Brood size influences patterns of DNA methylation in wild Zebra Finches (Taeniopygia guttata)

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Brood size influences patterns of DNA methylation in wild Zebra Finches (*Taeniopygia guttata*)

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ABSTRACT

Both natural and experimentally manipulated brood size can influence the competition dynamics among siblings in a nest and alter the environment during early development in birds. Brood size affects a variety of life-history and fitness-related traits, but relatively little is known about the mechanisms that might mediate these effects. There is accumulating evidence that early-life environments can influence adult phenotypes through epigenetic mechanisms such as variation in DNA methylation. Here, we profile DNA methylation in nestling Zebra Finches (*Taeniopygia guttata*) raised in naturally variable and experimentally manipulated brood sizes. We found that (1) natal brood size is significantly, positively correlated with percent DNA methylation in blood; and (2) individuals in manipulated broods experience significantly more demethylation events across early development than individuals from broods that remain the same size. Any manipulation of brood size creates fluctuations in early developmental conditions, potentially explaining why the frequency of demethylation events in these treatments was higher than in control broods. We also found that the specific loci that lost and gained methylation across early development differed between individuals in enlarged and reduced broods, which may reflect the different developmental pressures imposed by the different manipulations. Although the phenotypic consequences of reduced levels of methylation are yet to be elucidated, our findings support the hypothesis that brood size is associated with the prevalence and pattern of DNA methylation in wild birds.

Keywords: brood size, DNA methylation, early development, epigenetics, MS-AFLP, Zebra Finch

El tamaño de la nidada influye en los patrones de metilación del ADN en individuos silvestres de *Taeniopygia guttata*

RESUMEN

El tamaño de la nidada, tanto natural como experimentalmente manipulado, puede influir las dinámicas de competencia entre hermanos en un nido y alterar el ambiente durante el desarrollo temprano en las aves. El tamaño de la nidada afecta una variedad de rasgos de la historia de vida y de la adecuación biológica, pero se sabe relativamente poco sobre los mecanismos que podrían mediar en estos efectos. La evidencia acumulada sugiere que los ambientes de la vida temprana pueden influir en los fenotipos de los adultos a través de mecanismos epigenéticos como la variación en la metilación del ADN. Aquí, perfilamos la metilación del ADN en polluelos de *Taeniopygia guttata* criados en tamaños de nidada naturalmente variables y manipulados experimentalmente. Encontramos que i) el tamaño original de la nidada está correlacionado positiva y significativamente con el porcentaje de metilación del ADN en sangre, y ii) los individuos de nidadas manipuladas experimentan significativamente más eventos de desmetilación a lo largo del desarrollo temprano que los individuos de nidadas que permanecen del mismo tamaño. Cualquier manipulación del tamaño de la nidada crea fluctuaciones en las condiciones tempranas del desarrollo, explicando potencialmente por qué la frecuencia de los eventos de desmetilación en estos tratamientos fue mayor que en los polluelos de las nidadas control. También encontramos que los loci específicos que ganaron y perdieron metilación a lo largo del desarrollo temprano difirieron entre individuos de nidadas agrandadas y reducidas, lo que puede reflejar las diferentes presiones de desarrollo impuestas por las diferentes manipulaciones. Aunque aún deben dilucidarse las consecuencias fenotípicas de niveles reducidos de metilación, nuestros resultados apoyan la hipótesis de que el tamaño de la nidada está asociado con la prevalencia y el patrón de metilación del ADN en las aves silvestres.

Palabras clave: desarrollo temprano, epigenética, metilación del ADN, MS-AFLP, *Taeniopygia guttata*, tamaño de la nidada
INTRODUCTION

