez-Twinn et al., 2015). Indeed, in plants both heat and cold stress can affect global and site-specific DNA methylation, making temperature a candidate for studying how developmental stress shapes epigenetic variation (Liu et al., 2015).

Studies of temperature effects on DNA methylation in animals are more limited, but a recent study on polychaetes described a reduction in global levels of DNA methylation in individuals developing under unusually high temperature (Marsh and Pasqualone, 2014). The mechanisms underlying these effects are poorly understood, but they may be due to

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temperature-dependent expression of genes involved in de novo methylation and its maintenance, including DNA methyl transferases (DNMTs) and methyl-CpG binding domain proteins. For example, studies in maize demonstrated that expression of a DNA methyltransferase was downregulated during cold stress and correlated with genomewide demethylation (Steward et al., 2000). Other studies report upregulation of methyltransferases at low temperatures (e.g., Campos et al., 2012), but an increase in DNA methylation at low temperature may also be caused by the general tendency of genes with low expression to become highly methylated (Hu et al., 2012).

As a first step to assess the potential for temperature-dependent DNA methylation in vertebrates, we tested experimentally if incubation temperature affects DNA methylation in wall lizards, *Podarcis muralis*. Wall lizards have been introduced from Italy to England (Michaelides et al., 2015), exposing them to substantially cooler climate in their nonnative range compared to their native range (While et al., 2015). Low soil temperature generates strong selection for the ability to maintain developmental processes at temperatures below what is typically encountered in the native populations (While et al., 2015). Thus, modification of DNA methylation by temperature is not only of interest from a developmental perspective but also in terms of its potential consequences for individual variation and population processes, including adaption. Here we test if low-temperature stress can induce global changes in DNA methylation by characterizing the methylation status of CpG sites in brain tissue using methylation-sensitive AFLP.

MATERIALS AND METHODS

The common wall lizard, *Podarcis muralis*, is a small oviparous lizard native to southern and Western Europe. In spring 2013, we collected gravid female lizards from three locations in Tuscany, which belong to a mitochondrial lineage found in nonnative populations (Michaelides et al., 2013). Lizards introduced from this region to England have adapted to the lower incubation temperatures (While et al., 2015). The lizards were transported at 10°C to the facilities in Oxford, where they were kept individually in cages with a heat source, shelter, and food and water (see While et al. 2015 for details on husbandry). Eggs were collected within 24 hr of oviposition, and each clutch ($N = 25$) was split into two groups, each incubated at either 20 or 24°C ($N = 47$ and 50 eggs, respectively) in small plastic containers filled with a 5:1 vermiculite to water volume. Twenty degree centigrade is at the minimum constant temperature that allows successful hatching, whereas 24°C is within the range of optimal constant temperatures for hatching success (e.g., Vandamme et al., '92). Clutches were inspected daily for hatchlings. Hatchlings were measured for snout-to-vent length (SVL) and total length to the closest millimeters, weighed to the closest 0.01 g, and sexed using hemipene eversion. The incidence of malformations of the cephalic scales (pileus) was scored following a standardized scheme where we used the sum of the number of scale abnormalities (split scale or insertion of additional scale) as our estimate of the incidence of malformation and hence the severity of developmental stress (Zakharov, '89). Animals were sacrificed using concussion to the head followed by immediate freezing at −80°C.

Whole brains were dissected while frozen by cutting the skull open along sutures. The brains were split with a sterile scalpel along the sagittal line, and the tissue was immediately placed in 180 μL of ice-chilled ATL tissue lysis buffer plus 20 μL Proteinase K for final concentration of 2 mg/μL (Qiagen, California, USA) and mechanically dissociated. Tissue samples were incubated in lysis buffer for 72 hr at 65°C, and the DNA was isolated using a DNeasy blood and tissue kit (Qiagen, California, USA), resuspended in 200 μL of elution buffer, and stored in −20°C freezer. DNA concentration and purity were calculated using Nanodrop, and DNA integrity was verified by gel electrophoresis (1 hr, 50 V, TBE 0.5×, 0.7% v/v). We used CA solution (Microzone, Haywards Heath, UK) to remove RNA contamination, and only samples with a concentration above 10 ng/μL and no sign of DNA degradation were included in the study.

