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EPIGENETIC STUDIES IN ECOLOGY AND EVOLUTION

Sex-specific fitness effects of unpredictable early life conditions are associated with DNA methylation in the avian glucocorticoid receptor

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Abstract

Organisms can adapt to variable environments by using environmental cues to modulate developmental gene expression. In principle, maternal influences can adaptively adjust offspring phenotype when early life and adult environments match, but they may be maladaptive when future environments are not predictable. One of the best-studied 'maternal effects' is through modification of the offspring's hypothalamic–pituitary–adrenal (HPA) axis, the neuroendocrine system that controls responses to stress. In addition to the direct transfer of glucocorticoids from mother to offspring, offspring HPA function and other phenotypes can also be affected by epigenetic modifications like DNA methylation of the glucocorticoid receptor promoter. Here we examine how among-year variation in rainfall is related to DNA methylation during development and fitness in adulthood in the superb starling (*Lamprotornis superbus*), which lives in a climatically unpredictable environment where early life and adult environments are unlikely to match. We found that DNA methylation in the putative promoter of the glucocorticoid receptor gene is reduced in chicks – particularly in males – born following drier prebreeding periods. Additionally, DNA methylation is lower in males that become breeders than those that never breed. However, there is no relationship in females between DNA methylation and the likelihood of dispersing from the natal group to breed elsewhere. These results suggest that early life conditions may positively affect fitness in a sex-specific manner through chemical modification of an HPA-associated gene. This study is the first to show that epigenetic modifications during early life may influence the fitness of free-living organisms adapted to unpredictable environments.

Keywords: environmental predictability, epigenetics, glucocorticoids, maternal effects, Nr3c1, stress response

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Introduction

Most studies of ecological and evolutionary responses to climate change examine how mean values of climate variables (e.g. temperature, precipitation) influence organisms' distributions (Humphries *et al.* 2002; Moritz *et al.* 2008; Tingley *et al.* 2009; Chen *et al.* 2011; McCain & Colwell 2011), physiologies (Deutsch *et al.* 2008; Kearney *et al.* 2009), morphologies (Sheridan & Bickford 2011) and phenologies (Lane *et al.* (2012); Crick *et al.* 1997; Crick & Sparks 1999; Dunn & Winkler 1999; Both *et al.* 2004). Although changes in the average properties of climates are occurring as the earth continues to warm, environments are also becoming more unpredictable, as extreme weather events occur more

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frequently (Karl *et al.* 1995; Tsonis 1996; Easterling *et al.* 2000; Meehl *et al.* 2000; Trenberth *et al.* 2015). An increasing frequency of extreme climatic events means that many long-lived organisms are likely to face more variable and less predictable conditions during their lifetimes. For many species, such increases in climatic variation can have greater physiological effects than increases in mean climate (Vasseur *et al.* 2014). Thus, understanding how organisms adapt biologically to environmental variation will be important for predicting the consequences of global climate change.

Populations of organisms can adapt to environmental variation in a variety of ways (Bradshaw & Holzapfel 2006), but the type of adaptive response a species is likely to use is influenced by the timescale of environmental variation and its predictability (Botero *et al.* 2015). One of the primary ways that organisms living in unpredictable environments can respond to environmental cues is through developmental plasticity in gene expression early in life, which can have profound effects on fitness later in life (Levins 1968; Moran 1992). In many species, conditions during development can constrain fitness such that those individuals born in poor environments experience fitness disadvantages, whereas those born in good environments experience fitness advantages (Monaghan 2008). This 'developmental constraint' hypothesis, also referred to as the 'thrifty phenotype' (Hales & Barker 1992) or 'silver spoon' hypothesis (Grafen 1988), suggests that maladaptive outcomes result from a mismatch between conditions early and later in life.

Although poor early life conditions have the potential to result in maladaptive outcomes, some organisms can adapt their physiology and behaviour to overcome any potential developmental constraint. Such adaptive responses to developmental constraints can occur over the lifetime of an animal or across generations via maternal effects (i.e. where the phenotype of an organism is determined not only by the interaction between its own genotype and environment, but also by the interaction between its mother's genotype and environment). According to the 'predictive adaptive response' hypothesis (Gluckman & Hanson 2004; Gluckman *et al.* 2005a,b), mothers can influence their offspring's phenotypes during development to match conditions later in life. This idea has been extended into the 'maternal-match' hypothesis (Love *et al.* 2005; Love & Williams 2008), which suggests that developmental plasticity can have fitness advantages for both the offspring and the mother (Gluckman & Hanson 2004; Hayward & Wingfield 2004; Breuner 2008). Central to both of these hypotheses is the concept of 'environmental matching', or that any potential fitness advantages are only realized when early and later life environments are of similar quality (Monaghan 2008).

Empirical support for developmental plasticity and environmental matching comes primarily from species inhabiting predictable environments where conditions during early life match those in adulthood. For example, in snowshoe hares, where predation-driven population cycles occur in a predictable manner (Krebs *et al.* 1995), predation risk affects maternal body condition and glucocorticoid stress hormones (Boonstra *et al.* 1998), which in turn can affect offspring survival (Sheriff *et al.* 2009). When the maternal and offspring environments match, maternal effects in hares may be adaptive, but during transitional phases when the environments do not match, maternal effects may be maladaptive (Dantzer *et al.* 2013; Sheriff & Love 2013). When there is likely to be a mismatch between early and later conditions (e.g. species living in environments where climate varies unpredictably from year to year), the environmental matching hypotheses are unlikely to apply because individuals will experience both high and low quality environments as an adult, with limited ability to predict environmental quality from 1 year to the next. Indeed, there was no support for the predictive adaptive response hypothesis in savanna-living yellow baboons, which inhabit a highly unpredictable semi-arid environment in East Africa (Rubenstein & Lovette 2007); instead, individuals born in low quality years were developmentally constrained (Lea *et al.* 2015).

Although much of our current theory on maternally driven adaptive plasticity and environmental matching is based upon studies of species living in predictable environments, irreversible or developmental plasticity is likely to be common in species experiencing unpredictable climatic variation (Botero *et al.* 2015). In these environments, mothers may be able to adaptively programme offspring physiology during development (i.e. prior to birth or egg laying) – and independent of the conditions experienced as an adult – to overcome poor early life conditions even when there is likely to be a mismatch with adult conditions. Indeed, life history theory suggests that optimal maternal investment in offspring is predicted to increase as environmental quality decreases under some conditions (Rollinson & Hutchings 2013). One of the best-studied mechanistic examples of maternal influence on offspring physiology and fitness involves the endocrine system (Lessells 2008; Meylan *et al.* 2012; Love *et al.* 2013; Sheriff & Love 2013). In fact, hormone mediated maternal effects have been predicted to be critically important for species living in variable and unpredictable environments (Meylan *et al.* 2012) because hormones represent one of the primary links between the genome and the environment. Indeed, the hypothalamic–pituitary–adrenal (HPA) axis, the principal neuroendocrine system that

controls reactions to stress, is likely to be involved in developmental programming in many species (Love *et al.* 2013). Glucocorticoids are released following HPA activation in response to environmental stressors (e.g. predation risk, climatic perturbation, social conflict) (Wingfield & Kitaysky 2002; Goymann & Wingfield 2004; Dantzer *et al.* 2013) and trigger a series of behavioural and physiological responses to cope with environmental change (Sapolsky *et al.* 2000; Romero 2004). Numerous field studies in taxonomic groups as diverse as birds, reptiles, fish and mammals have demonstrated that glucocorticoid stress hormones passed from mother to offspring can be either adaptive or maladaptive depending upon the synchronization of early and later conditions (reviewed in Sheriff & Love 2013; Love *et al.* 2013).

