© 2020. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2020) 223, jeb217422. doi:10.1242/jeb.217422

RESEARCH ARTICLE

Changes in the expression of genes involved in DNA methylation and histone modification in response to daily food availability times in zebra finches: epigenetic implications

Ila Mishra*, Aakansha Sharma*, Abhilash Prabhat, Twinkle Batra, Indu Malik and Vinod Kumar‡

ABSTRACT

We hypothesised that daily food availability times serve as an 'epigenetic' factor and affect reproductive physiology in continuously reproducing species. This we tested by measuring mRNA expression of genes coding for enzymes involved in DNA methylation–demethylation (*dnmt*, *tet*) and histone modification (*hat1*, *hdac*) in the hypothalamus, liver and gonads of male and female zebra finches that were paired for a year under 12 h light:12 h dark conditions with food availability restricted to 4 h in the morning (morning FA group) or evening (evening FA group), with controls provided with food *ad libitum*. The overall hypothalamic and hepatic expression patterns of *hat1* and *hdac* were similar but those of *dnmt* and *tet* were different between males and females. Irrespective of the timing of food availability, both *hat1* and *hdac* mRNA levels were increased in the hypothalamus, but not in the liver, in which *hat1* mRNA levels were increased in the morning FA group. While hypothalamic *tet* levels were higher in evening FA males, hepatic *tet* levels were higher in morning FA birds (*tet1*, only males). Gonadal expression levels similarly varied and showed sex differences. Histone-modifying genes did not show food availability effects, except for elevated testicular *hdac3* levels. Similarly, testicular *dnmt3b* and *tet2* mRNA levels were increased and decreased in morning and evening FA groups, respectively, whereas ovarian *dnmt1* and *tet2* levels were reduced in morning FA and *tet1* levels were reduced in evening FA groups. The present results suggest that an enforced daily feeding schedule in the long term could serve as a conditioning environment that shapes overall hypothalamic regulation, and liver and gonadal function at the epigenetic level in diurnal vertebrates.

KEY WORDS: DNA methyltransferase, Epigenetics, Food availability, Histone modification, Zebra finch

INTRODUCTION

DNA methylation and histone modification (=epigenetic marks) modify local chromatin and affect DNA accessibility and DNA template processes during gene transcription (Allis et al., 2014). DNA methylation, which involves DNA methyltransferase (DNMT)-catalysed methylation of CpG dinucleotides, is an important mechanism of transcriptional repression (Moore et al., 2013). The functionality of DNA methylation remains ambiguous and debated, however (Bewick et al., 2016). As a counter-regulatory step to DNMT-mediated methylation, the ten–eleven translocase (TET) enzymes catalyse DNA demethylation (Ito et al., 2010). The complementary epigenetic modifier system includes processes that involve the acetylation and deacetylation of histone proteins by histone acetyltransferases (HATs) and de-acetyltransferases (HDACs), respectively. HATs promote transcription by chromatin unfolding, whilst HDACs repress transcription by chromatin condensation (Allis et al., 2014). The acetylated open-chromatin structure may also allow access to gene transcription repressors (Verdone et al., 2006).

Epigenetic marks, unlike fixed DNA sequences, are not stable and can undergo changes in response to exogenous stimuli, including diet (Zhang and Kutateladze, 2018), photoperiod (Stevenson and Prendergast, 2013), temperature (Vinoth et al., 2018; Yan et al., 2015), maternal effects (Bentz et al., 2016) and social interactions (Alvarado et al., 2014). In turn, epigenetic modification can influence the expression of genes without changing nucleotide sequences, and contribute to an altered phenotype. Much of the evidence for epigenetic modification among vertebrates comes from mammals and has been frequently reviewed (see especially Bohacek and Mansuy, 2015; Chen et al., 2017; Klemm et al., 2019). However, increasing evidence also suggests epigenetic changes as an important mechanism for phenotypic diversity in the face of environmental changes among birds (Sepers et al., 2019). DNA methylation levels of glucocorticoid receptor gene correlated with rainfall variation during the pre-breeding period, and with adult male reproductive behaviour in superb starlings (*Lamprotornis superbus*; Rubenstein et al., 2016). Similarly, DNA methylation levels of several loci positively correlated with the brood size of zebra finch (*Taeniopygia guttata*) nestlings (Sheldon et al., 2018).

Accumulated evidence from several taxa suggests a role of nutritional cues in epigenetic modification. DNA methylation reversibly marked the behavioural sub-castes of workers that forage and nurse in bees (Herb et al., 2012). Both food quality and quantity affected epigenetic modifications in rodents (Dunn and Bale, 2009; Zheng and Pan, 2010; Zheng et al., 2012). The maternal exposure of rat pups to a low-protein diet down-regulated the expression of the *p16* cell cycle gene through histone modification, and predisposed offspring towards certain diseases when adult (Zheng and Pan, 2010; Zheng et al., 2012). However, the functional linkage of time-restricted feeding with epigenetic control of physiology and behaviour is only beginning to emerge. A recent study showed a time-restricted feeding-induced reduction in HDAC activity, and hence increased histone H3 acetylation, in mouse hippocampus (Landgrave-Gómez et al., 2016). The question that we addressed here is: what is the consequence of long-term time-restricted feeding on epigenetic modification in a continuously reproducing diurnal

Department of Zoology, University of Delhi, Delhi 110 007, India.
*These authors contributed equally to this work

‡Author for correspondence (drvkumar11@yahoo.com)

I. Mishra, 0000-0002-9786-6358; A.S., 0000-0001-6838-0060; A.P., 0000-0001-7598-5519; T.B., 0000-0003-0176-6254; I. Malik, 0000-0001-9887-0108; V.K., 0000-0002-0523-8689

Received 27 October 2019; Accepted 23 December 2019

Journal of Experimental Biology (2020) 223, jeb217422. doi:10.1242/jeb.217422

vertebrate? We investigated this in diurnal zebra finches (*Taeniopygia guttata*) in which a previous study has shown time-restricted feeding-induced effects on reproductive performance, and the quality and quantity of offspring survivors, as assessed at multiple levels including circulating levels of hormones (sex steroids and mesotocin), egg laying latency and egg quality, reproductive fecundity, and offspring growth and size when adult (Mishra and Kumar, 2019). Importantly, time-restricted feeding adversely affected reproductive health, as suggested by reduced sex steroids and mesotocin levels compared with levels in birds that were fed *ad libitum* (Mishra and Kumar, 2019). Here, we examined whether time-restricted feeding affected the mRNA expression of epigenetic modifier genes, and whether the expression pattern differed between morning and evening FA groups. In particular, we measured the mRNA expression of genes involved in DNA methylation and demethylation (*dnmt1*, *dnmt3a*, *dnmt3b*, *tet1*, *tet2*), and histone modification (*hat1*, *hdac2*, *hdac3* and *hdac4*) in the hypothalamus (the overall regulatory centre), liver (the site of metabolic homoeostasis) and gonads (the reproductive gland involved in gamete production and sex hormone secretion, which hence represented the endpoint of reproductive activity).

