Causes and consequences of glucocorticoid variation in zebra finches
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DNA methylation and expression levels in the glucocorticoid receptor gene are affected by developmental conditions and predict corticosterone responses in zebra finches

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Submitted
ABSTRACT

Developmental conditions can induce phenotypic changes through epigenetic effects, modulating gene expression. In rodents and humans the glucocorticoid receptor gene \( Nr3c1 \) has been implicated as mediator of long-term effects of developmental conditions, but this has been little studied in other taxa. We experimentally tested for effects of developmental conditions (large vs. small natal brood size) on methylation and gene expression of the \( Nr3c1 \) promoter in adult zebra finches living in a benign or hard adult foraging environment, and tested for associations between \( Nr3c1 \) expression and glucocorticoid traits. We found that harsher conditions during development were associated with increased methylation of the \( Nr3c1 \) promoter. Gene expression was affected by methylation of the region closest to the coding region, showed lower values in the hard foraging environment and was strongly (\( r \geq 0.49 \)) correlated with all glucocorticoid traits (i.e. steps of the hypothalamus-pituitary-adrenal axis regulation). Our results show that epigenetic memory contributes to the emergence of long-term effects of developmental conditions in zebra finches.
Introduction

Environmental conditions experienced during development can induce phenotypic changes that last for life (Briga et al. 2017; reviewed in Monaghan 2008). One way in which early-life experiences can have a persistent impact on adult phenotype is via epigenetic mechanisms (i.e. changes in the gene expression through changes in DNA that do not involve changes DNA sequence; Perroud et al. 2011; Murgatroyd et al. 2009; Vaiserman & Koliada 2017). These mechanisms allow the integration of intrinsic and environmental signals in the genome and can lead to activation or suppression of gene function (Jaenisch & Bird 2003). Although several mechanisms of epigenetic modulation of gene function have been described, DNA methylation (i.e. the addition of a methyl group to the DNA) is one of the best studied. In vertebrates it mostly takes place in cytosines that occur before guanines (CpGs) and it is generally linked to repression of gene expression (Jones 2012; Szyf et al. 2005; Weaver 2014). DNA methylation has previously been related to long-term phenotypic changes due to adverse early-life conditions in mammals (Szyf et al. 2005; Murgatroyd et al. 2009; McGowan et al. 2009; Kundakovic et al. 2014), including long-term health consequences in humans (i.e. psychological disorders such as anxiety, depression or bipolar disorder, reviewed in Vaiserman & Koliada 2017). Many of these studies have pointed at changes in the hypothalamus-pituitary-adrenal (HPA) axis function as one of the mechanism mediating those processes. The HPA axis is a dynamic neuroendocrine system that regulates homeostatic responses, such as the ability to cope with challenge. It is highly sensitive to the impact of adverse experiences during early stages (Meaney 2001, Turecki & Meaney 2016), which can lead to long-term changes in its function during adulthood (reviewed in Turecki & Meaney 2016). The HPA axis regulates the production and secretion of glucocorticoids (GCs), involved in most of the behavioural and physiological regulatory processes that animals use to cope with environmental change, in particular when change affects energy expenditure (Jimeno et al 2017a). GC actions are regulated by the expression of two intracellular GC receptors: the mineralocorticoid (MR) and the glucocorticoid (GR) receptor. MR has a high affinity to GCs, and is saturated at lower circulating concentrations than the low affinity GR. MR is expressed only in a few organs (e.g. hypothalamus, liver), while GR is expressed in most tissues (Romero 2004).