Experiences during early development exert great influence on adult phenotypes in a range of taxa, including birds (Heijmans et al. 2009, Frésard et al. 2013). Brood size imposes long-term effects on individual fitness, physiology, and behavior, due to the elevated costs associated with sibling competition (resulting in less food per nestling and more energy expended on begging) and nest crowding (resulting in elevated nest temperature and parasite load) (Naguib and Gil 2005, Verhulst et al. 2006, Wegmann et al. 2015). Large brood sizes have been linked with a variety of offspring phenotypes, including decreased offspring weight (Nettle et al. 2013), reduced reproductive success in adults (de Kogel and Prijs 1996, Naguib et al. 2006), elevated metabolic rates (Verhulst et al. 2006), and shortened telomere lengths (associated with longevity and fitness; Mizutani et al. 2016). While many phenotypes associated with augmented brood size are deleterious, others may represent adaptive responses (Champagne and Meaney 2006, Zimmer et al. 2013). For example, Great Tits (Parus major) raised in large broods are known to be more exploratory and more aggressive than those raised in smaller broods, which could be adaptive in competitive conditions (Carere et al. 2005, Zimmer et al. 2013, Bloxham et al. 2014). Despite a wide range of studies documenting the relationship between brood size and phenotypic variation, we know little about the molecular mechanisms that potentially mediate these effects (Frésard et al. 2013). However, insight into these mechanisms would contribute to our understanding of how such widespread and persistent phenotypic variation emerges in response to early developmental conditions.

There is accumulating evidence that early life conditions can influence adult fitness though epigenetic mechanisms, such as DNA methylation (Meaney and Szyf 2005, McGhee and Bell 2014). DNA methylation is the addition of a methyl group to a cytosine, which may alter the transcriptional state of the DNA and can significantly affect gene expression (Weber et al. 2007). Genome-wide patterns of DNA methylation are established during embryogenesis; however, these patterns can be modified by environmental influences at sensitive phases during early development (Vickaryous and Whitelaw 2005, Heijmans et al. 2009, Feil and Fraga 2012). Environmentally induced changes to DNA methylation patterns have consequently been proposed to provide an avenue through which early developmental conditions can modulate individual phenotypes (Zhang et al. 2013, Roth et al. 2014). A well-studied example involves variation in maternal grooming behavior in rats (Rattus norvegicus), in which reduced grooming increases DNA methylation levels around offspring glucocorticoid receptors, resulting in decreased expression of this gene and elevated stress responses (Meaney and Szyf 2005).

Changes to DNA methylation patterns have been induced by a variety of developmental conditions, including the nutritional environment (Waterland et al. 2010, Snell-Rood et al. 2013, Lea et al. 2016), the level of parental care experienced (McGhee and Bell 2014, Roth et al. 2014), breeding density (Bentz et al. 2016), thermal regime (Renaudeau et al. 2012, Weyrich et al. 2016), environmental predictability (Leung et al. 2016), and climate conditions (Dimond and Roberts 2016, Rubenstein et al. 2016). Given that brood size exerts such a significant influence on early development in birds, we predicted that these effects could be mediated by modifications to DNA methylation patterns. Therefore, we compared nestling Zebra Finches (Taeniopygia guttata) raised in nests of naturally variable brood sizes to determine whether DNA methylation is influenced by natal brood size. We also experimentally manipulated brood size and used repeated blood samples to examine whether genome-wide and site-specific DNA methylation patterns changed with different brood-size alterations over developmental time. Previous studies of this species have shown both wild and captive Zebra Finches to be sensitive to brood manipulations, which have resulted in phenotypic changes at the nestling stage that persist throughout life (Tschirren et al. 2009, Griffith and Buchanan 2010, Mariette and Griffith 2015).

METHODS

Data Collection

Blood was collected from 133 nestlings from 48 Zebra Finch nests at Fowlers Gap Arid Research Station, New South Wales, Australia, in October 2016. All blood samples were taken from nestlings in nest boxes in their natural habitat (for details of field-site characteristics, see Griffith et al. 2008). Blood (<20 μL) was sampled from the metatarsal vein of young (3-day-old) nestlings and from the brachial vein of older nestlings (10–11 days). Blood was preserved in 95% ethanol and stored at room temperature. We used blood as a study tissue because it enabled repeated, nonlethal sampling across the early development of the Zebra Finch. Blood is also relevant when profiling DNA methylation, because it can be used to identify environmental effects on DNA methylation around phenotypically relevant genes (Frésard et al. 2013).