In addition to the direct transfer of glucocorticoids from mother to offspring, the HPA axis can influence developmental plasticity through epigenetic mechanisms (Weaver *et al.* 2004; McGowan *et al.* 2009). Epigenetic changes are molecular processes that alter gene expression, including the addition of a methyl group to cytosine DNA bases (termed DNA methylation), post-translational modification to histone proteins that shape the structure of chromatin (termed histone modification) or the regulatory actions of RNA molecules (Bird 2007). Studies in humans have shown that a lack of direct parental care (Naumova *et al.* 2011) or food availability (Heijmans *et al.* 2008; Tobi *et al.* 2012) can influence genomewide patterns of DNA methylation. DNA methylation within genes that regulate the HPA axis may be particularly important for controlling developmental plasticity. For example, laboratory studies suggest that regulation of DNA methylation of the Nr3c1 glucocorticoid receptor gene promoter is associated with prenatal stress in mice (Mueller & Bale 2008), maternal anxiety/depression during pregnancy in humans (Oberlander *et al.* 2008) and variation in postnatal maternal care in rats that ultimately shapes adult behaviour and response to stressors (Weaver *et al.* 2004).

Although laboratory studies with controlled manipulations are important for elucidating many of the complex molecular mechanisms related to stress, field studies have proven necessary for determining how the interactions between genes and the environment influence physiology, behaviour and ultimately fitness (Calisi & Bentley 2009). Despite their obvious importance (i.e. field studies are necessary to study the fitness effects of epigenetic modifications), epigenetic studies in free-living vertebrates are generally lacking (but see Pilsner *et al.* 2010; Tung *et al.* 2012; Schrey *et al.* 2012). Moreover, the taxonomic breadth of vertebrate studies of DNA methylation is also restricted primarily to mammals. Other than a few studies examining genomewide DNA methylation in chickens (Usui *et al.* 2009; Li *et al.* 2011; Gou *et al.* 2012) and house sparrows (Schrey *et al.* 2012; Liebl *et al.* 2013), there have been no targeted studies of DNA methylation in birds, and certainly none related to the glucocorticoid receptor promoter or to stress physiology in general. Thus, studies of free-living vertebrates are necessary for understanding the potential fitness consequences and epigenetic regulation of environmentally driven maternal effects on the HPA axis.

Here we explore the association between annual variation in rainfall and DNA methylation of the glucocorticoid receptor gene during development and the association between that DNA methylation profile and fitness in free-living birds inhabiting an unpredictable environment. Although there has been a great deal of work examining glucocorticoid-mediated maternal effects (reviewed in Lessells 2008; Meylan *et al.* 2012; Sheriff & Love 2013), there has been relatively little work on the molecular mechanisms underlying this form of adaptive plasticity. Our primary goal is to explore a potential epigenetic mechanism that could underlie maternal effects and physiological plasticity in coping with environmental change. We ask if DNA methylation in the putative promoter region of Nr3c1 – the gene encoding the glucocorticoid receptor – may be one potential molecular mechanism by which mothers living in variable and unpredictable environments could alter offspring phenotype for future environmental coping and to maximize fitness in adulthood. If developmental conditions are constraining, then individuals raised in low quality years will show reduced fitness later in life because they are negatively impacted by early life conditions. Alternatively, if mothers can adaptively compensate offspring born in poor early life conditions, then individuals born in low quality years will have higher fitness later in life. As we are interested in following individuals over the course of their lifetime, we use DNA extracted from blood sampled soon after hatching and then monitored the breeding life histories of these birds into adulthood. Because males tend to be philopatric and females tend to disperse from their natal areas in birds (Greenwood 1980), particularly cooperatively breeding ones, we quantified the potential fitness consequences as the likelihood of breeding in males and the likelihood of dispersing in females. Based on previous work that demonstrated sex-specific fitness effects of early life conditions in birds (e.g. Wilkin & Sheldon 2009), we also predicted that any fitness related effects of DNA methylation are more likely to be observed in males than in females.

We conducted this study in a population of plural cooperatively breeding superb starlings (*Lamprotornis superbus*) that are endemic to East African savannas where rainfall in this semi-arid environment is highly

variable and unpredictable within and among years (Rubenstein & Lovette 2007). We focused on rainfall during the prebreeding period (i.e. the three-month window prior to the long rains breeding season) because among-year variation in rainfall during this period influences a number of behavioural, physiological and life history traits in this species, including breeding roles (Rubenstein 2007b), helping behaviour (Rubenstein 2016), glucocorticoid levels (Rubenstein 2007b), immune function (Rubenstein *et al.* 2008), offspring sex ratio (Rubenstein 2007c), group structure (Rubenstein 2016) and reproductive success (Rubenstein 2011). Importantly, maternal effects have been predicted to be especially important in cooperative breeders because the division of reproductive labour (i.e. helping behaviour) could lead to the potential for reduced maternal care (Russell & Lummaa 2009). Additionally, as offspring in cooperatively breeding species vary in the potential for achieving reproductive success, developmental constraints or advantages conferred through maternal effects may be particularly important (Russell & Lummaa 2009). Finally, birds have proven to be a tractable model system for studying the mechanistic basis of maternal effects because most prenatal development occurs within the confines of the egg (Badyaev 2008; Groothuis & Schwable 2008). Ultimately, this work will have important implications for understanding not only the mechanistic bases and fitness consequences of developmental plasticity for species inhabiting unpredictable environments, but also for studying the epigenetic regulation of environmentally induced fitness effects in free-living vertebrates.

Materials and methods

Study system

A population of superb starlings has been monitored continuously since 2001 at the Mpala Research Centre, Laikipia, Kenya (0°17′ N, 37°52′ E). More than 97% of all birds in the population are banded with a numbered metal leg ring and a unique set of four coloured leg bands. Superb starlings breed twice a year during both the long and short rains (Rubenstein 2011), and breeding activities have been monitored for all 28 breeding seasons from 2001 through 2014. Multiple individuals of each sex breed within a social group, but not all individuals will breed during their lifetimes (Apakupakul & Rubenstein 2015). Males tend to be philopatric and breed in their natal groups, whereas females tend to disperse and join other groups to breed (Pollack & Rubenstein 2015).