The GR gene (Nr3c1) is sensitive to epigenetic changes (i.e. DNA methylation) attributed to the early-life environment (Yehuda et al. 2014; Weaver et al. 2004; Hompes et al. 2013; Perroud et al. 2011; reviewed in Zhang et al. 2013, Vaiserman & Koliada 2017). Adverse early-life environments have been associated with greater methylation in the Nr3c1 promoter in the majority of studies so far (reviewed in Turecki & Meaney 2016). Specifically, DNA methylation in the Nr3c1 promoter in mammals has been related to a reduction in the expression of this receptor and subsequent changes in HPA axis reactivity (Szyf et al. 2005; Meaney 2001). Many of the long-term phenotypic changes attributed to
methylation of *Nr3c1* include impaired glucocorticoid secretion and disruption of the homeostatic mechanisms that regulate the activity of the HPA axis (Weaver et al. 2004; Murgatroyd et al. 2009; Szyf 2013; Houtepen et al. 2016; Oberlander et al. 2008). However, as the majority of studies investigating methylation in the GR promoter did not measure glucocorticoid levels, conclusions on the overall impact of *Nr3c1* methylation patterns on basal and reactive glucocorticoid concentrations remain uncertain (Turecki & Meaney 2016). Moreover, all those studies have been mainly restricted to humans and captive rodents, and little is known about the occurrence and consequences of such processes in other taxa, or about their ecological relevance. In birds, few studies have looked at DNA methylation of target genes (Rubenstein et al. 2016; Riyahi et al. 2015; Verhulst et al. 2016; Saino et al. 2017), only one did so in the glucocorticoid receptor (Rubenstein et al. 2016), and none of them tested for DNA methylation and gene expression simultaneously.

While there are many evidences of long-term effects of developmental environment being influenced by the adult environment (Briga et al. 2017; Jimeno et al. 2017b), such interaction remains unexplored in the context of an epigenetic programming of HPA axis. Using captive zebra finches (*Taeniopygia guttata*), we previously showed long-term effects of developmental conditions (large vs. small broods) in combination with the adult environment (high vs. low foraging costs) on traits ranging from survival (Briga et al. 2017), social behaviour (Van der Bijl et al. in preparation), daily energy expenditure (our unpublished data) to HPA reactivity (Jimeno et al. 2017b; Table 1). An earlier study on the same species showed long-term effects of developmental conditions (deprivation of maternal care) on the expression of glucocorticoid receptors in the brain (Banerjee et al. 2012). We therefore predict that differences in the expression of the glucocorticoid receptor may explain the differences in HPA axis regulation between experimental treatments that we observe.

In this study, we investigated whether epigenetic mechanisms link early development to the adult phenotype in birds, and may thus be involved in the causation of the long-term developmental of early life environment previously shown in our population. To this end, we tested whether developmental adversity affects DNA methylation and expression of GR gene (*Nr3c1*), and whether such processes are affected by the adult environment. Based on the literature, we predicted harsh developmental conditions to increase methylation in the promoter region of the *Nr3c1* gene, presumably leading to reduced expression and differences in HPA axis reactivity (Oberlander et al. 2008; Hompes et al. 2013).
Materials and methods

Birds and experimental treatments

The study population was kept at the University of Groningen, the Netherlands, in the context of an ongoing long-term experiment described in detail in Briga et al. (2017). In brief, all birds were reared in either a small or large brood, and afterwards moved to outdoor aviaries in which foraging conditions were either easy or hard. Specifically, when the chicks were a maximum of 5 days old, all chicks in the nest were cross-fostered randomly to create experimentally small (two, sometimes three chicks) and large (six, sometimes five chicks) broods. These brood sizes are within the range observed in the wild (Zann 1996) and in captivity (Griffith et al. 2017). Chicks that grow up in large broods show increased begging and reduced food reward relative to those from small broods (Briga 2016); thus growing up in large broods implies increased foraging costs. We therefore interpret large broods as a harsh developmental condition. From 35 until c. 120 days of age, young birds were separated by sex and housed in indoor aviaries (153 × 76 × 110 cm) with about 40 other young and two male/female adults (tutors) to allow for sexual imprinting. Once they reached sexual maturity individuals were housed in eight outdoor aviaries (310 × 210 × 150 cm) for a long-term adult treatment in which they spent the rest of their lives. Four aviaries provided an easy foraging environment with low foraging costs and four provided a hard foraging environment with high foraging costs. In brief, food was offered in boxes that were suspended from the ceiling, and there were holes in the boxes through which the birds can reach the food. Underneath the holes, there was either a perch (easy foraging), or not (hard foraging), in which case the birds had to fly to the food box and back for every seed (see Koetsier & Verhulst, 2011 for details of the foraging manipulation). In each aviary, there was only one sex present, while numbers of birds reared in small and large broods were balanced. This is a long-term experiment and every year some young birds were added to the treatments to maintain c. 20 birds per aviary, and to keep the flock composition balanced with respect to brood size.

