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Research article

A potential role for epigenetic mechanisms enabling appropriate seasonal reproductive transitions of liver yolk-precursor production

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Animals breed at times of the year that ensure offspring production and growth during favorable periods. DNA methylation is one mechanism by which expression of genes necessary for reproduction may be regulated, enabling expression only at appropriate times. Much work on seasonal breeding in vertebrates has focused on the neuroendocrine system, however oviparous vertebrates, including birds, also rely on the liver for production of yolk precursors, such as vitellogenin (VTG) that will provide the nutrients necessary for development in ovo. We hypothesized that changes in DNA methylation in the promoter for VTG2 in the liver may be one mechanism ensuring appropriately timed seasonal breeding. DNA methyltransferases (DNMTs) facilitate de novo (DNMT3a) and maintenance (DNMT1) of DNA methylation. We observed that liver expression of VTG2 was lower in birds sampled during the pre-breeding compared with the early-breeding period, and also observed changes in liver expression of DNMT1 and DNMT3a between these two periods. Contrary to our predictions, we observed an increase in methylation from pre- to early-breeding at one of three CpG sites in the promoter of the VTG2 gene, with no differences at the other two CpG sites. Finally, we asked if patterns of DNA methylation of VTG2 in liver were similar in the blood. Although we observed strong correlations between blood and liver in two sites that did not change between pre- and early-breeding, there was only a trend for a significant association between blood and liver DNA methylation at the site that displayed an increase in liver DNA methylation between sampling periods. Together, these findings suggest that changes in DNA methylation in an important tissue outside of the reproductive endocrine axis (liver) may play a critical role in appropriate timing of seasonal clutch initiation, though it is unclear if these epigenetic changes are all reflected in blood.

Keywords: epigenetic, photoperiod, seasonal breeding, vitellogenin

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Introduction

In temperate environments, reproduction is often limited to a short window of time that ensures favorable conditions for rearing offspring (Baker 1938, Lack 1968, Bronson 1989, Dawson 2002). Photoperiod (i.e. daylength) provides a reliable predictive cue that influences seasonal changes in reproductive physiology and behavior (Bronson and Heideman 1994, Dawson et al. 2001, Goldman 2001). Although annual cycles are predictable, animals must also be able to modulate the timing of reproductive activities to match year-to-year variation in environmental conditions (e.g. late arrival of spring weather in one year, and an early spring the next). While a great deal has been learned regarding the processes by which male vertebrates alter seasonal reproductive physiology, much less is known about females, even though ultimately it is female timing decisions that dictate when rearing of offspring will occur (Caro et al. 2009, 2013, Caro 2012, Schaper et al. 2012, Williams 2012, Wingfield et al. 2016, Kimmitt 2020). Thus, it is vitally important to understand the mechanisms by which seasonal environmental information interacts with an individual's genome to allow for appropriate and flexible timing of reproduction in females.

One mechanism by which the environment can influence gene expression is via epigenetic modifications, including DNA methylation, or the addition of a methyl group to the nucleic acid cytosine when situated next to guanine (i.e. CpG sites) (Jaenisch and Bird 2003). DNA methylation can have a profound influence on the likelihood of gene transcription, particularly when found in coding DNA or regulatory regions upstream of a transition start site (Jaenisch and Bird 2003). Indeed, in the hypothalamus of the brain in the seasonally breeding Siberian hamster *Phodopus sungorus*, DNA methylation of the promoter region for a gene critical for seasonal transitions in reproduction (dio3) was found to be responsive to photoperiod (Stevenson and Prendergast 2013).

To enable appropriate seasonal changes in transcription of key genes, the changes of the epigenome should be dynamic. The DNA methyltransferase (DNMT) family of enzymes (DNMT1 and DNMT3a) are capable of adding a methyl group to the cytosine in DNA to alter DNA methylation. Specifically, DNMT3a is capable of de novo methylation of DNA in vertebrates, while DNMT1 acts to maintain DNA through cell division (Schübeler 2015). In Siberian hamsters, a vertebrate model of seasonal breeding, studies have uncovered that males housed in rooms with long summer-like days have altered activity of methyltransferases in the brain and reproductive tissue compared with males housed on short winter-like days (Stevenson and Prendergast 2013, Lynch et al. 2016). This suggests that the activity of DNMT enzymes may be responsive to seasonal cues such as photoperiod.