Active nests are checked every 1–3 days during the hatching and nestling stages. Chicks are bled and banded 7 days after hatching. In this study, we only used samples collected during the long rains breeding season. Parents are identified at nests during the nest building, incubation and chick-rearing stages using repeated 1–3 h focal observations. Parentage is confirmed using microsatellite markers (Rubenstein 2007a; Apakupakul & Rubenstein 2015; Weinman *et al.* 2015), and pedigrees are built from a combination of behavioural observations and parentage data (Pollack & Rubenstein 2015). Fieldwork was approved by Columbia University's Institutional Animal Care and Use Committee (IACUC No.: AAAE9606).

Rainfall

Daily rainfall data have been collected continuously since 1998 using an automated Hydrological Services TB3 Tipping Bucket Rain Gauge located at the Mpala Research Centre (Rubenstein 2011). Prebreeding rainfall was calculated as the sum of daily rainfall during December through February each year (Rubenstein 2007b,c, 2011). This period represents the primary dry season and the 3 months with the greatest variation in mean monthly rainfall (Rubenstein 2011, 2016).

Experimental design

Initial DNA methylation assessment of Nr3c1 exon 1 (hereafter coding region, i.e. CDS) and the 988 bp upstream region (hereafter regulatory region) was done using DNA extracted from blood collected from 11 male chicks in 2003 and 2007. To explore how DNA methylation varies across years and with different levels of prebreeding rainfall, we then assessed DNA methylation of the promoter region (see below; 33 CpG sites: −1092 to −775 with respect to the translation start site; Fig. 1) in 96 individuals that were born from 2001 to 2013 and survived to fledge the nest (N = 48 females and 48 males). We attempted to select five males and five females (each from a different nest) from each year, but this was not always possible because of low fledging success in some years (sample sizes are given in Table S1, Supporting information).

DNA amplification and sanger sequencing

Our initial goal was to amplify a segment of ~3.5 Kb of coding and noncoding sequence of the superb starling Nr3c1 gene. Unfortunately, there were methodological challenges in the optimization of the PCR amplification because the DNA sequence near the promoter is enriched by numerous CpG motifs. The abundance of these CpG sequences appears to affect the extension of polymerase by favouring the formation of unstable secondary structures during amplification and sequencing, even in the

presence of PCR and sequencing additives that often stabilize DNA structure. To avoid these latent secondary structures, we designed different sets of forward and reverse primers to be either inside or outside of the CpG islands. Using the genomic reference from the zebra finch (*Taeniopygia guttata*) (NW_002197294, NCBI), we effectively targeted three overlapping amplicons spanning the promoter and exon 1 of the superb starling Nr3c1 gene; each amplicon was extended from 0.7 to 1.5 Kb (Fig. S1, Table S2, Supporting information). We were then able to redesign more specific primers to target superb starling genomic DNA (Table S3, Supporting information) and obtain a final high quality sequence of ~2.5 Kb (GenBank Accession no.: KT201526). We note that a similar attempt to sequence this region in the zebra finch failed due to the apparent tertiary structure created by the CpG-rich regions near the putative promoter (Rubenstein *et al.* unpublished data). Thus, while our approach worked well for superb starlings, it may not succeed for all species.

All PCRs (20 μL) were prepared with 1X GoTaq® Hot Start Polymerase buffer (Promega), 1.0 mM of MgCl2, 0.2 mM of each dNTP, 0.5 μM of each primer, 1.25 U HotStart Taq Polymerase and 1.0 μL of diluted DNA. Amplifications were carried out on a Veriti Thermal Cycler (Applied Biosystems) using the following profile: initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 30 s, annealing at 55–60 °C for 30 s and extension at 72 °C for 90 s; and a final extension at 72 °C for 7 min. Amplicons were visualized on 1% agarose gels to check their band size and specificity, and then cleaned with Qiaquick PCR purification kits (Qiagen) according to the manufacturer's protocols. Sequencing was conducted at the University of Texas at Austin Institute for Cellular and Molecular Biology (ICMB). The resulting ABI Chromatograms were analysed using 'Map to Reference' parameters in GENEIOUS v6.1 (Biomatters).

Within the regulatory region of the resulting sequence, we identified the putative promoter of the superb starling Nr3c1 gene using PromoterInspecter Release 1.1.1 in the GENOMATIX SOFTWARE SUITE v2.6. The software identified a 220 bp region (Fig. 1) that was the homolog to, and partially overlapping with, the Nr3c1 promoter in the rat (Weaver *et al.* 2004). Thus, within the Nr3c1 regulatory region we identified sequence predicted to be the starling promoter (hereafter putative starling promoter) that partially overlapped with sequence that is the homolog to the rat promoter (hereafter rat promoter homolog).

DNA methylation and pyrosequencing

We assessed DNA methylation of exon 1 (52 CpG sites) and the 988 bp regulatory region that was upstream of the translation start site (68 CpG sites) (Fig. 1). Flanking and sequencing primers (Table S4, Supporting information) were designed for pyrosequencing using PYROMARK ASSAY DESIGN Software v2.0 (Qiagen). Genomic DNA was extracted from blood preserved in 2% SDS Queens's lysis buffer using a DNeasy Blood & Tissue Kit (Qiagen), and then 20ul of DNA was bisulphite-converted using an Epitect Fast Bisulfite Conversion Kit (Qiagen) according to the manufacturer's protocols. Carrier RNA was not used in the bisulphite reaction. As birds have nucleated erythrocytes and this is by far the most common cell type in avian blood, our DNA methylation data likely represent the Nr3c1 methylation status in red blood cells.

Annealing temperatures were optimized using a gradient of 54–60 °C. All PCRs (25 μL) were completed using a Pyromark PCR Kit (Qiagen) that included 12.5 μL PyroMark PCR Master Mix, 2.5 μL CoralLoad Concentrate, 0.1 μM of each primer and 1.0 μL bisulphite-converted DNA. Amplifications were carried out on Bio-Rad, Eppendorf or Applied Biosystems thermocyclers using the following profile: initial DNA polymerase activation at 95 °C for 15 min; 45 cycles of initial denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s; and a final extension at 72 °C for 10 min. Amplicons were visualized on a 2% agarose gel prior to pyrosequencing on a PyroMark Q24 (Qiagen).