MATERIALS AND METHODS

Animals and experiment

The Institutional Animal Ethics Committee, IAEC (DU/ZOOL/IAEC-R/2015/02) of the Department of Zoology, University of Delhi, India, approved the study. We used tissues harvested from our previous study (Mishra and Kumar, 2019), described in detail therein. Briefly, we used 36 adult birds of each sex (1–1.5 years of age with similar body mass: 12.4±0.1 g), born and raised in our indoor aviary and maintained at constant temperature (24±2°C) and photoperiod (12 h light:12 h dark; light 200±10 lx, dark 0 lx). Birds were housed in same-sex cages (54 cm×42 cm×30 cm; n=4 per cage) and kept on a 12 h light:12 h dark photoperiod, as before, for 3 weeks, during which they had no visual or acoustic contact with the opposite sex. This was done to break any pair bonds that were established in the aviary; we removed any eggs laid during this period to discount the carry-over effect of previous co-habitation. Birds had *ad libitum* access to food and water, and were fed daily on *Setaria italica* (Kakuni; 3.64 kcal g^{-1}) seeds as the primary diet, supplemented with hard-boiled eggs (1.47 kcal g^{-1}) mashed with crushed egg shell. Thereafter, for 24/36 birds of each sex, the food availability period was restricted by step-wise reduction of food availability times over 3 days to 8, 6 and 4 h, aligned with lights on (hour 0; morning FA) or lights off (hour 12; evening FA). Thus, time-restricted feeding deprived all birds of food for the same period of time but with a difference in the direction of food deprivation in relation to 4 h food availability. The remaining 12 birds of each sex continued to be given food *ad libitum*, and served as controls. Each day, we provided a food-filled cup at hour 0 to *ad libitum*-fed birds and the morning FA group, and at hour 8 to the evening FA group, and the cup was replaced by an empty food cup at hours 4 and 12 for the morning and evening FA group, respectively, and with another food-filled cup the next day at hour 0 for *ad libitum*-fed birds.

After a week of acclimation to the particular feeding condition, separately housed males and females were randomly paired, so each feeding condition had 12 breeding pairs. Each bird lived with the same partner for about a year, during which we assessed time-restricted feeding-induced effects on reproductive health and performance, as reported by Mishra and Kumar (2019). Thereafter, pairs were separated and returned to the same-sex groupings and maintained on identical lighting and feeding regimes to those during the breeding protocol, to minimise the influence of co-habitation. After 8 weeks, five randomly chosen birds from each group (n=5 per sex and food condition) were decapitated under dim red light half an hour before lights on so that they were 'starved' for the almost the entire night period, irrespective of the feeding regime (zebra finches do not eat during the period of darkness; I.M. and V.K., unpublished observations). Thus, in the current study, we performed gene expression analyses on a subset of animals from the larger year-long experiment. We preferred decapitation (an unanticipated quick procedure that lasts only ∼10 s from removal of the bird from its cage to its decapitation) over anaesthesia usage, as anaesthesia can influence mRNA expression levels in tissues (Hamaya et al., 2000; Staib-Lasarzik et al., 2014). The brain, liver and gonads were removed, the hypothalamus was quickly excised from the brain (Mishra et al., 2018), and all tissues were stored at −80°C until processed for gene expression assays.

Analysis of mRNA expression of epigenes in different tissues by qPCR

From each tissue, a 1 µg aliquot of total RNA extracted using Tri reagent (AM9738; Ambion, Austin, TX, USA) and treated with RQ1 RNase-free DNase (M6101, Promega, Madison, WI, USA) was reverse transcribed using Revert-Aid first strand cDNA synthesis Kit (K1622, Thermo Fisher Scientific, Waltham, MA, USA). The mRNA expression of *hat1*, *hdac2*, *hdac3*, *hdac4*, *dnmt1*, *dnmt3a*, *dnmt3b*, *tet1* and *tet2* genes was measured in the hypothalamus, liver and gonads using gene-specific primers (Table 1) and SYBR green chemistry, using protocols standardised and routinely used in our laboratory (Majumdar et al., 2015; Mishra et al., 2018; Sharma et al., 2018). We performed 2-step qPCR with an Applied Biosystems ViiA7 thermal cycler using 10 µl reaction mixture, with 1 µl each of cDNA (10 ng ml^{-1}) and forward and reverse primers (500 nmol l^{-1}; Table 1), 5 µl Power Syber Green PCR Mastermix (ABI 4387669; 1× in total reaction volume) and 2 µl of nuclease-free water, for a total of 40 cycles, each lasting for 75 s (15 s melting at 95°C+60 s annealing at 60°C). We used *β-actin* as the endogenous control (reference) gene, which was found to have high stability and low inter- and intra-group variability (Sharma et al., 2018). Here also, we assessed the stability of the *β-actin* gene using Best-Keeper (Pfaffl et al., 2004), and found that its values were stable and did not vary much between

Table 1. Gene-specific primers used for qPCR

Gene	Primer sequence	Accession no.
hat1	F: 5′ CTGAGGATTTGGAGGATGAGAA 3′. R: 5′ GAGGATCTTGAGTCCCTTGTAG 3′	XM_002195767.4
hdac2	F: 5′ TGCTGTCAACTTTCCAATGA 3′ R: 5′ TCTGCTCCACACTGCAATAC 3′	XM_002192494.3
hdac3	F: 5′ GTGTGTGGAGTACGTGAAGAG 3′ R: 5′ ACGAGCAAGGATGTTTCATA 3′	XM_012576240.2
hdac4	F: 5′ CGAGCACAGAAGTGAAGATGA 3′ R: 5′ TGGATATGCAGTGGTTGAGATT 3′	XM_030276973.1
dnmt1	F: 5′ CACAAAGTCACCTCCTTCAG 3′ R: 5′ TAGAGCTCGATGTTCCTCTC 3′	XM_030259939.1
dnmt3a	F: 5′ TGAACACACGGAATGAGAGAAG 3′ R: 5′ GCTAAAGCTGATCCTGGGAAA 3′	XM_030269406.1
dnmt3b	F: 5′CCTTCCACCCACTGTTCAA 3′ R: 5′CTGGTACCCATCTTCATCATAC 3′	XM_030288758.1
tet1	F: 5′ CAGGCCAAACAACAGGTAGA 3′ R: 5′ CCTCACCCATACACTCCATATTC 3′	XM_030275597.1
tet2	F: 5′ CTCCACGTGCTCCCATTATAC 3′ R: 5′ GATGCTGCCATTCCTCTTCT 3′	XM_030271641.1