Much of the recent work investigating the influence of seasonal cues like photoperiod on DNA methylation have been conducted on mammalian brain and gonadal tissues. In addition to brain and gonadial tissues, oviparous vertebrates like birds must also rely on the liver for production of yolk precursor proteins that will provide the nutrients necessary for developing young in ovo (Williams 2012, Johnson 2015). Thus, final maturation of follicles requires dramatic changes seasonally in liver function that enable large quantities of yolk precursor proteins to be produced and secreted into circulation for follicle uptake. One recent study in the great tit *Parus major* observed genome-wide changes in DNA methylation in liver and blood across the pre-breeding to breeding periods (Lindner et al. 2021b). However, much remains unknown about the processes influencing methylation of critical genes needed for liver production of yolk-precursors in birds. In general, regions of the genome that promote reproduction should be suppressed outside of the window of favorable breeding shaped by selection, potentially by increases in DNA methylation. Such a mechanism would ensure that transcription and downstream processes are not initiated in response to warm spring conditions when cold/freeze events are still likely to occur (Shipley et al. 2020). However, these same genomic regions, during the period of time favored by selection for reproduction, should be in an epigenetic state where transcription could be readily induced in response to favorable cues.

The liver is a critical component necessary for yolking of follicles in oviparous species. It produces lipoproteins like vitellogenin and yolk-targeted very low-density lipoproteins ($VLDL_y$), which enter circulation and are then taken up by developing follicles through specialized receptors (Williams 2012). The liver has also been implicated as a critical source of regulation of reproductive timing differences between groups of birds selected for or that naturally vary in timing of breeding (Caro et al. 2009, Kimmitt et al. 2019, Verhagen et al. 2019). Expression of the vitellogenin genes (VTG1, VTG2, and VTG3) are necessary for production of the derived lipoprotein vitellogenin (VTG) (Williams 2012). Transcript of VTG2 is the most abundant of the VTG genes in the liver, and VTG2 protein is the most abundant vitellogenin protein in chicken egg yolk (Wang et al. 2022). In poultry, the DNA methylation of the VTG2 promoter region vaies across life stages associated with different reproductive capabilities (Saluz et al. 1986, Gupta et al. 2006), making this a prime target for dynamic epigenetic changes to help enable proper timing of investment into yolking follicles. In young non-reproductive chickens, CpG sites in this promoter region are highly methylated, whereas these regions are unmethylated in adult laying hens (Saluz et al. 1986), suggesting facultative de-methylation associated with reproductive maturity. Additionally, more recent work has identified an increase in DNA methylation in the VTG2 promoter region in old quail that display reduced egg production (i.e. skip laying several days in a row; Gupta et al. 2006). These patterns across the life of poultry strongly suggest a regulatory relationship between methylation of the promoter region(s) of the VTG gene and maturation of follicles. Whether relevant seasonal information such as day length, in addition to age related information, is capable of altering epigenetic status of these DNA regions remains unknown.

While the liver is a critical site of regulation of reproduction in oviparous animals, sampling of this tissue is often