We quantified DNA methylation using a modified version of methylation-sensitive amplified fragment polymorphism (MSAP). The MSAP method based on the isoschizomers MspI and HpaII (in combination with EcoRI) has been extensively used on plants (e.g., Herrera and Bazaga, 2011), but we have established that existing protocols fail to produce reliable and repeatable results for at least some vertebrates, including wall lizards. The failure is mainly due to resistance of certain vertebrate genomes to be fully digested by restriction enzymes MspI and HpaII. If cleavage of DNA sites is not complete with either enzyme, it is not possible to determine whether the presence or absence of an amplified fragment in MspI or HpaII fingerprint profile is caused by the absence of cleavage site, methylation or hemimethylation. The application of MspI/HapII MSAP can therefore produce mismatches between MspI and HpaII profiles, which are often interpreted as hemimethylation, a type of epigenetic modification believed to be rare in vertebrate genomes (e.g., Laird et al., 2004). Additionally, the limited size distribution of CpG islands and correlation between MspI/HpaII and EcoRI cleavage sites increases the chances of producing polymerase chain reaction (PCR) amplification products of the same length, which reduces the reliability of inference about the methylation status of cleavage sites.

Instead of using HpaII+EcoRI/MspI+EcoRI to generate methylation and genetic fingerprints, we only used the combination of endonucleases NotI+MseI. Since NotI does not cleave methylated DNA, NotI-MseI MSAP produces repeatable, consistent, and unique methylation fingerprints and has therefore been used in vertebrates before (Yamamoto et al., 2001). NotI does not have an isoschizomere we could use to generate genetic

fingerprints of the same loci. On the basis that DNA synthetized via PCR (with a DNA polymerase) is devoid of methylated cytosines (Umetani et al., 2005), we generated a nonmethylated copy of each DNA sample.

In brief, we digested whole genomic DNA using the endonuclease MseI and ligated adapters to the cleavage ends. A portion of this ligated DNA was amplified by PCR. These two forms of DNA (naive and methylation free) were digested with NotI, thus producing genetic (from methylation free DNA) and epigenetic (from naive DNA) profiles of the same loci of the same sample only using one informative endonuclease. The rarity of NotI cleavage events (approximately 1 in every 64,000 bases) means that the PCR products are more likely to be representative of unique sites. Further information and the details of the laboratory procedures are found in the Supporting Information.

Genotyping was conducted in GeneMapper (version 3.7, California, USA) using default threshold intensities (50 relative fluorescent units) and a peak width of 1.5 bp. We calculated the methylation status per sample by counting presence or absence of locus-specific bands comparing naive and methylation free fingerprint profiles, similarly to standard MSAP (ReynaLopez et al., '97). When a band was found to be present in naïve and methylation-free DNA, this NotI site was considered unmethylated (scored as 1), absence in naïve DNA but presence in methylation-free DNA indicates methylation (scored as 2), and absence in methylation-free DNA but presence in naïve DNA are interpreted as inefficient PCR amplification of total DNA (scored as −1).

We calculated genotyping error per sample and repeatable loci per experiment using an AFLP script for R (Whitlock et al., 2008). Nine tissue samples were run and analyzed twice, and the data used to assess the repeatability of the assay and scoring of methylation across markers and individuals. We used the R script to select alleles with repeatable loci, and we excluded loci which were found in fewer than three individuals in the study, resulting in 28 suitable markers. Overall repeatability was estimated as the number of times; both analyses were in agreement (either methylated, unmethylated, or CpG site not present) divided by the total number of markers.

To test for the effect of temperature treatment on methylation status, we built a generalized linear mixed model explaining methylation status (methylated scored as 1 or unmethylated scored as 0) as a function of treatment and sex, while accounting for differences between clutches and markers (both random effects in the statistical model). For the nine tissue samples which were assayed twice, we randomly selected one of the assays. The model was run in R using the package MCMCglmm (Hadfield. 2010) with a binary error distribution and a logit link. We ran the models with commonly used priors ($V = 0.01$ and $nu = 2$, for both the error and random effects), for 500,000 iterations of which 50,000 were discarded as burn-in. To test whether individual markers were significantly hyper- or hypomethylated due to the temperature treatment, we also tested each marker separately. For each marker, we ran two generalized linear mixed models, one with and one without the treatment effect, and performed a chi square test to investigate the treatment effect. The models had binary error distributions and a logit link and had the factor treatment as fixed effect and clutch identity as random effect. The models were run in R using the package lme4. The distribution of χ^2 values was compared to the theoretically expected distribution given no effect of the treatment on methylation status, and P values were corrected for the false discovery rate.