Dataset

Individuals included in this study were selected to have a balanced and representative sample of all experimental groups and ranges of corticosterone concentrations. Baseline corticosterone is the trait showing stronger differences between developmental treatments (Jimeno et al. 2017b), and is also a component of the stress-induced corticosterone. For this reason, we balanced birds by experimental groups and their baseline corticosterone concentrations as follows: we extracted the residuals of the main male and female baseline corticosterone models showed in Jimeno et al. 2017b by experimental group (small broods and easy foraging treatment; small broods and hard foraging treatment; large broods and easy foraging treatment; large broods and foraging
Chapter 4

hard treatment). We calculated quantiles by experimental group and sex, and took one random individual from each group quantile. In total, we selected 32 birds (4 experimental groups x 2 sexes x 4 quantiles) from which we already had corticosterone data. To this dataset we added 5 new individuals in the 2017 sampling, where we also took second samples of 15 individuals of the ones sampled in 2015 (see below).

a) Corticosterone profile

The corticosterone data included in this study are a subset of the data presented in Jimeno et al. (2018), where detailed information of the sampling protocol is provided. In brief, blood samples were taken from the brachial vein in May in two consecutive years: 2014 and 2015. We determined baseline corticosterone concentrations by sampling within 2 min after the door of the aviary was opened. After the first sample, the birds were placed in an opaque cloth bag where they waited for 20 min (restraint), after which the stress-induced sample was collected. Immediately after the second sample, we tested the adrenal’s maximal ability to down-regulate the corticosterone response via negative feedback by administrating a dexamethasone (corticosterone analogue) injection into the pectoral muscle (1,000 µg/kg; MacDougall-Shackleton et al. 2013; Romero & Wikelski 2010) and taking a third blood sample at minute 80. Finally, we administered an adrenocorticotropic hormone (corticosterone precursor) injection (100 IU/kg; Hau et al. 2015) to determine the maximum corticosterone release capacity and took the final blood sample at minute 100 after first disturbance. Thus, for each bird, we took four blood samples (max total 150 µl) for corticosterone. Plasma was separated through centrifugation and stored at −20°C until analysed.

Repeatabilities of these corticosterone traits are high in our population (35-70 %; Jimeno et al. 2017b; Jimeno et al. 2018), indicating that individuals can be characterized by their corticosterone profile.

b) Epigenetics

We quantified DNA methylation and gene expression at the Nr3c1 promoter in whole blood samples. Previous research suggests that epigenetic biomarkers in peripheral tissues (e.g. blood), which are easily accessible in living animals, may be used to test for long-term environmental effects on DNA methylation patterns, also for the Nr3c1 gene (Hompes et al. 2013; Oberlander et al. 2008; Rubenstein et al. 2015; Ikegame et al. 2013; Heijmans et al. 2008; reviewed in Vaiserman & Koliada 2017). Furthermore, several studies have reported methylation patterns in blood to be highly correlated to methylation patterns in other target tissues (i.e. brain: Kurdakovic et al. 2014; Verhulst et al. 2011; Riyahi et al. 2015; Tyrka et al. 2012). Thus, blood methylation of target-genes rises as a good and less invasive potential biomarker for detection of long-term effects of
early life adversity (Kurdakovic et al. 2014), which is particularly convenient in bird studies, as bird erythrocytes are nucleated.