Cross-Fostering

A brood-manipulation experiment was carried out by cross-fostering nestlings. This entailed pairing 2 nests with nestlings at the same developmental age (day 3 after hatching) and cross-fostering a subset (1–3) of nestlings from each nest in order to manipulate the brood size. Parents and the natal nest also experienced a brood-size
change (i.e. an unequal number of chicks were translocated from each nest to achieve the brood-size manipulation). To mitigate the effects of handling, the chicks we used for DNA methylation profiling were not the translocated chicks, but rather the unmanipulated chicks that experienced a brood-size change from the effects of their siblings being removed and the fostered chicks being added.

Brood sizes naturally ranged from 2 to 8 nestlings, and manipulated broods changed by a minimum of $25\pm 0.246\%$ (SE). We took 2 blood samples from each nestling during early development to enable a within-individual comparison of methylation state. The first blood sample was taken on day 3 after hatching, immediately before the brood manipulation, and the second sample was taken $\sim 1$ wk after brood manipulation (days 10–11 after hatching). We were unable to take a later sample because the nestlings are prone to leave the nest prematurely if disturbed after $\sim 12$ days of age.

**MS-AFLP**

We attempted to use MS-AFLP (methylation sensitive-amplification fragment length polymorphism) on 96 individuals from 37 families (i.e. 192 blood samples including repeats). We excluded individuals that had only one blood repeat (due to natural mortality) or yielded a blood sample that was too low to extract a sufficient quantity of DNA. The MS-AFLP protocol was successful for only 62 blood samples (32.3% of the total collected) from 43 individuals and 25 nests. Logistic constraints (E.L.S. conducted the molecular work while visiting the laboratory of A.W.S., and the visit had a fixed duration that did not allow for repeated attempts at the protocol) meant that we were unable to rerun the failed samples. The scoring step was unsuccessful because of a failure at the restriction digest stage of the MS-AFLP procedure, where inconsistencies in the initial concentrations of DNA extract meant that the methylation-sensitive enzymes (see below) did not cut optimally in all samples. This meant that a significant subset of the subsequent polymerase chain reactions (PCRs) failed. Consequently, there are no underlying differences between the DNA samples that succeeded and failed in our study (and, thus, no bias in our dataset; however, we acknowledge that these failures decreased our sample size substantially).

In total, we successfully scored methylation in 30 individuals from 18 families at the natal stage (3 days after hatching), and in 28 individuals from 13 families at the older time-point (10–11 days after hatching). We successfully scored the methylation of 20 of these individuals, from 17 different nests at both time-points (days 3 and 11). For these individuals, we were able to compare DNA methylation before and after the brood manipulation. For these 20 individuals with successful repeated bleeds, broods were manipulated by an average ($\pm$ SE) of $37.5\pm 0.19\%$. We defined 3 brood-manipulation treatments (Table 1). Given our small sample sizes, we took the average level of DNA methylation from the one nest in each treatment for which we had data for 2 siblings.

**MS-AFLP Protocol**

DNA was extracted using the Gentra Puregene tissue kit and stored in 30 µL of DNA Hydration Solution. We screened samples for variation in DNA methylation using MS-AFLP, which modifies the standard AFLP protocol by substituting the MseI enzyme with the methylation-sensitive isoschizomeric enzymes MspI and HpaII (New England Biolabs, Ipswich, Massachusetts, USA). Enzymes MspI and HpaII have different sensitivities to cytosine methylation of their shared recognition sequence (CCGG) and together result in 4 types of variation that can be scored to indicate methylation status at particular loci (Richards et al. 2012; Table 2). Type 4 variation may represent epigenetic or genetic variation (Table 2); however, in some cases where individuals had repeated

### Table 1. Brood-manipulation treatments and their abbreviations, with sample sizes of 20 nestling Zebra Finches from 17 nests, bled at 2 time-points during postnatal development: before and after brood manipulation.