Each 24-well pyrosequencing assay contained 12 individuals run in duplicate (intra-assay variation = 23.1%). Duplicates that differed by more than 5% were rerun to check for accuracy (7.7% of sites). Additionally, all sites that the PYROMARK software marked as failed were rerun; sites that failed twice were omitted from the analysis (N = 579 of 6336 sites; 9.1%). Most of the sites that failed did so because of reference sequence errors, likely indicating the presence of a single nucleotide polymorphism (SNP) in the reference sequence. In total, data were collected for 2761 of 3168 (89.2%) sites across all 96 individuals run. Finally, we were unable to design hypermethylated controls because commercially available CpG Methyltransferase (M.Sss1) (Zymo Research) failed to hypermethylate avian DNA.

Temporal patterns of DNA methylation

As DNA methylation is known to change over the lifetime of an organism, we sampled 12 individuals as chicks that were then recaptured as adults. We chose individuals whose recaptures occurred at varying time points ranging from <1 year after initial capture (296 days) to nearly 12 years after initial capture (4314 days) (median time between captures = 1858 days). For this analysis, we only examined DNA methylation

at 12 CpG sites in the putative starling promoter (sites −899 to −775).

Statistics

General linear models with normal error structure were used to examine the relationships between the four correlated regions of DNA methylation (see Results) and prebreeding rainfall. After identifying the relevant region of DNA methylation, sex differences were examined in a separate model with DNA methylation as the dependent variable and prebreeding rainfall, sex and their interaction as predictor variables. To further explore sex differences, we also ran the models separately for males and females. Similarly, general linear models with binomial error structure were used to examine the relationship between DNA methylation and (i) the probability of breeding (for males) and (ii) the probability of dispersal (for females). In all cases, we assessed model fit using Akaike's information criterion (AICc) (Tables S5–7, Supporting information) (Burnham & Anderson 2002) and used likelihood ratio tests to determine significance. The likelihood of breeding for males was quantified by determining if males bred during the 14 year study period (from 2000 to 2014) in one of the marked social groups. The likelihood of dispersing for females was determined from their representation in annual census data. Although we could not distinguish between birds that dispersed and those that died, survival is high for birds that become independent, so we assume that most missing birds dispersed from their natal groups. For the models with breeding and dispersal, we only used individuals born over the 10-year period from 2001 through 2010 (N = 42 and 39 males and females, respectively) to ensure that most individuals were likely to have reached dispersal or breeding age; the median time to breeding in males in this population was 3 years, whereas the median time until a female disappeared (likely dispersed), excluding those individuals that were never seen again after fledging, was also 3 years. Consistent with this approximation, eye colour estimates of age suggest that most females appear to immigrate into our marked social groups between 2 and 4 years of age (Rubenstein 2006).

We examined temporal patterns of DNA methylation in three ways. First, paired tests were used to compare DNA methylation in birds sampled as chicks and then recaptured as adults. Second, to determine if individual differences in DNA methylation were maintained over time, we used a generalized linear mixed model with recapture DNA methylation as the dependent variable, original capture DNA methylation as a fixed effect and individual as a random effect to account for multiple CpG sites analysed in each bird. Finally, a regression was used to examine the relationship between the difference in DNA methylation at the two capture periods and the time elapsed between the two captures.

Results

Patterns of DNA methylation

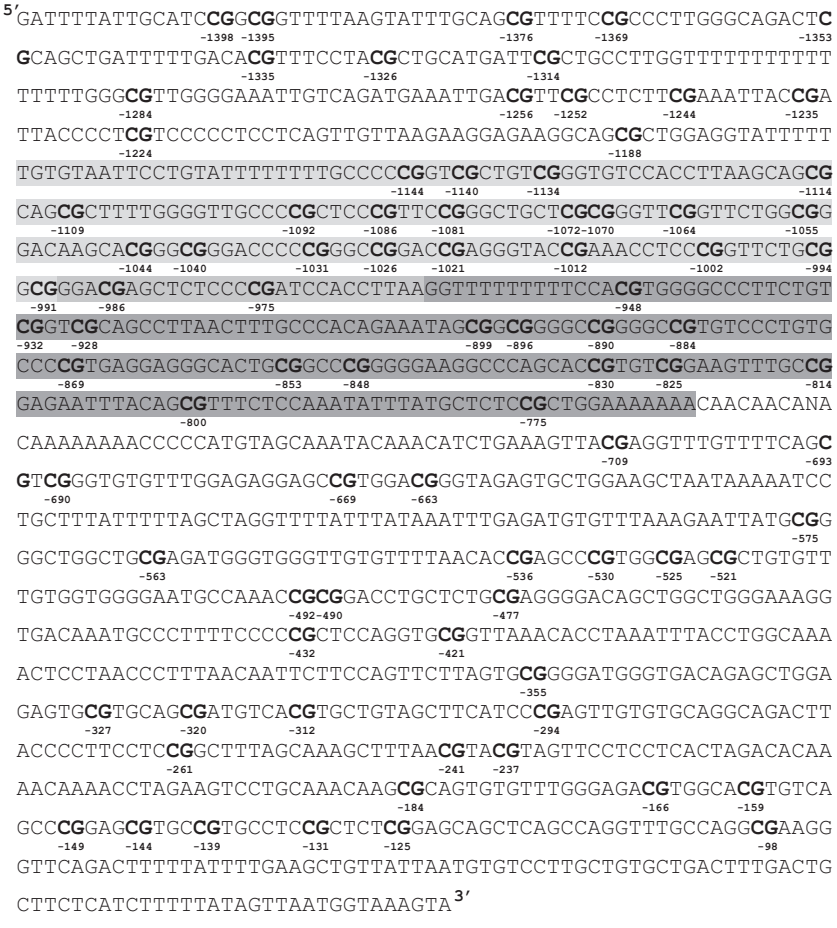
We initially assessed DNA methylation of exon 1 and the entire 988 bp upstream regulatory region for 11 individuals (Fig. 2A and Table 1). Consistent with documented patterns of DNA methylation within coding vs. upstream promoter genomic regions, DNA methylation in the coding region (mean ± SD = 48.74 ± 23.48) was significantly higher than in the regulatory region (mean ± SD = 3.21 ± 2.68) (Wilcoxon–Mann–Whitney test: $Z_1 = 9.12$, $N = 117$, $P < 0.0001$). Moreover, the range of DNA methylation at each CpG was much greater in the coding region (3.90–98.45%) than in the regulatory region (0.88%–16.89%). Within the regulatory region, DNA methylation was marginally but significantly lower in the promoter region (mean ± SD = 2.29 ± 0.20) than in the nonpromoter region (mean ± SD = 4.18 ± 0.61) (Wilcoxon–Mann–Whitney test: $Z_1 = 3.09$, $P = 0.0034$). Moreover, there was no difference in DNA methylation between the putative starling promoter (mean ± SD = 2.61 ± 0.32) and the starling's rat promoter homolog (mean ± SD = 2.04 ± 0.24) (Wilcoxon–Mann–Whitney test: $Z_1 = 1.39$, $P = 0.16$).