Journal of Experimental Biology (2020) 223, jeb217422. doi:10.1242/jeb.217422

the treatment groups (s.d.±crossover threshold, Ct<1.0; hypothalamus: 0.99, liver: 0.86, testis: 0.88, ovary: 0.92). Both sample and reference genes were run in duplicate, and the relative mRNA expression level was determined as 2^{-ddCt} (Livak and Schmittgen, 2001). Briefly, we first calculated ΔCt by subtracting the crossover threshold (Ct) of the reference gene from the target gene ($Ct_{target\ gene}-Ct_{reference\ gene}$). ΔCt value was then normalised against the Ct value of a pooled sample containing an equal quantity of cDNA from all samples; this gave the ΔΔCt value (Mishra et al., 2017; Sharma et al., 2018). For better visual resolution and presentation, data on mRNA expression were further transformed to define the sample with the lowest mRNA levels as a relative quantity of 1, which amounts to a $\log_2$ value of 0. On this scale, a difference of 1 unit reflects a 2-fold difference in the respective mRNA expression level (Mishra et al., 2018). The expression of *hdac2* was found to be below the detection limit in both the testes and ovary, and that of *dnmt3a* was below the detection limit the hypothalamus, liver and gonads and hence was excluded from the presentation.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism (version 6, San Diego, CA, USA) or SPSS Statistics software (version 20, IBM), as appropriate. All tests were two-tailed, and we report the results as means±s.e.m. We used generalised linear model (GLM, 2-factor analysis) followed by pairwise sequential Bonferroni *post hoc* test to test the effects of the feeding regime (factor 1), sex (factor 2) and their interaction (factor 1×factor 2) on mRNA expressions in the hypothalamus and liver, as values were not repeated measures. However, we did not compare the mRNA levels in the testes with those in the ovary, so we used Kruskal–Wallis (KW, 1-factor analysis) test followed by Dunn's *post hoc* test to test the effect of feeding regime on gene expression separately in the testes and ovary. We also calculated effect size estimates as ε^2=KW value/($N^2-1/N+1$) for 1-factor analysis (Kruskal–Wallis test) and $\eta^2_{partial}=SS_{effect}/(SS_{effect}+SS_{error})$ (where SS is sum of squares) for 2-factor analysis (GLM). We used log-transformed values for the statistics, and $P<0.05$ was considered a statistically significant difference.

RESULTS

Effects of time-restricted feeding on epigene expression

Hypothalamus

Time-restricted feeding significantly altered the hypothalamic expression of epigenetic modifier genes. There was a significant effect of feeding regime on all the measured epigenetic markers (*hat1*: Wald's χ^2=66.95, $P<0.0001$; *hdac2*: Wald's χ^2=53.85, $P<0.0001$; *hdac3*: Wald's χ^2=59.92, $P<0.0001$; *hdac4*: Wald's χ^2=164.49, $P<0.0001$; *dnmt1*: Wald's χ^2=25.53, $P<0.0001$; *dnmt3b*: Wald's χ^2=19.49, $P<0.0001$; *tet1*: Wald's χ^2=11.29, P=0.004; *tet2*: Wald's χ^2=29.11, $P<0.0001$; GLM; Table 2). We also found a significant sex effect on *dnmt3b* (Wald's χ^2=6.60, P=0.010; GLM; Table 2) and *tet2* (Wald's χ^2=3.91, P=0.048; GLM; Table 2) expression, and of the feeding regime×sex interaction on *hdac4* expression only (Wald's χ^2=6.59, P=0.037; GLM; Table 2). In particular, the mRNA level of histone modifiers (*hat1*, *hdac2*, *hdac3* and *hdac4*) was significantly increased in both sexes in time-restricted feeding groups, except for *hat1* levels in morning FA females ($P<0.05$; Bonferroni *post hoc* test; Fig. 1A–D). However, there were sex differences in the expression pattern of *dnmt* and *tet* genes. Whereas *dnmt1* mRNA expression was unaffected in males, the levels were significantly reduced in morning FA females ($P<0.05$; Bonferroni *post hoc* test; Fig. 1E). Similarly, we found significantly increased *dnmt3b*, *tet1* and *tet2* mRNA expression in males, but not in females, subjected to morning FA (*dnmt3b* alone) and evening FA regimes ($P<0.05$; Bonferroni *post hoc* test; Fig. 1F–H).

Liver

mRNA expression patterns in the liver showed a significant effect of feeding regime (*hat1*: Wald's χ^2=41.98, $P<0.0001$; *hdac2*: Wald's χ^2=9.27, P=0.010; *hdac4*: Wald's χ^2=93.20, $P<0.0001$; *dnmt1*: Wald's χ^2=47.27, $P<0.0001$; *dnmt3b*: Wald's χ^2=14.96, P=0.001; *tet1*: Wald's χ^2=16.57, $P<0.0001$; *tet2*: Wald's χ^2=69.02, $P<0.0001$; GLM; Table 2) and sex (*hdac3*: Wald's χ^2=4.40, P=0.036; *hdac4*: Wald's χ^2=4.02, P=0.045; *dnmt1*: Wald's χ^2=9.58, P=0.002; *dnmt3b*: Wald's χ^2=8.68, P=0.003; *tet1*: Wald's χ^2=18.20, $P<0.0001$; GLM; Table 2), and a feeding

Table 2. Results of generalised linear model analysis presented as Wald's χ^2, alpha (*P*-value) and effect size estimate

	Intercept			Feeding regime			Sex			Feeding regime×sex		
Gene	Wald's χ^2	*P*	$\eta^2_{partial}$	Wald's χ^2	*P*	$\eta^2_{partial}$	Wald's χ^2	*P*	$\eta^2_{partial}$	Wald's χ^2	*P*	$\eta^2_{partial}$
Hypothalamus												
hat1	**42.32**	**<0.0001**	**0.58**	**66.95**	**<0.0001**	**0.69**	0.85	0.351	0.03	4.58	0.101	0.13
hdac2	0.84	0.360	0.03	**53.85**	**<0.0001**	**0.64**	0.11	0.740	0.004	5.38	0.068	0.15
hdac3	**130.27**	**<0.0001**	**0.81**	**59.92**	**<0.0001**	**0.66**	0.76	0.381	0.02	3.61	0.164	0.11
hdac4	**108.44**	**<0.0001**	**0.78**	**164.49**	**<0.0001**	**0.84**	0.49	0.480	0.02	**6.59**	**0.037**	**0.18**
dnmt1	1.68	0.194	0.05	**25.53**	**<0.0001**	**0.46**	2.44	0.121	0.07	5.88	0.053	0.16
dnmt3b	**44.67**	**<0.0001**	**0.60**	**19.49**	**<0.0001**	**0.39**	**6.60**	**0.010**	**0.18**	5.20	0.074	0.15
tet1	**40.45**	**<0.0001**	**0.59**	**11.29**	**0.004**	**0.09**	0.69	0.401	0.000	4.71	0.095	0.02
tet2	3.73	0.053	0.11	**29.11**	**<0.0001**	**0.50**	**3.91**	**0.048**	**0.11**	1.22	0.543	0.04
Liver												
hat1	**30.98**	**<0.0001**	**0.48**	**41.98**	**<0.0001**	**0.59**	2.80	0.094	0.06	1.17	0.557	0.03
hdac2	**240.92**	**<0.0001**	**0.89**	**9.27**	**0.010**	**0.24**	0.66	0.416	0.003	1.13	0.569	0.008
hdac3	**81.86**	**<0.0001**	**0.73**	1.99	0.368	0.06	**4.40**	**0.036**	**0.13**	**7.51**	**0.023**	**0.20**
hdac4	0.44	0.507	0.01	**93.20**	**<0.0001**	**0.76**	**4.02**	**0.045**	**0.12**	2.22	0.330	0.071
dnmt1	**5.29**	**0.021**	**0.15**	**47.27**	**<0.0001**	**0.61**	**9.58**	**0.002**	**0.24**	2.65	0.266	0.08
dnmt3b	**74.92**	**<0.0001**	**0.76**	**14.96**	**0.001**	**0.37**	**8.68**	**0.003**	**0.28**	2.65	0.265	0.08
tet1	0.08	0.777	0.003	**16.57**	**<0.0001**	**0.36**	**18.20**	**<0.0001**	**0.38**	4.14	0.13	0.12
tet2	**102.23**	**<0.0001**	**0.77**	**69.02**	**<0.0001**	**0.69**	0.071	0.79	0.002	2.67	0.263	0.08