<table>
<thead>
<tr>
<th>Brood-manipulation treatment</th>
<th>Abbreviation</th>
<th>Nestlings (n)</th>
<th>Nests (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Developing brood greater than natal brood</td>
<td>Increased</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Developing brood smaller than natal brood</td>
<td>Decreased</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Developing brood equal to natal brood</td>
<td>Same size</td>
<td>7</td>
<td>6</td>
</tr>
</tbody>
</table>

### Table 2. Epigenetic variation in DNA methylation scored using MS-AFLP. "Y" indicates that the enzyme cut at the restriction site; "N" indicates that the enzyme did not cut at the restriction site.

<table>
<thead>
<tr>
<th>Variation</th>
<th>MspI</th>
<th>HpaII</th>
<th>Methylation status of restriction site</th>
<th>Methylation?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>Y</td>
<td>Y</td>
<td>No methylation</td>
<td></td>
</tr>
<tr>
<td>Type 2</td>
<td>Y</td>
<td>N</td>
<td>A methylated internal cytosine</td>
<td>Yes</td>
</tr>
<tr>
<td>Type 3</td>
<td>N</td>
<td>Y</td>
<td>A methylated outer cytosine</td>
<td>Yes</td>
</tr>
<tr>
<td>Type 4</td>
<td>N</td>
<td>N</td>
<td>Both cytosines methylated or a genetic mutation</td>
<td>Dependent</td>
</tr>
</tbody>
</table>
blood samples, we were able to distinguish between these two types of variation: if one sample had a Type 4 site and the repeated sample did not, we were able to identify this as epigenetic variation. If both repeated samples had Type 4, or in the cases without paired samples, we conservatively treated Type 4 as not methylated. Recently there has been a suggestion that Types 2 and 3 should be analyzed as separate states (Schulz et al. 2014); however, the actual source of these types of variation may be more complicated, based on nested fragments (Fúlneček and Kovařík 2014). As such, we combined Types 2 and 3 into one methylated category and treated all other states as not methylated. Throughout, we refer to an MS-AFLP locus to indicate a particular-sized band resolved in the selective PCR.

We performed MS-AFLP following the protocol used by Richards et al. (2012). We digested ~250 ng of genomic DNA at 37°C for 3 hr in paired reactions: one with EcoRI andMspI, the other with EcoRI and HpaII. We immediately followed the restriction digest with adapter ligation with EcoRI and MspI/HpaII adapters at 16–20 hr at 16°C (Appendix Table 5: all primer and adapter sequences). After adapter ligation, we conducted preselective PCR with EcoRI+1, MspI/HpaII+0 preselective primers (Appendix Table 5) at the following PCR conditions: 75°C for 2 min, 20 cycles of 94°C for 30 s, 56°C for 30 s, 75°C for 2 min, final extension at 60°C for 30 min, and 4°C hold. Following preselective PCR, we conducted selective PCR by multiplexing 6-FAM fluorescently labeled EcoRI+AGC primers and unlabeled primers HpaII/MspI+TCAT (Appendix Table 5) at the following PCR conditions: 94°C for 2 min, 8 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 2 min (dropping the annealing temperature 1°C each cycle), 31 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 2 min, final extension of 60°C for 5 min, and a 4°C hold. We sent the selective PCR products to Georgia Genomics Facility (USA) for fragment analysis on an ABI 3130XL.

We used PEAKSCANNER 1.0 (Applied Biosystems, Foster City, California, USA) to analyze resultant gel files and define fragment sizes and RAWGENO (Arrigo et al. 2012) to define bands. We eliminated bands that inconsistently amplified or occurred at highly variable intensities among individuals. We pooled data into 2 categories: methylated (Type II, Type III, and for paired samples appropriate Type IV cases) or not methylated (Type I and Type IV).

**Data Analysis**

We conducted all analyses using a binary haplotype-binding pattern (methylated 1, not methylated 0) for 107 verified consistent banding sites between 50 and 500 base pairs. Throughout, we used a sequential Bonferroni correction of α = 0.05 for multiple tests. We calculated percent methylation as the proportion of the 107 loci that were methylated for each individual.