RESULTS

Lizards incubated at 20°C developed slower, were lighter and smaller in terms of body size (SVL and total length), and showed an increase in the number of abnormal cephalic scales compared to lizards incubated at 24°C (Table 1).

Repeatability of the MSAP protocol based on nine individuals was 97.2%. Individuals showed large variation in estimated average methylation (from 0 to 62%), with an average of 12.6% methylated sites (Fig. S1 in the Supporting Information). Incubation temperature had a significant effect on methylation status

Table 1. Differences in the mean values for development time, body mass, snout-to-vent length (SVL), total length and head plate deformation for the two incubation temperatures.

Treatment:	20°C	24°C	Test statistic	df	*P* value
Development time (days)	121.8 (± 4.1)	54.7 (± 2.1)	$t = 97.1$	63.4	<0.001
Body mass (mg)	279 (± 38)	336 (± 34)	$t = -3.86$	10.9	0.003
SVL (mm)	24.6 (± 1.3)	25.9 (± 0.88)	$t = -5.32$	74.7	<0.001
Total length (mm)	60.7 (± 3.2)	66.3 (± 3.2)	$t = -7.98$	82.6	<0.001
Number of deformed scales	2.9 (0–10)	0.3 (0–2)	$W = 1727$		<0.001

For development time, body mass, SVL, and total length the means with standard deviations are reported, and the differences between the two temperatures were tested with the Welch two sample *t*-test.
Since head plate deformation is not normally distributed and skewed, means with the ranges of values are reported, and the difference between the two treatments was tested with the Wilcoxon rank sum test.

Table 2. Generalized linear mixed model explaining methylation of individual sites of individuals by treatment and sex, correcting for clutch and marker identity.

Effects	Posterior mean	Lower 95% CI	Upper 95% CI	P_{MCMC}
Random				
Clutch ID	0.255	0.057	0.493	
Marker ID	2.028	0.939	3.379	
Residuals	0.037	0.002	0.159	
Fixed				
Intercept	−2.437	−3.057	−1.790	<0.001
Treatment	0.620	0.327	0.889	<0.001
Sex	−0.122	−0.510	0.198	0.524

with individuals from 20°C treatment showing hypomethylation ($P < 0.001$; Table 2). On average, 7.7% (confidence interval: 4.2–13.5%) of the sites were methylated for the 20°C treatment compared to 13.1% (CI: 7.4–21.7%) of the sites for the 24°C treatment. Sex did not significantly affect methylation status (Table 2); however, one out of the 28 markers was significantly hypermethylated in females compared to males ($P = 0.0004$; Fig. S2 in the Supporting Information). In the same model, 9.0% of the variance in methylation status was explained by clutch of origin and 89.8% was explained by marker identity, suggesting modest variation in DNA methylation among families and large variation in methylation status amongst markers (Table 2; see also Fig. S1 in the Supporting Information). Nevertheless, 23 of 28 markers (82.1%) showed hypomethylation under stress, a significant deviation from 50% ($\chi^2_1 = 10.3$, $P = 0.0013$). The QQ-plot of χ^2-test statistics shows that the χ^2 tends to be higher than expected for many of the markers (Fig. 1), but no individual marker was significant following correction for false discovery rate.

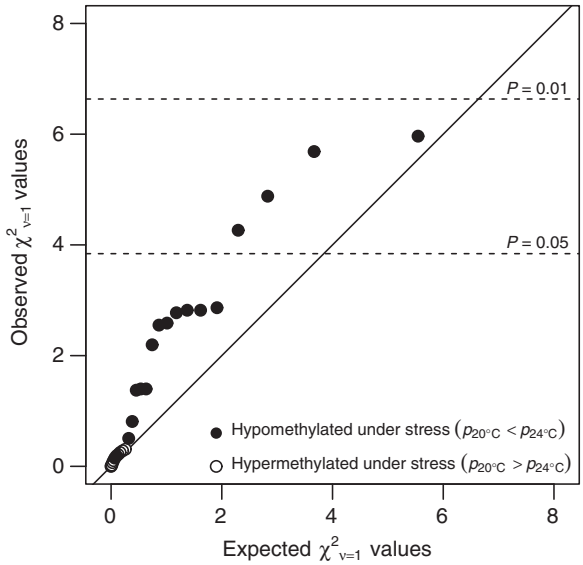

Figure 1. QQ plot for observed versus expected χ^2-test statistics when testing all markers separately. Observed values tend to be higher than expected, but none of the individual markers were significantly different after correction for false discovery rate.