terminal, making it difficult to relate findings with reproductive timing phenotypes. Blood can be repeatedly sampled and if patterns are similar between blood and liver, then DNA collected from blood may serve as a biomarker for liver methylation state. Encouragingly, studies of the great tit observed change in blood DNA methylation across the genome related with timing of laying, however the functional significance and relationship of changes in blood methylation with reproductive physiology remains unclear (Lindner et al. 2021a). In another study comparing genome-wide methylation in the great tit, researchers observed significant correlations between changes in DNA methylation in the liver and in the blood (Lindner et al. 2021b). However, when only looking at a few candidate genes, methylation change in these candidate genes was not related with expression patterns of these genes (Lindner et al. 2021b). A study investigating the effects of developmental stress on methylation in house sparrows *Passer domesticus* observed that some genomic regions displayed similar methylation patterns between blood and brain, while methylation of other genomic regions were tissue specific (Siller Wilks et al. 2024), suggesting that methylation in blood at specific sites may serve as a marker of methylation in other tissues, while methylation in blood at other genomic regions may not be a reliable marker. These recent findings combined with further investigation of seasonal changes in methylation of key tissues and regions of the genome may provide novel insight into the mechanisms by which animals appropriately regulate seasonal timing of key life-history transitions in variable environments.

Here we test the hypothesis that female dark-eyed juncos *Junco hyemalis*, seasonally reproducing birds common to North America (Nolan et al. 2002), will upregulate expression of VTG2 in their liver as they near breeding. Further, we predict that expression of the DNMT enzymes DNMT1, DNMT3a, and DNMT3b, which promote and maintain DNA methylation, will change as the breeding season approaches. Additionally, we predict that the putative promoter region of the VTG2 gene will be differentially methylated in liver tissues collected from females several weeks prior to the expected first egg laid in the population and tissue collected at the time that the earliest females in the population were beginning to lay. Finally, we ask if DNA methylation of the VTG2 promoter in blood, which can be sampled repeatedly from the same individual, shows similar patterns to that in the liver. As birds transition from a reproductively quiescence winter state into a reproductive state, we predict that the promoter region for the VTG2 gene will display reductions in DNA methylation, and that this will be observed in both liver and blood tissue.

Material and methods

Study species and capture

We captured dark-eyed juncos passively in continuously monitored seed-baited walk-in traps on their breeding grounds in the Black Hills National Forest (44°14′35″N, 103°52′56″W) in the spring of 2016 at two time points: the "pre-breeding" stage: (25–27 April 2016, n = 14) and again later during the "early-breeding" stage, the period of time when the first known eggs were found near the sampling site (12–14 May 2016; n = 10). Other aspects of these bird's physiology have been reported previously (Needham et al. 2019). The daylength increased from 13 h 58 min (sunrise 04:55; sunset 18:53) on the first day of sampling on 26 April 2016 to 14 h 46 min (sunrise 4:29; sunset 19:15) on the last day of sampling, 14 May 2016 (sunrise sunset times obtained from https://gml.noaa.gov/grad/solcalc/sunrise.html)

Blood and tissue collection

After capture, a blood sample was collected by puncturing the alar wing vein using a sterile 26 g needle and collected into heparinized microhematocrit capillary tubes. Blood samples were kept on ice until centrifugation. Plasma was separated and collected, and the remaining red blood cells were immediately frozen at −20°C for up to one month until they were transferred back to North Dakota State University (NDSU) in Fargo, ND. Immediately after collection of the blood sample, individuals were euthanized by an overdose of isoflurane followed by rapid decapitation. Liver tissue was collected and rapidly frozen on powdered dry ice and temporarily frozen at −20°C for up to one month until they were transferred back to North Dakota State University (NDSU) in Fargo, ND. Tissue and blood were transported to NDSU on dry ice and were then transferred and stored at −80°C until RNA and DNA extraction.

RNA extraction

RNA was extracted from liver tissue using RNAzol®RT isolation reagent (Sigma Aldrich) following manufacturer's instructions. The extracts were treated with DNAse (TURBO DNAse Life Technologies) following manufacturer's guidelines. We quantified RNA concentration and purity using a Nanodrop 8000 spectrophotometer (Thermo Scientific) after DNAse treatment. We reverse transcribed 500 ng of RNA using qScript reverse transcriptase and oligo (DT) primers (Quanta Biosciences) in a total reaction volume of 5 µl. To verify that samples were not contaminated with DNA, we ran a portion of our samples without reverse transcriptase.