**Does brood size influence percent DNA methylation across the genome?** For the 30 individuals (from 18 families) that had at least the natal bleed, we analyzed the relationship between percent DNA methylation and natal brood size using a linear mixed model (LMM). The LMM was run in R 3.3.1 (R Core Team 2015) using the package “lmer” (Bates et al. 2015) with the package “lmerTest” (Kuznetsova et al. 2016) to calculate degrees of freedom and P values. We modeled the effects of brood size (fixed effect) on percent DNA methylation. We included nest ID as a random factor. We calculated the marginal $R^2$ and intraclass correlation coefficient (ICC) using the method described by Nakagawa and Schielzeth (2013).

We used a nonparametric Spearman’s correlation analysis (as we were limited by sample size) to test for a relationship between brood size and the genome-wide level of DNA methylation in nestlings at day 11 after hatching. For this analysis, we used only the nestlings whose brood size remained the same throughout development (i.e. individuals in the “no-change” treatment; we used nest averages for the one case where there was >1 chick per nest).

**How do brood-size manipulations affect genome-wide percent DNA methylation across early development?** For the paired samples (20 individuals from 17 nests), we compared the methylation state of the earlier sample to the later sample at the same 107 variable loci. In the one case in each brood-manipulation treatment that had more than one chick per nest, we took nest averages to account for familial effects. Because of low sample sizes, we used a Wilcoxon signed-ranks analysis to compare percent DNA methylation between days 3 and 11 after hatching in chicks from each brood-manipulation treatment.

To test whether the percent change in DNA methylation levels between days 3 and 11 after hatching differed significantly between individuals in different brood-manipulation treatments, we used a one-way analysis of variance (ANOVA) and subsequent Tukey post hoc analyses.

**How do brood-size manipulations affect the number of cases of locus hypomethylation and hypermethylation?** Individual loci can lose methylation (hypomethylation) and gain methylation (hypermethylation) across development. We used a one-way ANOVA and subsequent Tukey post hoc analyses to compare the frequency of cases of locus hypermethylation and hypomethylation (from day 3 to day 11 after hatching) in individuals from each brood-manipulation treatment. This analysis allowed us to test, for example, whether a small decrease in percent DNA methylation across the development of an individual was caused by the hypomethylation of a small number of loci.
or by the hypomethylation of a large number of loci and the hypermethylation of a smaller subset of loci.

Do brood-size increases and decreases elicit methylation-state changes in different loci? Different brood-manipulation treatments may target the methylation-state change of different loci. For example, consistent hypermethylation at “locus 29” across all individuals in the brood-increase treatment, vs. consistent “no methylation state change” at the same locus across all individuals in the brood-decrease treatment, would highlight the potential for directed hypomethylation of locus 29 by brood-size increases. To illustrate how the methylation state of individual loci may respond differently to each brood manipulation, we calculated the percentage of individuals that underwent hypomethylation or hypermethylation at each of the 107 individual loci. For illustrative purposes, we then selected the 10 loci that responded most differently among individuals from brood-increase and brood-decrease treatments. We plotted the percentage of individuals that were hypomethylated or hypermethylated at those 10 loci, and compared those percentages between chicks that experienced a brood-size increase or decrease (Figure 1).

RESULTS

Natal Brood Size Is Positively Correlated with DNA Methylation Levels

The LMM showed a significant positive relationship between percent DNA methylation and brood size, such that the larger the natal brood size, the more loci were methylated (Table 3 and Figure 2). The random factor of nest ID did not explain a significant portion of variance in the data (ICC = 0.01; Table 3; however, increasing the number of individuals within each random factor level [nest] may reveal an influence of nest ID on DNA methylation levels. The Auk: Ornithological Advances 135:1113–1122, © 2018 American Ornithological Society

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>SE</th>
<th>df</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.133</td>
<td>0.067</td>
<td>1</td>
<td>1.987</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Brood size</td>
<td>0.054</td>
<td>0.012</td>
<td>1</td>
<td>4.633</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Marginal $R^2 = 0.417$

<table>
<thead>
<tr>
<th>Variable</th>
<th>Variance</th>
<th>SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nest ID</td>
<td>0.015</td>
<td>0.121</td>
<td>18</td>
</tr>
<tr>
<td>ICC</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 1. Variation in the number of individual Zebra Finches undergoing (A) hypomethylation or (B) hypermethylation across development (days 3 to 11 after hatching) at specific loci in different brood-manipulation treatment (e.g., 5 chicks experiencing a brood increase were hypomethylated at locus 107, whereas only 1 chick experiencing a brood decrease was hypomethylated at this locus).