The effect size estimate was calculated as the partial eta^2 [$\eta^2_{partial}=SS_{effect}/(SS_{effect}+SS_{error})$, where SS is sum of squares]. Bold indicates the presence of significant differences in mRNA expression levels. $P<0.05$ was considered statistically significant.
Model: intercept, feeding regime, sex, feeding regime×sex.

Journal of Experimental Biology (2020) 223, jeb217422. doi:10.1242/jeb.217422

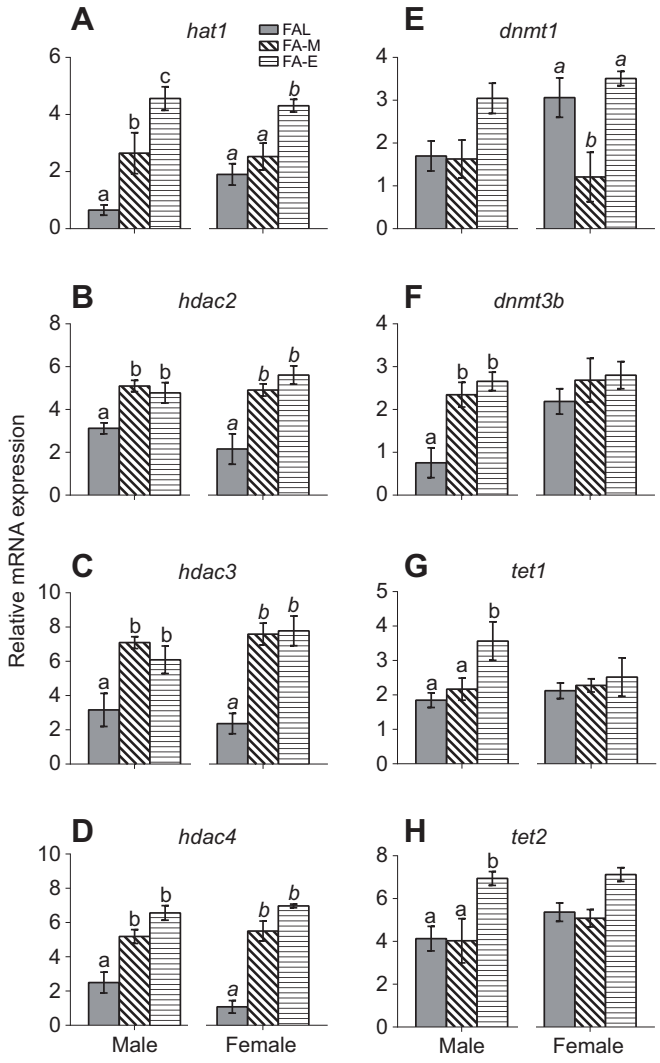

Fig. 1. Effects of daily food restriction on mRNA expression of genes involved in histone modification and DNA methylation in the hypothalamus of adult male and female zebra finches. (A–D) Histone modification genes *hat1*, *hdac2*, *hdac3* and *hdac4*. (E–H) DNA methylation genes *dnmt1*, *dnmt3b*, *tet1* and *tet2*. Birds were subjected to 4 h of food availability in the morning (morning FA group, FA-M) or evening (evening FA group, FA-E), with controls provided with food *ad libitum* (FAL). Data are means ±s.e.m. Different letters indicate a significant difference between food restriction groups as determined by generalised linear model (GLM) test followed by Bonferroni *post hoc* test. $P<0.05$ was considered statistically significant.

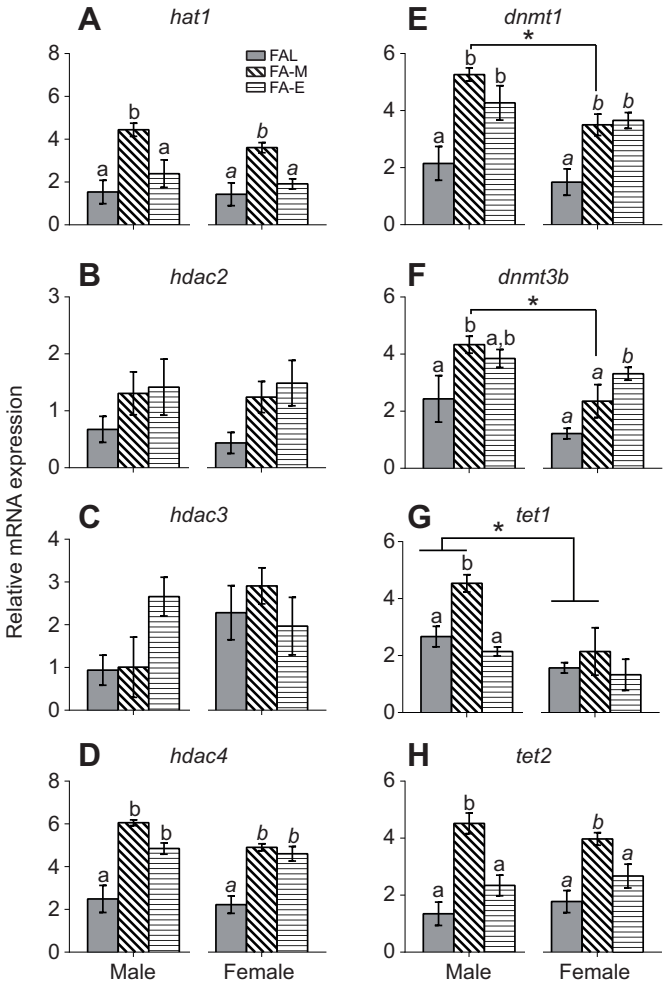

Fig. 2. Effects of daily food restriction on mRNA expression of genes involved in histone modification and DNA methylation in the liver of adult male and female zebra finches. (A–D) Histone modification genes *hat1*, *hdac2*, *hdac3* and *hdac4*. (E–H) DNA methylation genes *dnmt1*, *dnmt3b*, *tet1* and *tet2*. Birds were subjected to 4 h of food availability in the morning (morning FA group, FA-M) or evening (evening FA group, FA-E), with controls provided with food *ad libitum* (FAL). Data are means±s.e.m. Different letters indicate a significant difference between food restriction groups and asterisks indicate differences between male and female zebra finches as determined by GLM test followed by Bonferroni *post hoc* test. $P<0.05$ was considered statistically significant.