For DNA methylation analyses, we used samples from the 32 individuals selected previously which were taken as part of the long-term experiment protocol (i.e. twice a year). These blood samples were taken with a maximum of 4 weeks gap between the date in which we sampled the individuals for corticosterone in 2014 and 2015. In order to test the association between DNA methylation and gene expression, this data set was complemented with DNA and RNA samples from 20 additional individuals in 2017: 15 were the ones still alive from 2015, and we added 5 new ones, keeping the balance between treatments and sexes. This design also allowed us to control for temporal differences in methylation patterns over time. At this moment we also took simultaneous samples of baseline corticosterone for these 20 birds, but unfortunately the assay failed; therefore we tested for the association between gene expression and CORT traits using the data available from 2014-2015 on the 15 birds sampled in both time-points only. Blood samples in 2017 were taken following the protocol described above for baseline corticosterone samples. Samples for DNA were stored cooled (2-8°C) in 500μl 2% EDTA buffer. In the lab, this buffer was replaced by 500μl glycerol-storage buffer (50 mM TRIS, 5 mM MgCl, 0.1 mM EDTA, 40% Glycerol) carefully mixed and snapfrozen in liquid nitrogen to be stored at -80°C until analysed. Samples for RNA were stored in TRI Reagent®, immediately snap-frozen in liquid nitrogen and stored at -80°C until analysed. DNA extraction was done using InnuPREP Blood DNA Mini Kit (Westburg), following manufacturer’s protocol. Extracted DNA and samples for RNA extraction were further analyzed at the Estación Biológica de Doñana (Sevilla, Spain).

Hormone analyses

Detailed information of the corticosterone analyses are provided in Jimeno et al. 2017b. We determined plasma corticosterone concentrations using an enzyme immunoassay kit (Cat.No. ADI-900-097, ENZO Life Sciences, Lausen, Switzerland), following previously established protocols (Ouyang et al., 2015). Aliquots of either 10 μl (for baseline and feedback response) or 7 μl plasma (stress-induced and response to ACTH) along with a buffer blank and two positive controls (at 20 ng/ml) were extracted twice with diethylether. After evaporation, samples were re-dissolved in 280 μl assay buffer. On the next day, two 100 μl duplicates of each sample were added to an assay plate and taken through the assay. Buffer blanks were at or below the assay’s lower detection limit (27 pg/ml). In 2014, intra-plate coefficient of variation (CV; M ± SE) was 9.63 ± 5.1% and inter-plate CV was 15.23 ± 3.2% (n = 10 plates). In 2015, the intra-plate CV was 11.43 ± 7.05% and inter-plate CV was 9.99 ± 2.67% (n = 16 plates).
Table 1. Phenotypic effects of experimental treatments (brood size manipulation during development, foraging costs manipulation during adulthood) on HPA axis regulation in our study population. The different traits correspond to the different steps of the endocrine response. Baseline CORT: corticosterone concentrations sampled within 2 minutes after disturbance; Stress-induced CORT: corticosterone concentrations sampled after 20 minutes of restraint; Feedback response: corticosterone concentrations after induction of negative feedback via dexamethasone injection; ACTH-induced CORT: corticosterone concentration after acute increase induced via ACTH (adenocorticotropic hormone) injection. For details on the HPA axis characterization, see Jimeno *et al.* 2018.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Brood size effect</th>
<th>Foraging costs effect</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline CORT</td>
<td>Yes (females)</td>
<td>Yes (females)</td>
<td>Females from large broods had higher BasCORT in hard and lower BasCORT in easy environment</td>
</tr>
<tr>
<td>Stress-induced CORT</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Feedback response</td>
<td>No (trend)</td>
<td>Yes</td>
<td>Birds in hard foraging treatment had weaker feedback response. Older birds show weaker responses, especially those from large broods in hard treatment</td>
</tr>
<tr>
<td>ACTH-induced CORT</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

**DNA methylation analyses**

Extracted DNA samples (20μl, 20ng/ml) were subjected to bisulphite treatment using EZ DNA Methylation-Gold kit (Zymo Research) following manufacturer’s protocols. As birds have nucleated erythrocytes, our DNA methylation data likely represent the *Nr3c1* methylation status in blood cells (Rubenstein *et al.* 2016).