FIGURE 2. A significant, positive correlation between natal brood size and nest averages of percent DNA methylation (among 107 loci) for 18 families and 30 nestling Zebra Finches sampled before cross-fostering to day 3 after hatching.
methylation). Genome-wide percent DNA methylation differed by an average of 19.04% between natal broods with 4 nestlings (average ± SE = 27.73 ± 0.19%, range: 15.89–52.34, n = 6 nests) and broods with >6 nestlings (46.77 ± 0.25%, 23.36–61.37%, n = 8 nests).

We detected no significant correlation between brood size and genome-wide percent DNA methylation at day 11 after hatching (Spearman's correlation: r^2 = 0.821, P = 0.089). However, there was a trend for larger broods to have greater levels of genome-wide methylation, which is consistent with the pattern observed at day 3 (we acknowledge that this analysis is limited by sample size).

**Individuals from Manipulated Broods Are Associated with a Loss of DNA Methylation across Early Development**

Chicks that experienced a decline in brood size had significantly higher genome-wide percent DNA methylation at day 3 after hatching, compared to day 11 after hatching (Z = −2.201, P = 0.028; Wilcoxon signed-ranks analysis), losing an average (± SE) of 16.67 ± 0.22% methylation through development. Chicks that experienced no brood-size change had higher percent DNA methylation at day 3 compared to day 11 after hatching (Z = 1.992, P = 0.046), gaining an average of 7.79 ± 0.16% methylation through development. Chicks that experienced a brood-size increase had significantly more methylation at day 3 compared to day 11 (Z = −2.366, P = 0.018), losing an average of 10.37 ± 0.33% methylation through development (Figure 3).

The degree to which percent DNA methylation changed from day 3 to day 11 after hatching significantly differed between the 3 brood-manipulation treatments (one-way ANOVA: F_{2,16} = 6.771, P = 0.009). A Tukey post hoc test showed that individuals from the brood-decrease treatment lost significantly more DNA methylation than individuals from the no-change treatment (P = 0.008), individuals from brood-increase treatment lost more DNA methylation than individuals from the no-change treatment (however, this was not significant; P = 0.060). There was no difference in percent DNA methylation change between the brood-decrease and brood-increase treatments (P = 0.665).

**TABLE 4.** Mean numbers of cases, with 95% confidence intervals in parentheses, of locus hypomethylation and hypermethylation between days 3 and 11 after hatching in individual Zebra Finches that experienced different brood-manipulation treatments.

<table>
<thead>
<tr>
<th>Brood manipulation</th>
<th>Hypomethylated loci per individual</th>
<th>Hypermethylated loci per individual</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brood increase</td>
<td>14.5 (9.9–17.4)</td>
<td>3.0 (1.2–4.4)</td>
<td>6</td>
</tr>
<tr>
<td>Brood decrease</td>
<td>17.7 (13.4–19.6)</td>
<td>9.8 (6.9–11.4)</td>
<td>5</td>
</tr>
<tr>
<td>No change</td>
<td>5.7 (3.4–7.2)</td>
<td>16.2 (19.7–27.3)</td>
<td>6</td>
</tr>
</tbody>
</table>

**FIGURE 3.** Changes in genome-wide levels of DNA methylation in Zebra Finches between days 3 and 11 after hatching in each brood-manipulation treatment.
Brood Increases and Decreases Elicit Methylation State Changes at Different Loci

While the overall pattern of change identified the most common outcome as greater locus hypomethylation in the brood-increase and brood-decrease treatments compared to the “no change” treatment (Table 3), all loci did not respond in the same way. While the methylation state change of some loci were consistent between treatments, each brood-manipulation treatment also had unique changing loci (i.e. some loci that were hypermethylated or hypomethylated across all individuals in the brood-decrease treatment remained unchanged in individuals from the brood-increase treatment, and vice versa). We have illustrated the 10 most extreme cases of variation in hypermethylation and hypomethylation between individuals experiencing brood-size increases and decreases (in Figure 1).