regime×sex interaction for *hdac3* mRNA expression alone (Wald's $\chi^2=7.51$, $P=0.023$; GLM; Table 2). In both sexes, *hat1* mRNA levels were significantly higher in the morning FA group than in the evening FA or *ad libitum*-fed group ($P<0.05$; Bonferroni *post hoc* test; Fig. 2A). There was no difference in mRNA expression of *hdac3*, but *hdac4* mRNA levels in both sexes were significantly higher in time-restricted feeding groups ($P<0.05$; Bonferroni *post hoc* test; Fig. 2C,D). Similarly, in both sexes, *dnmt1* mRNA levels were significantly higher in time-restricted feeding groups, as compared with the *ad libitum*-fed group ($P<0.05$; Bonferroni *post hoc* test; Fig. 2E). However, *dnmt3b* mRNA levels were significantly higher in morning FA males and evening FA females, as compared with the *ad libitum*-fed birds ($P<0.05$; Bonferroni *post hoc* test; Fig. 2F). Within the time-restricted feeding groups, *dnmt1* and *dnmt3b* mRNA expression showed sex-dependent differences. For example, both *dnmt1* and *dnmt3b* mRNA levels were significantly higher in males than in females in the morning FA group ($P<0.05$; Bonferroni *post hoc* test; Fig. 2E,F). We also found that *tet1* mRNA levels in male birds were significantly higher in morning FA birds than in evening FA and *ad libitum*-fed birds; mRNA levels were also higher in males than in females in the *ad libitum*-fed and morning FA groups ($P<0.05$; Bonferroni *post hoc* test; Fig. 2G). Likewise, *tet2* mRNA levels in both sexes were significantly higher in morning FA birds than in *ad libitum*-fed and evening FA birds ($P<0.05$; Bonferroni *post hoc* test; Fig. 2H).

Gonads

We found a significant time-restricted feeding-induced effect on testicular mRNA expression of *hdac3* (KW statistic: 9.50, $P=0.002$, $N=15$, $\varepsilon^2=0.68$, Kruskal–Wallis; Fig. 3B), *dnmt1* (KW statistic:

Journal of Experimental Biology (2020) 223, jeb217422. doi:10.1242/jeb.217422

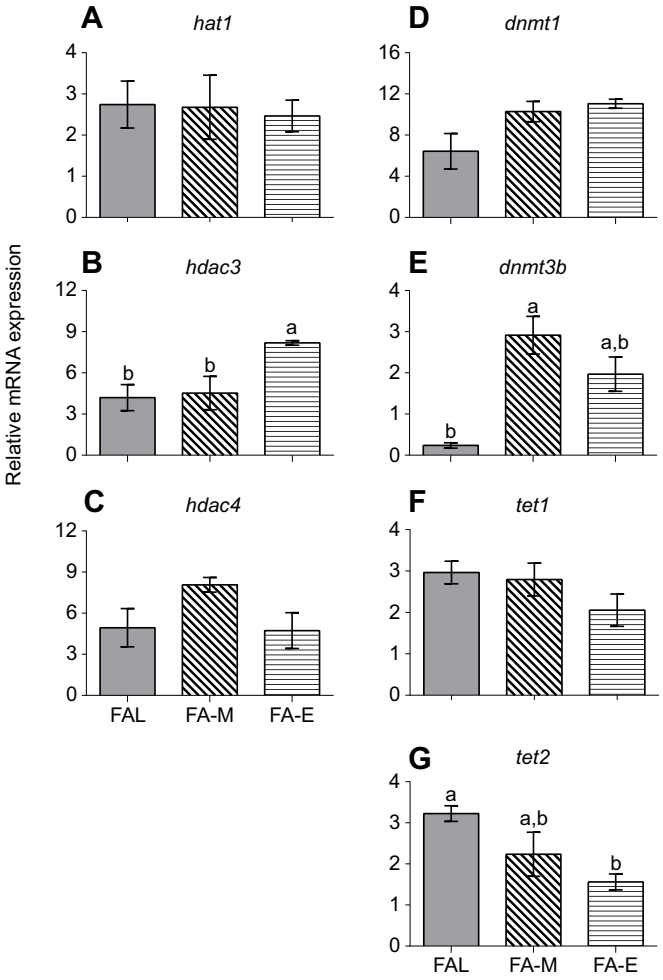

Fig. 3. Effects of daily food restriction on mRNA expression of genes involved in histone modification and DNA methylation in testes of adult male zebra finches. (A–C) Histone modification genes *hat1*, *hdac3* and *hdac4*. (D–G) DNA methylation genes *dnmt1*, *dnmt3b*, *tet1* and *tet2*. Birds were subjected to 4 h of food availability in the morning (morning FA group, FA-M) or evening (evening FA group, FA-E), with controls provided with food *ad libitum* (FAL). Data are means±s.e.m. Different letters indicate a significant difference as determined by Kruskal–Wallis test followed by Dunn's *post hoc* test. $P<0.05$ was considered statistically significant.