We first carried out the set-up of a methylation profiling approach in the zebra finch. We designed a battery of new primers to amplify the promoter region of the *Nr3c1* gene based on published zebra finch nucleotide gene sequences, using bisulfite primer seeker tool (Zymo Research). We checked for the appropriateness of the primers based on conventional nested PCR. PCR products were sequenced and we confirmed the expected fragment size in an agarose gel. We selected the following primer pair with the highest specificity: forward 5´ GTT TTT TAT TGY GGG GAT GGT GAT AGA GTT GGA GAG TG, and
DNA methylation in the glucocorticoid receptor gene

reverse 5’ AAA AAT AAA AAA CAA TCA AAA TCA ACA CAA CAA ACA C. The amplified fragment (350 bp) was located on the promoter region closest to the exon, and it contained 10 CpG sites, from which 8 (CpG2 to CpG9) could be quantified in all the samples (Fig. 1). We named those CpG sites starting from the closest to the exon (CpG1) to the farthest (CpG10).

PCRs (25 μL) were prepared with 1.25 μL of 10x ImmoBuffer (10x, Bioline), 0.5 μL of MgCl₂ solution (50mM), 0.5 μL of dNTP (100mM), 0.5 μL of each primer (20 μM), 0.25 μL of Immolase DNA Polymerase (5u/μL, Bioline) and 0.75 μL of DNA (20 ng/ml). Amplifications were carried out as follows: initial denaturation at 95 °C for 10 min; 30 cycles of 95 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. Amplicons were visualized on 2% agarose gels to confirm expected fragment size, and sent out for sequencing (Macrogen). Sequences were analyzed using FinchTV (Geospiza), and we quantified the number of CpG sites methylated and percentage of methylation per CpG site. Final sample size for methylation analyses after discarding samples that did not pass quality standards was 43 samples of 31 individuals (original, 52 samples of 37 individuals).

**Gene expression analyses**

RNA was extracted using Tri Reagent® (MRC) following standard protocol specification. RNA quantity and quality was checked using nano drop. Reverse transcription was performed using Superscript III RT (Invitrogen), according to the manufacturer’s instructions. Quantitative real-time PCR (Applied Biosystems) was carried out on cDNA using previously published gene-specific primer pairs for the glucocorticoid receptor (Banerjee et al. 2012) and the house-keeping gene β-actin (Tagirov & Rutkowski 2014). Reactions (12.5 μL) were performed in duplicate and consisted of 1 μL of cDNA (1:10), 1 μL of forward and reverse primers (10 μM), and 5 μL of KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems). Cycling conditions were 10 min initial denaturation at 95°C, followed by 45 cycles of 15 sec denaturation at 95°C, 30 sec at 58°C annealing , 30 sec extension at 72°C, and a final extension at 72 °C for 5 min. The raw cycle threshold (Ct) values were converted to copy number with a standard curve. Each target gene copy number was normalized using the house keeping copy number from the same sample. Normalized data are reported as ratio of copy numbers of glucocorticoid receptor to house-keeping. Product-specific amplification was confirmed by performing melting curves for each reaction, gel electrophoresis of expected sizes, and direct sequencing of amplification products. Expression levels were obtained for 20 individuals, of those only 10 had the corresponding methylation data.
Statistics

We used General Linear Mixed Models (GLMM) to test for the effect of experimental treatments on the number of CpG sites exhibiting methylation. We ran analogous models for the average percentage of methylation (as the sum of the % methylation per site divided by the number of quantified sites, including those with zero methylation), sex and their interactions. Individual identity was included as random factor. Year of sampling was not included because it explained a negligible part of the variance (<2%). To test the effect of treatments on expression levels, we ran a linear model with experimental treatments as predictors.
We calculated Pearson correlations for the percentage of methylation at the different CpG sites. To test for the correlation between methylation and expression we used linear regressions, first using total number of CpG sites methylated and average percentage of methylation, and then doing the same for each of the 8 CpG sites quantified. We also used linear regression to test for the association between gene expression and all four corticosterone traits.