Across the 33 total CpG sites in the putative starling promoter and the starling's rat promoter homolog (Fig. 2B), we found groups of CpG sites that were significantly correlated with each other. Within the rat promoter homolog, there were 10 CpG sites whose DNA methylation levels were positively correlated with each other (Fig. 3A, Table S4, Supporting information). Within the putative starling promoter, there were three groups of CpG sites whose DNA methylation levels were positively correlated with each other (Fig. 3B, Table S4, Supporting information). Thus, as there were natural clusters of correlated CpG sites in these four regions, we summed the DNA methylation and treated each region separately in the remaining analyses.

Temporal patterns of DNA methylation

There were no differences in DNA methylation at any of the 12 CpG sites examined in individuals captured after hatching and then again later in life (Table 2). Moreover, individual differences in DNA methylation were maintained over time, as individual DNA methylation levels were correlated across all CpG sites

Regulatory Region



Coding Region

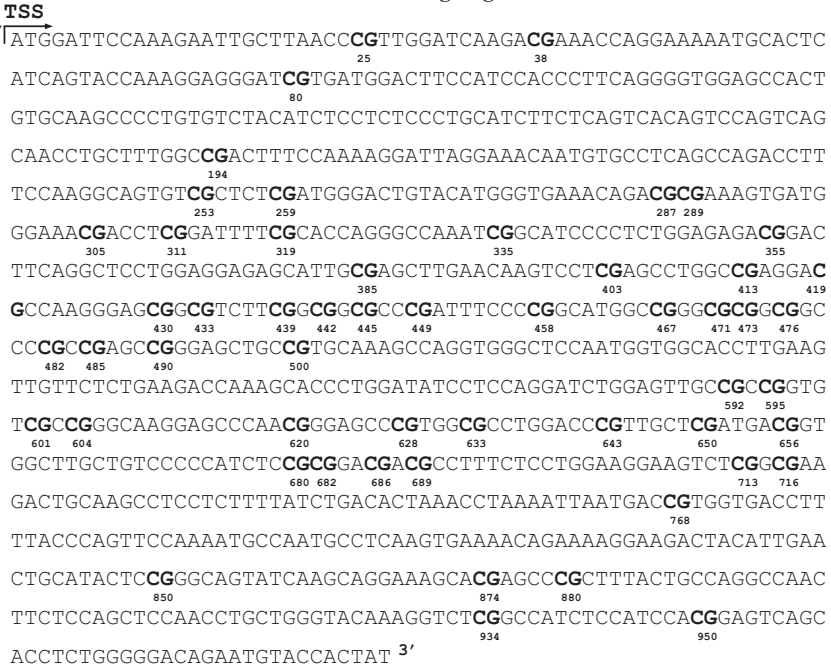


Fig. 1 Partial sequence of the Nr3c1 glucocorticoid hormone receptor gene, including exon 1 and 988 bp of the upstream regulatory region. There are 86 CpG sites in the regulatory region and 54 sites in the coding region of exon 1 indicated in bold. Each CpG site is given an identifying number relative to its distance from the translation start site (TSS); positive numbers proceed into the exon and negative numbers proceed into the regulatory region. The putative starling promoter is highlighted in dark grey, the rat promoter in light grey and the overlap of the two regions in medium grey.

($F_{1,123.7}$ = 237.57, $P < 0.0001$, $r^2 = 0.68$). Finally, there was no relationship between the difference in DNA methylation at the two time points and the time elapsed between recapture ($F_{1,9.88}$ = 2.54, $P = 0.14$, $r^2 = 0.21$).

Environmental variation and DNA methylation

In nearly all cases, our best fit AIC models only tended to include at most 2 of 4 regions of correlated CpG sites: sites −830 to −775 and sites 869 to −848 (Tables S5–S7, Supporting information). Across all birds, prebreeding rainfall was positively correlated with total DNA methylation over CpG sites −830 to −775 ($\chi^2_{1,89}$ = 4.21, $P = 0.04$; Fig. 4, Table 2A), but not over CpG sites −869 to −848 ($\chi^2_{1,89}$ = 1.71, $P = 0.19$; Fig. 4, Table 2A). Males and females exhibited the same positive relationship between prebreeding rainfall and DNA methylation over CpG sites −830 to −775 (prebreeding rainfall: $\chi^2_{1,90}$ = 3.82, $P = 0.05$; sex: $\chi^2_{1,90}$ = 0.041, $P = 0.52$; interaction: $\chi^2_{1,90}$ = 0.25, $P = 0.62$). However, when we examined males and females separately, prebreeding was positively correlated with total DNA methylation in males over CpG sites −830 to −775 ($\chi^2_{1,43}$ = 4.33, $P = 0.038$) and CpG sites −869 to −848 ($\chi^2_{1,43}$ = 3.90, $P = 0.048$), but there were no relationships between prebreeding rainfall and DNA methylation in females (CpG sites −830 to −775: $\chi^2_{1,43}$ = 0.90, $P = 0.34$; CpG sites −869 to −848: $\chi^2_{1,43}$ = 0.73, $P = 0.79$).

DNA methylation and fitness

To determine the potential fitness consequences of differential DNA methylation, we examined two correlates of fitness: the likelihood of breeding (in males) and the likelihood of dispersing (in female). The likelihood of breeding in males was positively related to DNA methylation over CpG sites −830 to −775 ($\chi^2_{1,39}$ = 5.11, $P = 0.024$; Fig. 5, Table 2B), but not over sites −869 to −848 ($\chi^2_{1,39}$ = 0.026, $P = 0.087$; Fig. 5, Table 2B). However, the likelihood of dispersing in females was not related to DNA methylation over CpG sites −869 to −848 ($\chi^2_{1,37}$ = 1.38, $P = 0.24$), nor any of the regions of correlated CpG sites (Table 2C).

Discussion

Among-year variation in prebreeding rainfall influences many aspects of superb starling behaviour, physiology and reproductive life history (reviewed in Rubenstein 2016). Here we show that (i) prebreeding rainfall is cor-