DISCUSSION

Wall lizards introduced to England experience stressfully low temperatures during development (While et al. 2015). Our morphological data show that this is manifested in developmental abnormalities and small body size, both of which are likely to reduce survival (e.g., Olsson et al., '96). Although impaired gross development under thermal stress is commonly described in lizards (Deeming, 2004), we show for the first time that incubation temperature also affects the incidence of DNA methylation. This result is consistent with recent studies demonstrating that environmental stress can affect both global and site-specific DNA methylation plants, birds, and mammals, which may have consequences for gene regulation and genome stability in thermally stressed individuals.

Methylation-sensitive AFLP generates data on the methylation status of a sample of CpG sites throughout the genome. The cleavage sites for our restriction enzyme NotI coincide with CpG islands, and large tandem repeats and previous studies have found that these are often associated with functional genes (Costello et al., 2009). Although the causes of variation in DNA methylation during development are complex, the consistent reduction in methylation across loci suggests a generic mechanism is at play. One plausible mechanism is that low temperature limits the expression of genes involved in the establishment and maintenance of DNA methylation. This mechanism appears to explain low methylation in cold-stressed maize (Steward et al. 2000), cotton (Fan et al., 2013), and *Arabidopsis* (Naydenov et al., 2015). Furthermore, recent studies in ducks and zebra fish have demonstrated that DNMTs during embryonic development are responsive to temperature (Campos et al., 2012; Yan et al., 2015). Unfortunately, we did not measure expression of DNMTs in this study, so we cannot test this hypothesis. Patterns of DNA methylation are of course to some extent tissue dependent, but studies nevertheless often find concordance between tissues (e.g., Masliah et al., 2013; Walton et al., 2016). Our study was restricted to brain tissue. This has potentially important implications for understanding temperature effects on lizards as it has been shown, for example, that incubation temperature has short- and long-term effects on cognition (Amiel et al., 2014). However, brain tissue is only one of many tissues where hypomethylation could be of biological significance. Indeed, we predict that these effects will be shared across tissues in wall lizards if they,

as suggested above, represent a direct consequence of the rate of transcription or translation of genes involved in the DNA methylation machinery rather than a mechanism that evolved to fine-tune expression of particular genomic regions or specific tissues. Analyses of total methylation content of DNA, for example, using ELISA, would be useful to test this hypothesis. It would also be interesting to see if similar changes occur in germ cells and hence possibly could be transmitted to the next generation.

The overall impact of incubation temperature on the level of methylation was quite substantial, representing about 40% lower methylation. This magnitude of change, particularly during embryonic development and/or organogenesis, appears large enough to affect gene regulation and perhaps even genome stability, for example by increased activity of transposable elements (Slotkin and Martienssen, 2007). There is evidence that incubation temperature can organize behavior in lizards (Sakata and Crews, 2004), which may occur partly through changes in gene expression. Low incubation temperature is often associated with increased mortality, which could be one outcome of reduced genome stability. However, whether low incubation temperature actually has such consequences is currently speculative and can only be addressed using more sophisticated molecular techniques.

Since we have previously shown that nonnative lizards have adapted to cool incubation conditions by increasing the rate of development at low temperatures (which allows them to hatch before the onset of autumn; While et al. 2015), it would be interesting to test if the reduction in DNA methylation is smaller in nonnative populations compared to their native counterparts. If so, this could indicate that the evolutionary response in developmental rate at low temperatures has been accompanied by increased robustness of developmental processes more generally. This is not necessarily the case since fast growth could evolve independently of developmental robustness, perhaps resulting in long-term costs (Metcalfe and Monaghan, 2001).

In summary, the cool incubation temperature experienced by nonnative populations of wall lizards causes developmental stress manifested as smaller size, increased scale malformation, and a hypomethylated genome in brain tissue. These results suggest that further studies of DNA methylation in lizards could reveal epigenetic mechanisms that contribute to widely observed short- and long-term effects of incubation temperature on physiology and behavior.

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