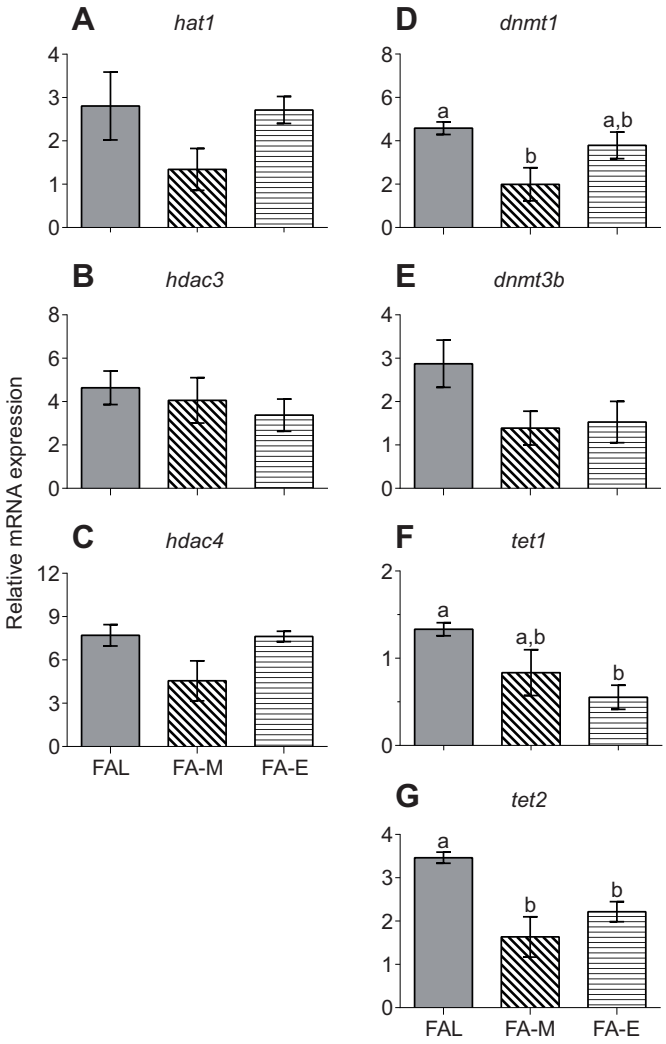

Fig. 4. Effects of daily food restriction on mRNA expression of genes involved in histone modification and DNA methylation in the ovary of adult female zebra finches. (A–C) Histone modification genes *hat1*, *hdac3* and *hdac4*. (D–G) DNA methylation genes *dnmt1*, *dnmt3b*, *tet1* and *tet2*. Birds were subjected to 4 h of food availability in the morning (morning FA group, FA-M) or evening (evening FA group, FA-E), with controls provided with food *ad libitum* (FAL). Data are means±s.e. Different letters indicate a significant difference as determined by Kruskal–Wallis test followed by Dunn's *post hoc* test. $P<0.05$ was considered statistically significant.

6.62, $P=0.028$, $N=15$, $\varepsilon^2=0.47$, Kruskal–Wallis; Fig. 3D), *dnmt3b* (KW statistic: 9.14, $P=0.0035$, $N=15$, $\varepsilon^2=0.65$, Kruskal–Wallis; Fig. 3E) and *tet2* genes (KW statistic: 7.46, $P=0.014$, $N=15$, $\varepsilon^2=0.53$, Kruskal–Wallis; Fig. 3G). In the ovary, however, there were significant time-restricted feeding-induced effects on *dnmt1* (KW statistic: 6.14, $P=0.038$, $N=15$, $\varepsilon^2=0.44$, Kruskal–Wallis; Fig. 4D), *tet1* (KW statistic: 7.46, $P=0.014$, $N=15$, $\varepsilon^2=0.53$, Kruskal–Wallis; Fig. 4F) and *tet2* (KW statistic: 9.62, $P=0.001$, $N=15$, $\varepsilon^2=0.69$, Kruskal–Wallis; Fig. 4G), but not on *hdac3* and *dnmt3b* mRNA expression. Further, testicular *hdac3* mRNA levels were significantly higher in evening FA and those of *dnmt3b* were higher in morning FA groups than in the *ad libitum*-fed group ($P<0.05$, Dunn's *post hoc* test; Fig. 3B,E). However, testicular *tet2* mRNA levels were significantly higher in *ad libitum*-fed than in evening FA birds ($P<0.05$, Dunn's *post hoc* test; Fig. 3G). In the ovary, *dnmt1* and *tet2* mRNA levels were significantly lower in the morning FA group and *tet1* levels were significantly lowed in evening FA birds, as compared with levels in the *ad libitum*-fed group ($P<0.05$, Dunn's *post hoc* test; Fig. 4D,F,G).

DISCUSSION

We show differential effects of time-restricted feeding on the mRNA expression of genes coding for histone-modifying enzymes and DNA methyltransferases/translocases in tissues that are involved in the neuroendocrine regulation (hypothalamus), metabolic homeostasis (liver) and reproduction (gonad) in diurnal zebra finches. Intriguingly, we found tissue-specific sex-dependent genetic responses to time-restricted feeding and its timing during the day. For example, the overall hypothalamic and hepatic expression patterns of *hat1* and *hdac* were similar but those of *dnmt* and *tet* were different between males and females. More specifically, irrespective of the timing of food restriction, both *hat1* and *hdac* mRNA levels were increased in the hypothalamus but not in the liver, in which *hat1* mRNA levels were increased only in the morning FA group. Increased hypothalamic expression of *hdac* under the time-restricted feeding regime in zebra finches was consistent with its

expression in the medial hypothalamus of adult mice in response to fasting or a high fat diet (Funato et al., 2011). Likewise, hypothalamic *dnmt1* mRNA levels were decreased in morning FA females, but those of *dnmt3b*, *tet1* and *tet2* were increased in morning FA and/or evening FA male zebra finches. This is inconsistent with the reported increase in *dnmt1* and *dnmt3b* expression in response to 12 h food deprivation in chickens, which was reversed after 36 h of food deprivation (Kang et al., 2017). However, DNA methylation was significantly decreased in pregnant rats subjected to a reduced protein diet, suggesting the hypomethylation of specific genes (Lillycrop et al., 2005). We speculate that differences in the epigene expression pattern were linked to the daily food times and tissue-specific regulatory mechanism(s) in zebra finches. We would not discount that the diet-induced effects on epigenetic modification also affected the egg components, and resulted in a time-restricted feeding-dependent effect on reproductive performance (Groothuis et al., 2005). Indeed, time-restricted feeding significantly affected egg quality, as assessed in the measures of primary maternal investment and other parameters, egg mass and volume, and the percentage lipid and yolk testosterone content (Mishra and Kumar, 2019). In particular, eggs were significantly smaller in size and lower in mass in time-restricted feeding than in *ad libitum*-fed pairs, and eggs from morning FA pairs had a significantly higher percentage of lipid and lower yolk testosterone content, as compared with eggs from the evening FA or *ad libitum*-fed pairs (Mishra and Kumar, 2019).