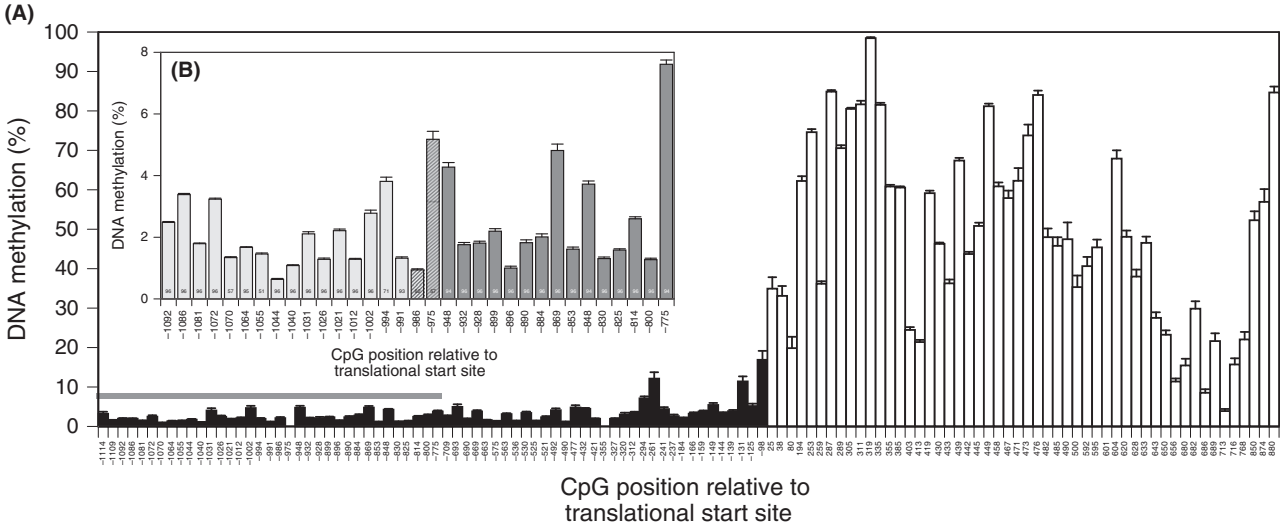


Fig. 2 Mean ± SE levels of DNA methylation in the superb starling Nr3c1 glucocorticoid receptor gene. (A) The entire coding (white bars) and regulatory regions (black bars) from 11 chicks. The grey line indicates the partially overlapping rat promoter homolog and putative starling promoter. (B) The rat promoter homolog (light grey bars) and putative starling promoter (dark grey bars) from 96 chicks; hatched bars indicate regions of promoter overlap. Sample sizes are indicated at the base of each bar.

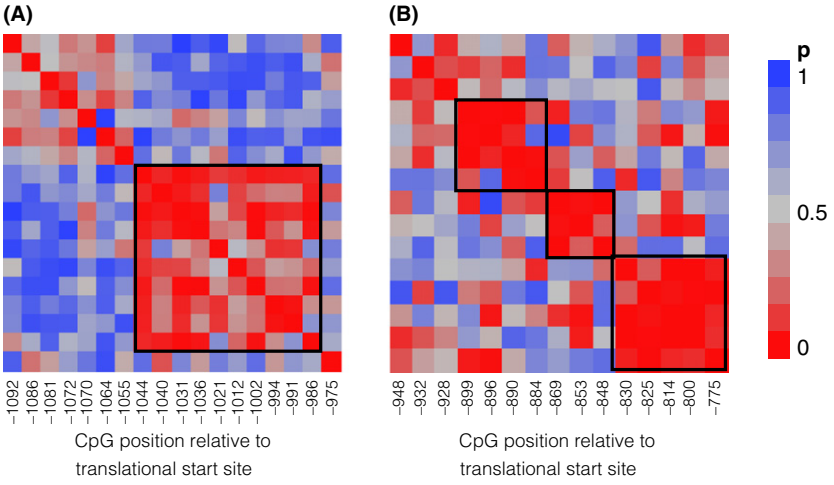


Fig. 3 Map showing CpG sites whose levels of DNA methylation were correlated in the (A) rat promoter homolog and (B) putative starling promoter. Black boxes indicate groups of CpG sites that were positively correlated. These regions were summed for further analysis. As sites −991, −986 and −975 in the rat promoter homolog often failed, they were excluded. Samples sizes are indicated in Fig. 2B.

related with DNA methylation in the putative promoter of the glucocorticoid receptor (and more strongly in males than females) such that chicks have lower DNA methylation in poorer quality years, and (ii) DNA methylation in the same promoter region predicts the likelihood of breeding in males, but not the likelihood of dispersing in females. Thus, early life conditions influence DNA methylation levels, which in turn, are related to adult fitness in a sex-specific manner. This finding is similar to results from great tits, where early life rearing conditions impact male but not female fitness (Wilkin & Sheldon 2009). In contrast, however, in blue-footed boobies that experience unpredictable environments resulting from the El Niño Southern Oscillation (ENSO), female offspring that experienced harsh ENSO conditions had improved breeding success, whereas male offspring had reduced breeding success (Ancona & Drummond 2013). Finally, this result is consistent with the hypothesis that some individuals can overcome poor early life conditions to achieve higher fitness later in life, even when living in unpredictable environments where adult conditions are unlikely to match those in early life. This result is also consistent with life history theory predicting that optimal maternal investment in offspring should increase as environmental quality decreases under some conditions (Rollinson & Hutchings 2013). As environmental conditions are unrelated to mean fecundity in superb starlings and fledging success in equally low in all years (Rubenstein 2011), the trade-off between current and future reproduction that would normally favour reduced maternal investment in low quality years is no longer an issue (Horn & Rubenstein 1984).

Table 1 Results of paired *t*-tests comparing DNA methylation at 12 CpG sites (−899 to −775) in individual samples after hatching and then again later in life. After controlling for multiple comparison ($\alpha = 0.0042$), there were no differences in DNA methylation at any site

CpG site	*t*	d.f.	*P*
−899	1.28	11	0.23
−896	0.23	11	0.82
−890	0.14	11	0.89
−884	−0.43	11	0.67
−869	0.04	11	0.97
−853	0.95	11	0.36
−848	−0.39	4	0.72
−830	1.33	11	0.21
−825	1.06	11	0.31
−814	2.18	11	0.052
−800	2.08	11	0.062
−775	0.73	8	0.49

Previous studies examining the phenotypic consequences of DNA methylation in the glucocorticoid receptor promoter in mammals have found patterns opposite to what we observed here, namely that high DNA methylation in offspring impairs their stress responses as adults rather than conferring some adaptive benefit (Weaver *et al.* 2004, 2005). In superb starlings, baseline and stressed-induced glucocorticoid levels in adults are related to among-year variation in prebreeding rainfall, but only in subordinate individuals (Rubenstein 2007b). Theses glucocorticoid levels are likely influenced as much by social factors as they are by ecological ones (Rubenstein 2007b; Rubenstein & Shen 2009). It remains unclear whether higher or lower levels of glucocorticoids are adaptive in this species, and how developmental conditions influence stress responses later in life. Additionally, it is important to emphasize that we examined DNA methylation in blood, while other studies have looked at tissue in the hippocampus. Although correlations in DNA methylation between blood and brain have been observed in a

Table 2 Results of GLM models examining the relationships between DNA methylation in the putative starling promoter of Nr3c1 and (A) prebreeding rainfall (in both sexes), (B) the likelihood of breeding in males and (C) the likelihood of dispersing in females. AICc values were used to identify these from a full set of models including all four regions of correlated CpG sites (Tables S5–7, Supporting information). Parameter estimates and standard errors (SE) are given along with lower and upper confidence limits, likelihood ratio chi-square statistics and *P*-values