Between brood-increase and brood-decrease treatments, there was no variation in the number of individuals that underwent hypomethylation at 49 (of 107) loci. Similarly, there was no variation in the number of individuals that underwent hypermethylation at 47 loci between the 2 treatments. At 11 loci, the number of individuals that underwent hypermethylation differed by >3 individuals (50%) between the 2 brood-manipulation treatments; and at 5 loci, the number of individuals that underwent hypomethylation differed by >3 individuals between the 2 brood-manipulation treatments.

DISCUSSION

Like many avian species (Hegner and Wingfield 1987, Wegmann et al. 2015), Zebra Finches are extremely sensitive to brood-size manipulations, which induce phenotypic changes that can persist throughout life (de Kogel 1997, Griffith and Buchanan 2010, Mariette and Griffith 2015). Our results show that (1) natal brood size is correlated with percent DNA methylation in blood of nestling Zebra Finches and (2) individuals in manipulated broods experience significantly more demethylation events across early development than individuals from broods that remain the same size. We also note that the specific loci that lost and gained methylation across early development tended to be different in each brood-manipulation treatment. Our results support the hypothesis that early life conditions associated with brood size can influence the prevalence and pattern of DNA methylation in wild Zebra Finches.

We detect a significant, positive correlation between percent DNA methylation 3 days after hatching and natal brood size, such that large natal brood sizes were associated with higher levels of DNA methylation. The relationship between percent DNA methylation at hatching and natal brood size could occur through prenatal epigenetic programming, via maternal effects (Champagne et al. 2006, Murgatroyd et al. 2009). For example, females are known to allocate fewer resources to eggs when they produce larger clutches (Williams 2001), and Bentz et al. (2016) recently identified a positive correlation between maternal testosterone allocation to embryos and percent DNA methylation in wild nestling Eastern Bluebirds (Sialia sialis). We also identified a positive (yet statistically insignificant) relationship between DNA methylation level and brood size 11 days after hatching, indicating that the positive relationship between brood size and DNA methylation levels may be maintained through developmental time, and this may be detected with greater sample sizes.

Although most epigenetic programming has been considered to occur prenatally (Vickaryous and Whitelaw 2005), postnatal experiences have also been shown to influence DNA methylation patterns in a range of taxa (Weaver et al. 2004, Renaudeau et al. 2012). We found a significant trend for individuals that experienced a postnatal reduction or increase in brood size to show a greater loss of methylated loci than individuals from broods that remained the same size. Our results suggest that brood-size manipulations in general induce more demethylation events than those induced in nonmanipulated cases. Leung et al. (2016) found that the rate of stochastic epimutations rises when fish (Chrosomus eos-neogaeus) are exposed to stress imposed by fluctuating environments. Stress is known to induce stochastic variation in DNA methylation because it amplifies the error rate of methyltransferase in the establishment of new methylation patterns during DNA replication (Riggs et al. 1998). Broods that either increase or decrease in size create fluctuations in early developmental conditions (brood enlargements may increase competition for food, and brood-size reductions may decrease competition for provisions or reduce provisioning if parents suspect reductions to be caused by predation (Martin et al. 2011). Consequently, the influence of environmental fluctuations on the lability of DNA methylation may explain why methylation patterns were similar between the 2 experimental treatments in the present study, despite potentially imposing or alleviating different developmental pressures (Herman et al. 2014). Given that stress-induced DNA methylation changes can affect individual phenotypes (Cubas et al. 1999, Miura et al. 2009), further work should examine the potential for stochastically established DNA methylation patterns to facilitate an individual’s survival in fluctuating environments via diversified bet-hedging strategies (Piggot 2010, Herman et al. 2014).