Interestingly, we found clear differences in the mRNA expression pattern of genes in gonads, as compared with that in the hypothalamus and liver. This was particularly evidenced by the non-detection *hadc2* mRNA and no-difference in *hat1* and *hdac4* mRNA levels in gonads between the three feeding regimes. There were also differences in the mRNA expression pattern of genes between the testes and ovary. In response to time-restricted feeding, there was increased testicular expression of *hdac3* and *dnmt3b* in the evening and morning, respectively, and decreased expression of *tet2* in the evening; however, mRNA levels of *dnmt3b* and *tet2* were relatively high and low in evening FA and morning FA birds, respectively, compared with those in *ad libitum*-fed birds. In ovary, in contrast, we found decreased *dnmt1* in morning FA birds, decreased *tet1* in evening FA birds, and decreased *tet2* in both morning and evening FA birds. We suggest a differential time-restricted feeding-induced effect at the gonadal level in male and female zebra finches. Perhaps, testicular *hdac3* was associated with delayed reproductive performance, as reported by the delayed egg laying onset and reduced reproductive success in response to evening FA in zebra finches (Mishra and Kumar (2019). A direct functional link between histone deacetylase activity and testicular function has indeed been suggested by an adverse effect of the histone deacetylase inhibitor Trichostatin A in murine spermatogenesis (Fenic et al., 2004). A linkage of *hdac3* to the local inhibition of reproductive function has also been suggested in the Siberian hamster, *Phodopus sungorus* (Lynch et al., 2016). The lack of change in ovarian *hdac* expression in zebra finches is also consistent with results in Siberian hamsters (Lynch et al., 2016). We interpret that high *dnmt3b* and low *tet1* and *tet2* mRNA levels indicate an elevated methylation state in response to time-restricted feeding in zebra finches. At the same time, reduced *tet2* expression in both testes and ovary might suggest a commonality in time-restricted feeding-induced effects on reproductive tissues in zebra finches, as suggested by studies in Siberian hamsters (Lynch et al., 2016).

In conclusion, we suggest that time-restricted feeding could potentially modulate overall hypothalamic regulation, liver functions and gonadal activity at the genetic level in diurnal zebra finches. Probably, the food availability and its timing served as an 'epigenetic' factor in the regulation of gene expression and, in turn, affected reproductive physiology via the hypothalamus–liver–gonad axis in zebra finches. However, this is purely speculative and needs further investigation. A role for diet via its effects on DNA methylation has been suggested in the regulation of reproductive performance in mammals, including humans (Cisneros, 2004). Although we have shown changes in the mRNA expression of genes involved in the epigenetic modification in response to restricted food availability, the mechanism(s) of epigenetic modification may need to be further confirmed in a future study. The overall implication of the present results is that an enforced daily feeding schedule in the long term could serve as a conditioning environment that shapes the activity of the genome by chromatin activation/silencing, which in turn could affect reproductive physiology and performance in vertebrates.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: V.K.; Methodology: I. Mishra, A.P., V.K.; Formal analysis: I. Mishra, A.S., T.B., I. Malik; Investigation: I. Mishra, A.P., T.B., I. Malik, V.K.; Data curation: I. Mishra, A.S.; Writing - original draft: A.S., V.K.; Writing - review & editing: A.S., V.K.; Visualization: V.K.; Supervision: V.K.; Project administration: V.K.; Funding acquisition: V.K.

Funding
A research grant (EMR/2015/002158) from Science and Engineering Research Board (SERB) to V.K. provided funding. The experimental facility used for this study was built under Intensification of Research in High Priority Area (IRHPA) grant support (IR/SO/LU-2005) by the Department of Science and Technology and SERB.

References
Allis, D. C., Jenuwein, T., Reinberg, D. and Caparros, M. L. (2014). *Epigenetics*. Cold Spring Harbour, NY, USA: Cold Spring Harbour Laboratory Press.

Alvarado, S., Fernald, R. D., Storey, K. B. and Szyf, M. (2014). The dynamic nature of DNA methylation: a role in response to social and seasonal variation. *Integr. Comp. Biol.* **54**, 68-76. doi:10.1093/icb/icu034

Bentz, A. B., Sirman, A. E., Wada, H., Navara, K. J. and Hood, W. R. (2016). Relationship between maternal environment and DNA methylation patterns of estrogen receptor alpha in wild Eastern Bluebird (*Sialia sialis*) nestlings: a pilot study. *Ecol. Evol.* **6**, 4741-4752. doi:10.1002/ece3.2162

Bewick, A. J., Ji, L., Niederhuth, C. E., Willing, E.-M., Hofmeister, B. T., Shi, X., Wang, L., Lu, Z., Rohr, N. A., Hartwig, B. et al. (2016). On the origin and evolutionary consequences of gene body DNA methylation. *Proc. Natl. Acad. Sci. USA* **113**, 9111-9116. doi:10.1073/pnas.1604666113

Bohacek, J. Mansuy, I. M. (2015). Molecular insights into transgenerational non-genetic inheritance of acquired behaviours. *Nat. Rev. Genet.* **16**, 641-652. doi:10.1038/nrg3964

Chen, Z., Li, S., Subramaniam, S., Shyy, J. Y.-J. and Chien, S. (2017). Epigenetic regulation: a new frontier for biomedical engineers. *Annu. Rev. Biomed. Eng.* **19**, 195-219. doi:10.1146/annurev-bioeng-071516-044720

Cisneros, F. J. (2004). DNA methylation and male infertility. *Front. Biosci.* **9**, 1189-1200. doi:10.2741/1332

Dunn, G. A. and Bale, T. L. (2009). Maternal high-fat diet promotes body length increases and insulin insensitivity in second-generation mice. *Endocrinology* **150**, 4999-5009. doi:10.1210/en.2009-0500

Fenic, I., Sonnack, V., Failing, K., Bergmann, M. and Steger, K., (2004). In vivo effects of histone-deacetylase inhibitor trichostatin-A on murine spermatogenesis. *J. Androl.* **25**, 811-818. doi:10.1002/j.1939-4640.2004.tb02859.x

Funato, H., Oda, S., Yokofujita, J., Igarashui, H. and Kuroda, M. (2011). Fasting and high-fat diet alter histone deacetylase expression in the medial hypothalamus. *PLoS ONE* **6**, e18950. doi:10.1371/journal.pone.0018950

Groothuis, T. G. G., Müller, W., von Engelhardt, N., Carere, C. and Eising, C. (2005). Maternal hormones as a tool to adjust offspring phenotype in avian species. *Neurosci. Biobehav. Rev.* **29**, 329-352. doi:10.1016/j.neubiorev.2004.12.002

Hamaya, Y., Takeda, T., Dohi, S., Nakashima, S. and Nozawa, Y. (2000). The effects of pentobarbital, isoflurane, and propofol on immediate-early gene expression in the vital organs of the rat. *Anesth. Analg.* **90**, 1177-1183. doi:10.1097/00000539-200005000-00034

Herb, B. R., Wolschin, F., Hansen, K. D., Aryee, M. J., Langmead, B., Irizarry, R., Amdam, G. V. and Feinberg, A. P. (2012). Reversible switching between epigenetic states in honeybee behavioral subcastes. *Nat. Neurosci.* **15**, 1371-1373. doi:10.1038/nn.3218

Ito, S., D'Alessio, A. C., Taranova, O. V., Hong, K., Sowers, L. C. and Zhang, Y. (2010). Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* **466**, 1129-1133. doi:10.1038/nature09303