Parameter	Estimate	SE	Lower CL	Upper CL	LR chi-square	*P*-value
(A) Prebreeding rainfall vs. DNA methylation						
Intercept	−33.96	49.78	−132.56	64.64	0.46	0.5
CpG sites −830 to −775	**5.77**	**2.78**	**0.27**	**11.27**	**4.21**	**0.04**
CpG sites −869 to −848	3.04	2.31	−1.54	7.623	1.71	0.19
(B) Likelihood of breeding in males vs. DNA methylation						
Intercept	−5.34	4.99	−18.57	2.77	1.51	0.22
CpG sites −830 to −775	**0.51**	**0.3**	**0.06**	**1.37**	**5.11**	**0.024**
CpG sites −869 to −848	0.027	0.17	−0.3	0.38	0.026	0.87
(C) Likelihood of dispersing in females vs. DNA methylation						
Intercept	2.46	1.39	−0.156	5.44	3.39	0.066
CpG sites −869 to −848	−0.15	0.13	−0.427	0.1	1.38	0.24

Bold indicates statstical significance.

number of recent studies (e.g. Masliah *et al.* 2013; Farre *et al.* 2015), these correlations are often weak or not seen in all regions of the genome (Walton *et al.* 2015). Therefore, it will be important to validate this relationship in brain tissue from free-living birds to determine the utility of this method in future studies. These types of validation studies will also have direct implications for humans, as clinical studies need noninvasive ways to measure functionally relevant DNA methylation for studying disease risk.

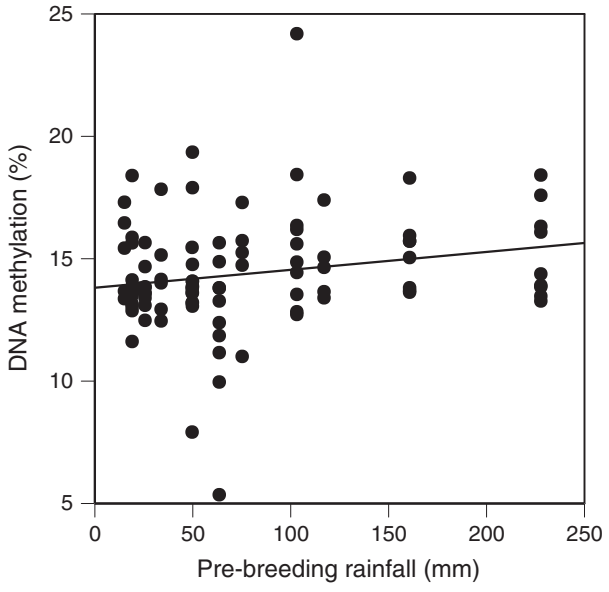


Fig. 4 Relationship between prebreeding rainfall and DNA methylation in the superb starling Nr3c1 glucocorticoid receptor gene. Only DNA methylation at sites −830 through −775 showed a significant, positive relationship with rainfall. There were 92 individuals used from the 13-year period from 2001 to 2013. Sample sizes in each year are given in Table S1 (Supporting information).

Epigenetic modifications are thought to be both heritable and plastic over the course of an individual's lifetime (Bird 2007; Bollati & Baccarelli 2010; Ledon-Rettig *et al.* 2012). In a subset of our samples, we found that DNA methylation levels in week-old chicks did not change over the course of their lives, even those sampled up to 12 years later. Despite small sample and effect sizes, this result suggests that DNA methylation of the putative promoter of the avian Nr3c1 gene is not a reversibly plastic trait in this species. However, we do not yet know if DNA methylation levels are heritable and can be passed from mother to offspring in superb starlings or other birds. We know from previous work in this species that environmental conditions during the prebreeding period affect maternal body condition (Rubenstein 2007c), which could in turn influence DNA methylation in their offspring at the time of inception or during development in the egg. Although we do not yet know the role of such mechanisms in birds, the transfer of egg contents like hormones, micronutrients and other chemicals is known to influence embryo development in birds, particularly in cooperative breeders (Russell *et al.* 2007). Further work should examine the possible inheritance of maternal DNA methylation and explore how epigenetic modifications might be passed from mother to offspring via egg contents.

As we sampled chicks after only 1 week of life, levels of DNA methylation at the glucocorticoid receptor seem likely to be influenced by maternal effects prior to egg laying and not to levels of parental care in the first week of life. Superb starlings mothers can influence the sex of their offspring based on prebreeding rainfall,

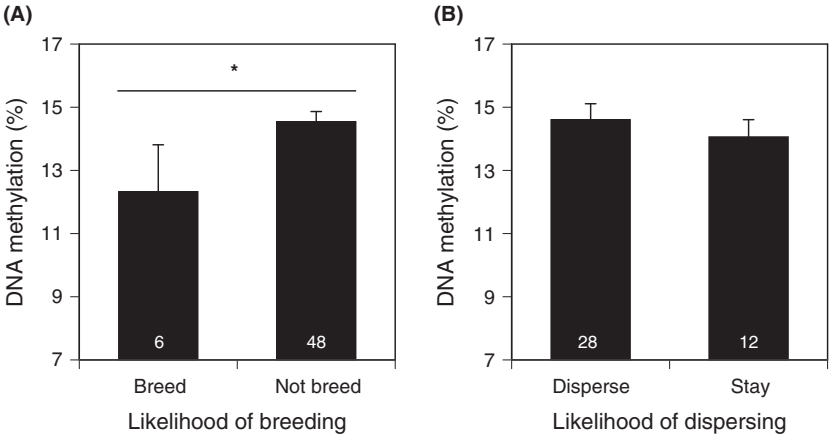


Fig. 5 DNA methylation at sites −830 through −775 in the superb starling Nr3c1 glucocorticoid receptor gene (A) predicted whether-or-not males were likely to breed as adults, but (B) not whether-or-not females were likely to disperse from their natal group. The asterisk indicates significance at $P < 0.05$. Sample sizes are indicated at the base of each bar.