All statistical analyses were performed using R version 3.3.2 (http://www.R-project.org/) with the functions ‘lmer’ of the R package lme4 (Bates et al. 2015) and lm of the R package nlme (Pinheiro et al. 2017). Logarithmic transformations were performed to normalize the residuals of the CORT variables. CORT increase and ACTH response were calculated as ln(stress-induced CORT)−ln(Baseline-CORT) and ln(ACTH-induced CORT)−ln(CORT after feedback), respectively. Residuals of the final models showed a normal distribution.

Results

**DNA methylation patterns at the Nr3c1 promoter**

Results for 12 individuals sampled twice (2015 and 2017) showed consistency of methylation levels (number of CpG sites showing methylation) over years with no apparent time effects (r=0.60, Fig. S1). The number of methylated CpG sites (F\textsubscript{1,23.68} =7.19; p=0.01) and the average methylation percentage per site (F\textsubscript{1,16.8} =4.38; p=0.05) were higher in individuals reared in large broods (Fig.2), with no effect of foraging treatment (number of CpG sites: F\textsubscript{1,16.93} =0.22, p=0.64; average percentage of methylation: F\textsubscript{1,18.31} =0.36, p=0.85) or sex (number of CpG sites: F\textsubscript{1,18.08} =2.19, p=0.16; average percentage of methylation: F\textsubscript{1,19.06} =0.23; p=0.64, Fig. S3). The brood size effect was consistent over CpG sites, although the difference between treatments was significant only at 3 sites: 3, 6 and 9 (Fig. 3).

We found significant positive correlations between percentage of methylation at CpG sites located close to each other, and lower correlations between sites at a greater distance (Fig.S2).

**DNA methylation and gene expression**

Nr3c1 expression was not significantly correlated with the number of methylated CpGs (F\textsubscript{1,12} =0.03; p=0.83) or with average methylation level (F\textsubscript{1,9} =0.95; p=0.36) (Table 2). However, this association varied between CpG sites and approached significance at two CpG sites (CpG2: r= 0.58, F\textsubscript{1,9} =4.18, p=0.07; CpG3: r=0.54; F\textsubscript{1,9} =3.22; p=0.1). Combining the two P-values using Fisher’s combined probability test yields a significant association (Chi\textsubscript{2} =9.9, d.f.=4, P<0.05), and thus higher methylation at these sites combined was associated with lower expression (Table 2, Fig.4).
Environmental manipulations and gene expression

Foraging treatment was the only factor showing a significant effect on GR expression (Sex: $F_{1,15} = 0.008$, $p=0.93$; Brood size: $F_{1,15}=0.89$, $p=0.36$), which was lower in hard foraging treatment ($F_{1,17}=5.38; p=0.03$). Although brood size manipulation did not have a significant effect on gene expression ($F_{1,17}=1.50; p=0.24$), the correlation coefficient between brood size treatment and GR expression ($r=0.28$) was at the expected level given the effect of brood size treatment on methylation ($r=0.46$), and the correlation between methylation at sites 2 and 3 and gene expression ($r=0.58$; product of $r=0.25$). Therefore, we attribute the lack of significance of the brood size treatment to our low sample size, as data suggests an additive effect of brood size and foraging treatments on gene expression (Fig. 5).

*Nr3c1* expression was strongly correlated with all four HPA axis reactivity traits (Fig. 6). Lower receptor promoter expression was associated with higher baseline CORT ($r=0.65$; $F_{1,13}=9.25; p=0.01$), weaker CORT increase in response to restraint ($r=0.49$; $F_{1,13}=4.21; p=0.06$) and ACTH ($r=0.51$; $F_{1,13}=4.55; p=0.05$), and weaker negative feedback ($r=0.50$; $F_{1,13}=4.23; p=0.06$).
Figure 3. Percentage of methylation on each of the 8 CpG sites (mean ± s.e.m) quantified in birds coming from either small or large broods.

Figure 4. Relationship between the percentage of methylation of CpG 2 (R²=0.34) and CpG 3 (R²=0.29) and gene expression.
Table 2. Correlation coefficients of the association between expression levels and percentage of methylation per CpG site (2 to 8), average percentage of methylation (% met), or number of CpG sites showing methylation (nºCpG).