Kang, S. W., Madkour, M. and Kuenzel, W. J. (2017). Tissue-specific expression of DNA methyltransferases involved in early-life nutritional stress of chicken, *Gallus gallus*. *Front. Genet.* **8**, 204. doi:10.3389/fgene.2017.00204

Klemm, S. L., Shipony, Z. and Greenleaf, W. J. (2019). Chromatin accessibility and the regulatory epigenome. *Nat. Rev. Genet.* **20**, 207-220. doi:10.1038/s41576-018-0089-8

Landgrave-Gómez, J., Mercado-Gómez, O. F., Vázquez-García, M., Rodríguez-Molina, V., Córdova-Dávalos, L., Arriaga-Ávila, V., Miranda-Martínez, A. and Guevara-Guzmán, R. (2016). Anticonvulsant effect of time-restricted feeding in a pilocarpine-induced seizure model: metabolic and epigenetic implications. *Front. Cell. Neurosci.* **10**, 7. doi:10.3389/fncel.2016.00007

Lillycrop, K. A., Phillips, E. S., Jackson, A. A., Hanson, M. A. and Burdge, G. C. (2005). Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *J. Nutr.* **135**, 1382-1386. doi:10.1093/jn/135.6.1382

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408. doi:10.1006/meth.2001.1262

Lynch, E. W. J., Coyle, C. S. and Stevenson, T. J. (2017). Photoperiodic and ovarian steroid regulation of histone deacetylase 1, 2, and 3 in Siberian hamster (*Phodopus sungorus*) reproductive tissues. *Gen. Comp. Endocrinol.* **246**, 194-199. doi:10.1016/j.ygcen.2016.12.008

Majumdar, G., Rani, S. and Kumar, V. (2015). Hypothalamic gene switches control transitions between seasonal life history states in a night-migratory photoperiodic songbird. *Mol. Cell Endocrinol.* **399**, 110-121. doi:10.1016/j.mce.2014.09.020

Mishra, I. and Kumar, V. (2019). The quantity–quality trade-off: differential effects of daily food availability times on reproductive performance and offspring quality in diurnal zebra finches. *J. Exp. Biol.* **222**, jeb196667. doi:10.1242/jeb.196667

Mishra, I., Bhardwaj, S. K., Malik, S. and Kumar, V. (2017). Concurrent hypothalamic gene expression under acute and chronic long days: implications for initiation and maintenance of photoperiodic response in migratory songbirds. *Mol. Cell. Endocrinol.* **439**, 81-94. doi:10.1016/j.mce.2016.10.023

Mishra, I., Singh, D. and Kumar, V. (2018). Temporal expression of *c-fos* and genes coding for neuropeptides and enzymes of amino acid and amine neurotransmitter biosynthesis in retina, pineal and hypothalamus of a migratory songbird: Evidence for circadian rhythm-dependent seasonal responses. *Neuroscience* **371**, 309-324. doi:10.1016/j.neuroscience.2017.12.016

Moore, L. D., Le, T. and Fan, G. (2013). DNA methylation and its basic function. *Neuropsychopharmacology* **38**, 23-38. doi:10.1038/npp.2012.112

Pfaffl, M. W., Tichopad, A., Prgomet, C. and Neuvians, T. P., (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: bestkeeper – excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **26**, 509-515. doi:10.1023/B:BILE.0000019559.84305.47

Rubenstein, D. R., Skolnik, H., Berrio, A., Champagne, F. A., Phelps, S. and Solomon, J. (2016). Sex-specific fitness effects of unpredictable early life conditions are associated with DNA methylation in the avian glucocorticoid receptor. *Mol. Ecol.* **25**, 1714-1728. doi:10.1111/mec.13483

Sepers, B., Heuvel, K., Lindner, M., Viitaniemi, H., Husby, A. and Oers, K. (2019). Avian ecological epigenetics: pitfalls and promises. *J. Ornithol.* **160**, 1183-1203. doi:10.1007/s10336-019-01684-5

Sharma, A., Singh, D., Malik, S., Gupta, N. J., Rani, S. and Kumar, V. (2018). Difference in control between spring and autumn migration in birds: insight from seasonal changes in hypothalamic gene expression in captive buntings. *Proc. R. Soc. Lond. B Biol. Sci.* **285**, 20181531. doi:10.1098/rspb.2018.1531

Sheldon, E. L., Schrey, A. W., Ragsdale, A. K. and Griffith, S. C. (2018). Brood size influences patterns of DNA methylation in wild Zebra Finches (*Taeniopygia guttata*). *Auk* **135**, 1113-1122. doi:10.1642/AUK-18-61.1

Staib-Lasarzik, I., Kriege, O., Timaru-Kast, R., Pieter, D., Werner, C., Engelhard, K. and Thal, S. C. (2014). Anesthesia for euthanasia influences mRNA expression in healthy mice and after traumatic brain injury. *J. Neurotrauma* **31**, 1664-1671. doi:10.1089/neu.2013.3243

Stevenson, T. J. and Prendergast, B. J. (2013). Reversible DNA methylation regulates seasonal photoperiodic time measurement. *Proc. Natl Acad. Sci. USA* **110**, 16651-16656. doi:10.1073/pnas.1310643110

Verdone, L., Agricola, E., Caserta, M. and di Mauro, E. (2006). Histone acetylation in gene regulation. *Brief. Funct. Genomics* **5**, 209-221. doi:10.1093/bfgp/ell028

Vinoth, A., Thirunalasundari, T., Shanmugam, M., Uthrakumar, A., Suji, S. and Rajkumar, U. (2018). Evaluation of DNA methylation and mRNA expression of heat shock proteins in thermal manipulated chicken. *Cell Stress Chaperones* **23**, 235-252. doi:10.1007/s12192-017-0837-2

Yan, X.-P., Liu, H.-H., Liu, J.-Y., Zhang, R.-P., Wang, G.-S., Li, Q.-Q., Wang, D.-M., Li, L. and Wang, J.-W. (2015). Evidence in duck for supporting alteration of incubation temperature may have influence on methylation of genomic DNA. *Poult. Sci.* **94**, 2537-2545. doi:10.3382/ps/pev201

Zhang, Y. and Kutateladze, T. G. (2018). Diet and the epigenome. *Nat. Commun.* **9**, 3375. doi:10.1038/s41467-018-05778-1

Zheng, S. and Pan, Y.-X. (2010). Histone modifications, not DNA methylation, cause transcriptional repression of p16 (CDKN2A) in the mammary glands of offspring of protein-restricted rats. *J. Nutr. Biochem.* **22**, 567-573. doi:10.1016/j.jnutbio.2010.04.013

Zheng, S., Li, Q., Zhang, Y., Balluff, Z. and Pan, Y.-X. (2012). Histone deacetylase 3 (HDAC3) participates in the transcriptional repression of the *p16^{INK4a}* gene in mammary gland of the female rat offspring exposed to an early-life high-fat diet. *Epigenetics* **7**, 183-190. doi:10.4161/epi.7.2.18972