DNA extraction

We extracted DNA from red blood cells and liver tissue using Macherey-Nagel Nucleospin® blood and tissue kits (Macherey-Nagel) following the manufacturer's instructions. DNA concentration and purity were verified using a Nanodrop 8000 spectrophotometer.

Expression of VTG2 and DNMTs

We quantified expression of VTG2, DNMT1, DNMT3a and DNMT3b (see the Supporting information for primer sequence) on a Stratagene Mx3000P with MxPro software (ver. 4.10, Agilent). VTG2 was assessed before DNA extractions (pre-breeding n = 14; early-breeding n = 10), while DNMT1, DNMT3a and DNMT3b we analyzed from

samples with tissue remaining after pyrosequencing (pre-breeding n = 10; early-breeding n = 6). We ran reactions (10 µl) in triplicate, with 5 µl Perfecta SYBR green low ROX (Quanta Biosciences, no. 95056-100), 3 µl cDNA (diluted 1:40), and primers at a concentration of 0.3 µM. We also included no template controls on each plate to confirm that there was no contamination of the cDNA or the production of primer dimers.

We included a sample of pooled liver on every plate, and used this reference sample when calculating relative expression using the $2^{-\Delta\Delta Ct}$ method of quantification (Livak and Schmittgen 2001); the abundance of each gene of interest is expressed as the fold change expression relative to the pooled reference sample, normalized by the expression of the reference genes (PPIA and RPL4). To make the liver pool, we combined 3 µl of RNA from each liver sample, and this pool was reverse transcribed as described above. We assessed amplification efficiencies for each gene using a 5-point standard dilution curve in MxPro; efficiencies ranged from 89 to 103%. After running VTG2 there was not enough product remaining for some individuals for DNMT analysis.

Thermocycling conditions for VTG2 reactions were 10 min at 95°C, 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min. Thermocycling conditions for DNMT1, DNMT3a reaction cycling conditions were 10 min at 95°C, 50 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s. For DNMT3b, thermocycling conditions were 10 min at 95°C, 50 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Conditions for PPIA and RPL4 were 10 min at 95°C, 50 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. A final dissociation curve of 95°C for 1 min, 55°C for 30 s to 95°C for 30 s was added to each thermoprofile to confirm single-product specificity of each sample.

Pyrosequencing of VTG2 promoter

We identified the putative promoter region for the vitellogenin 2 (VTG2) gene in a draft dark-eyed junco genome and further confirmed sequence using the published dark-eyed junco reference genome as described below. First, we aligned Ensembl references for chicken VTG2 (gene_id: ENSGALG00000001863) and presumable zebra finch VTG2 sequence. We also aligned white-throated sparrow sequence against chicken VTG2 to confirm sequence is decently conserved (> 80%). We used LASZT 1.02.00 using Geneious 10.0.4 plugin wrapper, and also confirmed alignments with Mauve (Darling et al. 2004). We used the VTG2 alignments to identify the region of above junco reference that corresponded to VTG2 promoter. Then this junco sequence was used in pyrosequencing assay design. Once a new junco reference became available on Ensembl (http://useast.ensembl.org/Junco_hyemalis/Info/Annotation), we confirmed that the sequence from the draft Junco reference used for Pyrosequencing Assay Design is in fact identical to region preceding the identified junco "gene" ENSJHYG00000003478 in this new junco genome reference. This annotated "gene" aligns with chicken reference ENSGALG00000001863.

Primers were designed for a targeted region that contained 3 CpG sites similar to sites identified in the chicken that display developmental changes in DNA methylation (Saluz et al. 1986) using Pyromark Assay Design Software ver 2.0 (Qiagen). The primers were designed to amplify the following sequence 900–990 base pairs upstream from the start codon:

5′TTCACACAGGAGCTCTCTTCTAGAAGCC TCAGGGCCA**CG**GAGG**CG**AGTGGAGATGCACAA AACACTCTTGGCCTGGTTGAGTA**CG**GTGAG3′.