<table>
<thead>
<tr>
<th>r</th>
<th>CpG2</th>
<th>CpG3</th>
<th>CpG4</th>
<th>CpG5</th>
<th>CpG6</th>
<th>CpG7</th>
<th>CpG8</th>
<th>CpG9</th>
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<td></td>
<td>-0.57</td>
<td>-0.52</td>
<td>-0.01</td>
<td>0.14</td>
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<td>0.05</td>
<td>0.06</td>
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<td>-0.05</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Figure 5. Gene expression levels (mean ± s.e.m) in the four treatment groups (small or large broods during development; easy or hard foraging treatment during adulthood).
Discussion

Early life environment affected methylation at the *Nr3c1* promoter, with birds reared in large broods (i.e. harsher conditions) showing higher levels of methylation. This is in accordance to our predictions based on similar findings reporting associations between early life adversity and higher levels of GR methylation in mammals (e.g. Meaney 2001; Oberlander *et al.* 2008; Zhang *et al.* 2013; Perroud *et al.* 2011; Vaiserman & Koliada 2017). Our results suggests that early life experiences can lead to lifelong differences in the epigenome of individual zebra finches, independently of the degree of challenge faced in adulthood. We also found methylation at the *Nr3c1* promoter to be associated with gene expression at 2 of the 8 CpG sites analyzed. Interestingly, these 2 CpG sites were closest to the transcription region (~120 bp from the end of the promoter), and therefore more likely to be located within transcription factors binding sites, and thus directly regulating gene expression (see Meaney 2001; Weaver *et al.* 2004; Murgatroyd *et al.* 2009). These findings raise the question of how early environment-triggered epigenetic signals may appear in peripheral blood cells during adulthood. Performing DNA methylation analyses on a broader range of genomic targets, including genome-wide analyses, would be required to investigate this. On the other hand, the nature of specific epigenetic marks might be context-specific, with only environmental conditions of wide biological impact leading to coordinated changes that would enhance epigenetic changes across multiple tissues and cell types (see Turecki & Meaney 2016).

Expression of the glucocorticoid receptor was reduced in individuals living in a hard foraging environment. We had predicted lower expression to be associated to increased methylation at the promoter. Not finding an effect of the foraging treatment on methylation while finding such effect on expression could be explained by other epigenetic processes, such as histone acetylation/deacetylation, contributing to a greater extent to the differences in gene expression we observe (Miller *et al.* 2008). Histone acetylation leads to the alteration of chromatin domains from a condensed (heterochromatin) to a transcriptionally accessible (euchromatin) state. These chromatin changes are considered a primary molecular mechanism underpinning the early developmental programming and long-lasting maintenance of many homeostatic responses (Weaver *et al.* 2017). In contrast, DNA methylation promotes the formation of heterochromatin. Investigating between-individual differences in both chromatin modifications and DNA methylation marks simultaneously may allow to further study the impact of early life experiences on adult phenotype (Weaver *et al.* 2017).

Our results suggest the manipulation effects during development and in adulthood to be additive (i.e. birds from large broods in hard treatment showing the lowest expression levels). However our limited sample size prevented us from proving or refuting such hypothesis. Further research should focus on confirming these results with larger sample sizes that allow for more complex tests on interactions between early and adult
environments, which have remained overlooked so far. Investigating the changes in DNA methylation and gene expression at different time points (i.e. before, during and after environmental changes) would also provide relevant insights on the temporal progression and additive effects of epigenetic processes.

Figure 6. HPA axis regulation traits in relation to Nr3c1 expression (Table 1). Bas-COR: Baseline corticosterone; CORT after DEX: Corticosterone concentrations after dexamethasone injection (i.e. after feedback response); CORT increase: Corticosterone concentrations after increase due to restraint; ACTH response: Corticosterone concentrations after increase due to ACTH injection.