overproducing sons after drier prebreeding periods and daughters following wetter periods (Rubenstein 2007c). Consistent with the 'Trivers–Willard' hypothesis (Trivers & Willard 1973), we have argued that maternal body condition – which is positively related to prebreeding rainfall – influences sex allocation decisions; mothers seem to favour the sex with higher variance in reproductive success (females in this and other cooperatively breeding vertebrates) following years when mothers were in good condition (Rubenstein 2007c; Apakupakul & Rubenstein 2015). As superb starlings are long-lived birds that can survive a decade or more in the wild, adults are likely to experience both high and low quality conditions throughout their breeding lives, with no ability to predict environmental quality from year to year. In such a variable and unpredictable environment where prebreeding rainfall affects so many aspects of the birds' reproductive life histories (Rubenstein 2007b,c, 2016; Rubenstein *et al.* 2008) and ultimately fitness (Rubenstein 2011), maternal influence over offspring phenotype may give some sons born in low quality years a competitive advantage in adulthood when the reproductive success of most males is constrained by their poor early life conditions. Studies from fish are consistent with this idea, demonstrating that under poor environmental conditions mothers in some species will invest more to produce higher quality offspring, which ultimately increases their fitness (Rollinson & Hutchings 2013; Stahlschmidt & Adamo 2015). Such a pattern of increased maternal investment in low quality years may be common in environments like this one where conditions vary unpredictability from year to year, but mean reproductive success in superb starlings is unaffected by environmental variation (Rubenstein 2011). Our results also emphasize that this pattern of life history investment may be sex-specific, much like has been predicted for sex ratio investment (Trivers & Willard 1973) in this and other species (Rubenstein 2007c). Comparing offspring born in different years to the same parents, as well as relating maternal body condition to offspring DNA methylation, will help tease apart these interactions.

For species living in temperate regions where climatic conditions are fairly predictable from year to year, and early and later life conditions are likely to match, glucocorticoid-mediated maternal effects have been shown to be adaptive in many species (Sheriff & Love 2013). Predictable environments include both the social/demographic environment (Krebs *et al.* 1995; Dantzer *et al.* 2013) as well as the ecological environment (Love *et al.* 2005; Love & Williams 2008). In contrast, when environments are unpredictable and early and later life conditions are unlikely to match, as they are in the semi-arid tropics, developmental conditions may either be constraining to adult fitness (Lea *et al.* 2015) or confer some adaptive benefit (Ancona & Drummond 2013). Consistent with our results from superb starlings living in an unpredictable environment, a recent laboratory study in mice demonstrated that unpredictable levels of maternal stress can have positive behavioural consequences later in life for male but not female offspring – effects that are mediated by epigenetic changes involving histone modifications at the mineralocorticoid receptor gene (Gapp *et al.* 2014). Like the glucocorticoid receptor, the mineralocorticoid receptor also plays a substantial role in HPA activity (Breuner & Hahn 2003). Although DNA methylation at the mineralocorticoid receptor promoter did not appear to regulate behavioural changes in this experiment (Gapp *et al.* 2014), this study further demonstrates that unpredictable early life environments – in this case social conditions – can influence adult phenotypes via epigenetically regulated maternal effects of the HPA axis.

This study represents a first attempt to link early life conditions to fitness in adulthood in a free-living vertebrate via epigenetic regulation of a candidate gene. We

recommend interpreting our results with caution, as this is a correlational study with both small effect and sample sizes. We chose to take a targeted gene approach and study the Nrc3c1 glucocorticoid receptor gene because it has been shown to be important in laboratory studies (Weaver *et al.* 2004; McGowan *et al.* 2009), and because previous work in this species has demonstrated an important role for the HPA axis in influencing fitness (Rubenstein 2007b; Rubenstein & Shen 2009). Although the results described here are intriguing, it will be important to determine (i) whether DNA methylation can be passed across generations in this species, (ii) the mechanism by which early life environmental conditions alter DNA methylation on the promoter of the glucocorticoid receptor, (iii) whether DNA methylation in this region influences receptor gene expression and (iv) how DNA methylation affects HPA function in adults. While a candidate gene approach can be useful, taking whole genome approach to look at global patterns of DNA methylation will also be important in future studies.

In summary, we have shown that early life conditions – climatic conditions prior to egg laying that apparently affect maternal condition (Rubenstein 2007c) – are related to offspring DNA methylation in the putative promoter of the glucocorticoid receptor, which in turn is related to fitness later in life. However, as has also been shown in laboratory studies examining epigenetic regulation of the HPA axis (Gapp *et al.* 2014) and in field studies linking early life conditions to adult fitness in other species of birds (Wilkin & Sheldon 2009), the fitness effects are only observed in males. Such sex-specific effects mimic the pattern of sex ratio investment observed in superb starlings (Rubenstein 2007c) and should be further considered in maternal effects theory. Hormonally induced maternal effects are likely to be one of the primary mechanisms that facilitate phenotypic changes in the face of changing climates (Meylan *et al.* 2012). Although the direct transmission of hormones – particularly glucocorticoids (Lessells 2008; Love *et al.* 2013; Sheriff & Love 2013) – from mother to offspring may be important for providing an organizational role during development, epigenetic transmission through chemical (DNA methylation) or structural modifications (histone modification) of the genes related to the HPA axis may also be important for developmental plasticity (Weaver *et al.* 2004; Gapp *et al.* 2014). To our knowledge, no one has yet studied the potential relationship between maternally transferred egg contents and epigenetic modification of offspring in birds. Although we can only speculate on a potential mechanism at this point, cooperatively breeding species offer ideal systems within which to study the epigenetic transmission of developmentally plastic maternal effects on the HPA axis in unpredictable environments because (i) maternal effects are likely to be important in cooperative breeders because of how reproduction and parental care are shared (Russell & Lummaa 2009), and (ii) cooperative breeders tend to inhabit climatically variable environments where both early and later life conditions are going to vary unpredictably from year to year (Rubenstein & Lovette 2007; Jetz & Rubenstein 2011). Our results demonstrate that studying the molecular mechanisms underlying adaptive plasticity of free-living organisms is essential for determining the fitness consequences of epigenetic modifications. Moreover, only by studying animals in the wild will we begin to understand how chemical modifications of the genome may help organisms cope with changing climates.

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D.R.R. and H.S. contributed equally to the project. D.R.R. designed the study, oversaw the project and wrote the manuscript. H.S. collected pyrosequencing data, and A.B. sequenced the Nr3c1 gene. F.A.C. and J.S. oversaw pyrosequencing, and S.P. oversaw sequencing of the Nr3c1 gene. All authors helped revise the manuscript.

Data accessibility

DNA sequences: GenBank Accession no. KT201526.

Raw data (DNA methylation, behaviour and rainfall): Dryad DOI: 10.5061/dryad.4s61t/.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Sample design for examination of DNA methylation in the regulatory region.

Table S2. Sanger sequencing primer sequences designed to be either inside or outside CpG islands within the Nr3c1 gene.

Table S3. Sanger sequencing primer sequences and PCR annealing temperatures.

Table S4. Pyrosequencing primer sequences and PCR annealing temperatures.

Table S5. Full set of GLM models examining the relationships between DNA methylation and prebreeding rainfall.

Table S6. Full set of GLM models examining the relationships between DNA methylation and the likelihood of breeding in males.

Table S7. Full set of GLM models examining the relationships between DNA methylation and the likelihood of dispersing in females.

Fig. S1. Diagram of amplification of overlapping sequences of the Nr3c1 gene.