Forward and reverse primers and sequencing primers for pyrosequencing were designed using the with the following sequence (Table 1).

Bisulfite treatment of DNA and pyrosequencing

DNA extracted from blood and liver samples was bisulfite converted using the EpiTect Fast Bisulfite kit (Qiagen). Bisulfite treatment of DNA converts unmethylated cytosine to uracil, while methylated cytosine in not altered, enabling differentiation of methylated from unmethylated cytosines. Annealing temperatures were optimized using a gradient from 56–62°C. We tested the primers by conducting PCR on bisulfite converted DNA; products were run on a gel to confirm a single band of ~ 300 bp in size.

We performed polymerase chain reactions (PCRs) (25 µl) using the Pyromark PCR kit (Qiagen), with 12.5 µl PyroMark PCR Master Mix, 2.5 µl CoralLoad Concentrate, 0.5 µl of each primer, and 0.5 µl bisulfite-converted DNA. Amplifications were carried out on an Eppendorf PCR cycler using the following conditions: initial denaturation at 95°C for 15 min; 45 cycles of 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 2% agarose gels prior to pyrosequencing. All samples were run in duplicate and randomized across two plates; both blood and liver from the same individual were included in the same plate. Bisulfite-converted PCR products were sequenced on a Q96 sequencer by EpigenDx (Hopkinton) and analyzed using the Q96 software. Samples that were marked as failed by the Pyromark software in the initial pyrosequencing run (error message returned) were re-run. Samples that returned a 'failed' message for the quality check on the second run were excluded from further analysis. The final sample size of samples included in this analysis were pre-breeding n = 10 and early-breeding n = 8.

Statistical analyses

Because this was necessarily a terminal sampling, which limits sample size (for ethical and practical reasons), we

Table 1. VTG2 promoter region pyrosequencing primer set for bisulfite converted DNA.

Top strand _Forward	AGGAATGGGTTTGGTTAATTT
Top strand Reverse	CAACAAAAACCCAAACACACAT
Top strand Sequencing	GGGTTTGGTTAATTTTTTAGTT

designed our sampling protocol to minimize the need for covariate inclusion (e.g. sampling over a 3-day window in each sampling period). Thus, data were analyzed with linear models. A t-test was performed to ask if liver VTG2 expression changed over the three weeks between the pre-breeding and the early-breeding sampling periods; VTG2 was log transformed to meet model assumptions. Next, we asked if DNMT1, DNMT3a and DNMT3b expression in the liver differed between the pre-breeding and early-breeding season using t-tests. We also asked if the percent of methylation of the three identified CpG sites in the promoter region of VTG2 differed between the pre-breeding and early-breeding season using t-tests, and if variation in percent methylation at these sites was related with VTG2 expression using a Pearson correlation. Finally, we used Pearson correlation to ask if percent methylation at each of the three identified CpG sites in DNA from blood and liver were related. All model assumptions were assessed, and analyses were conducted using JMP Pro 17.0.

Results

Birds sampled during the pre-breeding period had significantly lower expression of liver VTG2 compared with birds sampled 3 weeks later during the early-breeding period, when females in the population were just beginning to build nests and lay, ($t_{22} = 2.25$, $p = 0.03$; Fig. 1). All individuals in the pre-breeding period had undeveloped small white follicles (< 0.1 mm), while in the early-breeding sampling period VTG2 expression was significantly related with the size of the largest follicle (see the Supporting information).

Expression of liver DNMT1 was greater during the pre-breeding compared with the early-breeding period ($t_{14} = -2.36$, $p = 0.03$; Fig. 2A), while expression of liver DNMT3a was lower during the pre-breeding compared with the early-breeding period ($t_{14} = 2.85$, $p = 0.01$; Fig. 2B). While testing of our DNMT3b primers using blood did amplify and produce product of the predicted size, our liver samples did not amplify DNMT3b, potentially because there was a splice variant that our primers did not amplify (Robertson et. al., 1999).