In addition to stochastic epigenetic changes, several studies have shown that environmentally induced DNA methylation changes can be coordinated to translate environmental signals to directed phenotypic changes (Rubenstein et al. 2016, Weyrich et al. 2016). For example,
baboons (*Papio cynocephalus*) exposed to unpredictable compared with stable food accessibility were found to have different patterns of DNA methylation in loci that differentially affect the expression of genes related to metabolism (Lea et al. 2016). Although we cannot interpret the significance of DNA methylation changes for perceptive gene regulation in the present study, we did identify that the overall direction of change in methylation state for a small subset of loci was different in each treatment. With larger sample sizes, it may be possible to detect whether hypomethylation at these particular loci is associated with brood-manipulation treatment—potentially reflecting the different pressures imposed by brood enlargements and reductions—and perhaps indicates that locus-specific DNA methylation changes are, to some degree, tailored to different brood-manipulation experiences.

Our findings contribute to a growing body of research that is revealing a pivotal role for early developmental conditions in modulating epigenetic variation (Renaudeau et al. 2012, Bentz et al. 2016, Rubenstein et al. 2016). Other studies that have exploited sequence approach methods, such as bisulphite sequencing (Bentz et al. 2016, Lea et al. 2016, Weyrich et al. 2016), have demonstrated the significance of environmentally induced DNA methylation changes in shaping offspring phenotypes via effects on gene regulation (Szyf and Bick 2013). The MS-AFLP technique used in the present study is limited in its ability to provide information about the sequence and function of methylated loci; thus, correlations between the methylation patterns observed in our study and their phenotypic effects have yet to be elucidated (Shaham et al. 2016). However, the advantage of this MS-AFLP method is that it can be applied across species as a universal method that will provide a preliminary examination of genome-wide DNA methylation patterns in response to different environmental and biological parameters. In a very young field, this is useful to further identify the key parameters that affect epigenetic process at an early stage of development in birds.

Our results demonstrate a clear effect of natal brood size on percent DNA methylation at 107 loci across the genome, yet our brood-manipulation experiments have been constrained by small sample sizes due to the sensitivity of the MS-AFLP procedure. We were able to detect significant levels of hypomethylation after experimental brood manipulations, and the consistency of these results warrants further investigation into the mechanisms underlying DNA methylation state changes in response to fluctuations in early developmental conditions.

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**Ethics statement:** All work was carried out in compliance with the Animal Research Authority (2015/017-4) issued by Macquarie University.

**Author contributions:** E.L.S. and S.C.G. conceived the idea for the project. E.L.S. collected data in the field. A.W.S., A.K.R., and E.L.S. carried out the MS-AFLP analyses. E.L.S. and A.W.S. analyzed the data. E.L.S. wrote the manuscript, which was revised by A.W.S. and S.C.G.

**Conflict of interest:** No authors have a conflict of interest to declare.

**Data deposits:** Our data will be deposited in the Dryad Digital Repository.

**LITERATURE CITED**


**APPENDIX TABLE 5.** All primer and adapter sequences used for the MS-AFLP protocol.

<table>
<thead>
<tr>
<th>Primer/adapter name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI Adapter Forward</td>
<td>CTCGTATACCTGCCTACC</td>
</tr>
<tr>
<td>EcoRI Adapter Reverse</td>
<td>AATTGTTACGCAGTA</td>
</tr>
<tr>
<td>MspI/HpaII Adapter Forward</td>
<td>GATCATGAGTCCTGCT</td>
</tr>
<tr>
<td>MspI/HpaII Adapter Reverse</td>
<td>CGAGCAGGACTCATGA</td>
</tr>
<tr>
<td>EcoRI Preselective Primer</td>
<td>TACTGCGTACCAATTCA</td>
</tr>
<tr>
<td>MspI/HpaII Preselective Primer</td>
<td>ATCATGAGTCCTGCTCGG</td>
</tr>
<tr>
<td>EcoRI Selective Primer</td>
<td>6-FAM-TACTGCGTACCAATTCCAGC</td>
</tr>
<tr>
<td>EcoRI Selective Primer</td>
<td>SHEX-TACTGCGTACCAATTCCAGC</td>
</tr>
<tr>
<td>MspI Selective Primer</td>
<td>ATCATGAGTCCCTGCTCGGTCA</td>
</tr>
</tbody>
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