*Nr3c1* expression was a strong predictor of all four glucocorticoid traits we tested (i.e. HPA axis reactivity). Birds with lower gene expression had higher baseline corticosterone levels and weaker responses (i.e. stress-induced, feedback and after ACTH). Thus we would predict birds in hard treatment to show a “flatter” HPA regulation profile. Although studies testing GR methylation or expression and basal and reactive glucocorticoid levels are scarce (Turecki & Meaney 2016), some previous studies in mammals report early life adversity or reduced expression of *Nr3c1* to be related to higher baseline glucocorticoid
levels, heightened acute responses and disruption of the feedback mechanisms (Oberlander et al. 2008; Weaver et al. 2004; Murgatroyd et al. 2009, reviewed in Turecki & Meaney 2016). In contrast, we did not find so for the acute responses in our zebra finch population, which were weakened in the individuals with lower gene expression. We can only speculate about the cause of this difference, but birds and mammals may differ in the way in which HPA regulation is affected by environmental challenge (incl. epigenetic marks). In addition, we have not investigated the interactions that may be taking place between the glucocorticoid receptor and the high affinity mineralocorticoid receptor, as well as whether methylation and gene expression changes occur within that gene (Nr3c2). Overall, the high correlations between expression of the GR in peripheral blood and corticosterone phenotype may help understanding the mechanistic associations between different steps of the glucocorticoid regulation. We further believe they will provide a novel perspective towards the development of integrative and less invasive measures of glucocorticoid traits and HPA reactivity.

An open question arising from the treatment differences in Nr3c1 expression is whether such differences (and the subsequent effects on HPA regulation) are adaptive, in the sense that fitness would be lower without treatment effects on Nr3c1 expression. In our case, challenge experienced during either development (large broods) or adulthood (hard foraging treatment) seemed to affect expression in the same direction, but we can still say little about whether the phenotypic outcome is beneficial or detrimental, and how this depends on the circumstances. Although most of the research on developmental effects and epigenetic processes has focused on detrimental medical outcomes (e.g. see Feinberg & Tycko 2004; Vaiserman & Koliada 2017), greater emphasis is being placed on the role of epigenetic mechanisms in facilitating the adaptation of organisms to changing environments (Murgatroyd et al. 2009). Under this interpretation, methylation induced during early life may also remain as a “footprint” for a faster or more efficient response as prompt changes in expression when facing specific challenges later in life (see Murgatroyd et al. 2009; Weaver et al. 2017). Testing for effects of gene-specific methylation and expression on survival, and whether they depend on developmental and adult environments, could shed more light on the consistency and evolutionary relevance of epigenetic programing.

In this study we provide, to our best knowledge, the first report showing both epigenetic and gene expression changes in relation to early life adversity in birds. The similarity between our findings and earlier findings in laboratory mammals suggest epigenetic programming to be a mechanism underlying long-term effects of early life experiences also in other taxa. We believe that our work opens new perspectives towards the study of epigenetic processes as pathways linking environmental conditions experienced throughout life and coping strategies, which is of fundamental and applied interest in the context of a changing world.
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SUPPLEMENTARY INFORMATION to:

DNA methylation and expression levels in the glucocorticoid receptor gene are affected by developmental conditions and predict corticosterone responses in zebra finches.

Figure S1. Correlation between methylation levels of 12 individuals sampled in both 2015 and 2017.
Figure S2. Pearson correlation matrix for the percentage of methylation on the 8 CpG sites included in the analyses. Percentage of methylation at CpG2, the most often methylated site (and closest to the transcription region), was only significantly correlated with methylation at CpG3. Methylation at sites 3, 4, 5, 6 and 7 where significantly correlated with each other.

Figure S3. Methylation levels (number of CpG sites methylated, mean ± s.e.m.) in males and females reared on small or large broods (developmental treatment).
DNA methylation in the glucocorticoid receptor gene

Figure S4. Expression levels of 5 individuals measured on 2 different qPCR assays ($r=0.95$).

Figure S5. Melting curves of the housekeeping (left) and Nr3c1 (right) gene products.
Part II

Glucocorticoids and metabolic rate