Our analysis of the three CpG sites in the VTG2 promoter region found no difference in methylation at site 1 ($t_{16} = -0.12$, $p = 0.90$) or site 2 ($t_{16} = 1.37$, $p = 0.19$) between pre-breeding and early-breeding stages. However, at site 3, methylation was significantly lower in the pre-breeding than in the early-breeding stage ($t_{16} = 2.40$, $p = 0.03$; Fig. 3). We did not find any association between VTG2 expression and methylation at any of the three sites (all $p > 0.05$).

DNA methylation in the blood and liver were significantly positively correlated at the 1st ($n = 17$, $R^2 = 0.96$, $p < 0.01$; Fig. 4A) and 2nd ($n = 17$, $R^2 = 0.90$, $p < 0.01$; Fig. 4B) CpG site in the VTG2 promoter. However, there was only a marginally significant relationship in DNA methylation between blood and liver at the 3rd site ($R^2 = 0.21$, $p = 0.06$; Fig. 4C).

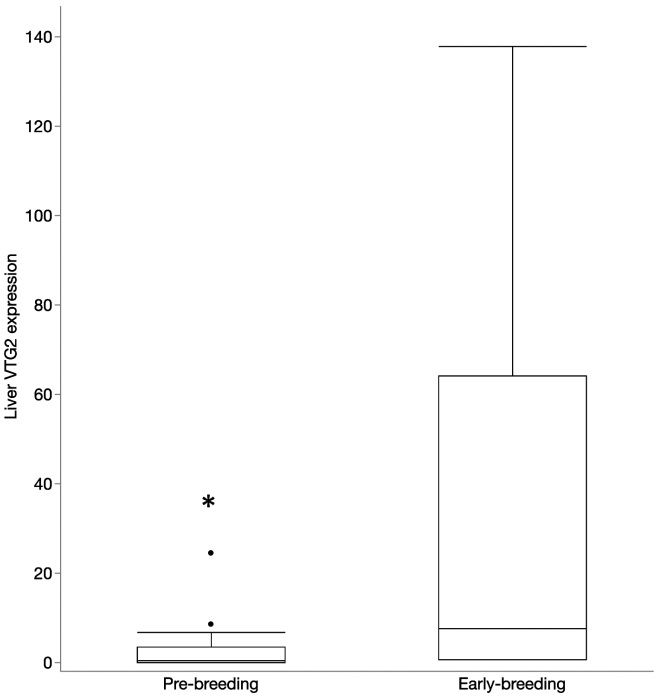

Figure 1. Liver expression of the vitellogenin gene (VTG2) was greater in birds sampled during the early-breeding period compared with the pre-breeding period. In the boxplots the median is indicated by the center line within the box, with the 25th and 75th quartiles forming the bottom and top of the box; the whiskers extend 1.5 times the inter-quartile range with outliers indicated by black dots; *indicates groups that differ significantly, $p < 0.05$.

Discussion

As predicted, liver expression of the vitellogenin gene (VTG2) was low in birds sampled during the pre-breeding period and significantly greater in individuals sampled during the early-breeding period. We also observed significant changes between these two sampling periods in liver expression of DNMT1 and DNMT3a, enzymes that maintain or add methyl groups at CpG sites. Contrary to our predictions, however, we observed reduced methylation at one of the three CpG sites in the promoter region of the VTG2 gene (site 3) in the pre-breeding period compared with greater methylation observed during early-breeding period, with no differences at the other two CpG sites. Percent methylation at these sites was not related with VTG2 expression. Below we discuss the potential implications of these data and the possible role that seasonal epigenetic changes may play in altering liver function for breeding.

Transition in yolk-precursor protein

Production of yolk-precursor proteins produced by the liver is a critical and necessary step for follicular maturation in birds (Williams 2012). We investigated expression of liver VTG2, the most abundant vitellogenin found in chicken yolk (Wang et al. 2022). We observed significantly greater VTG2 expression in liver samples collected from females during the

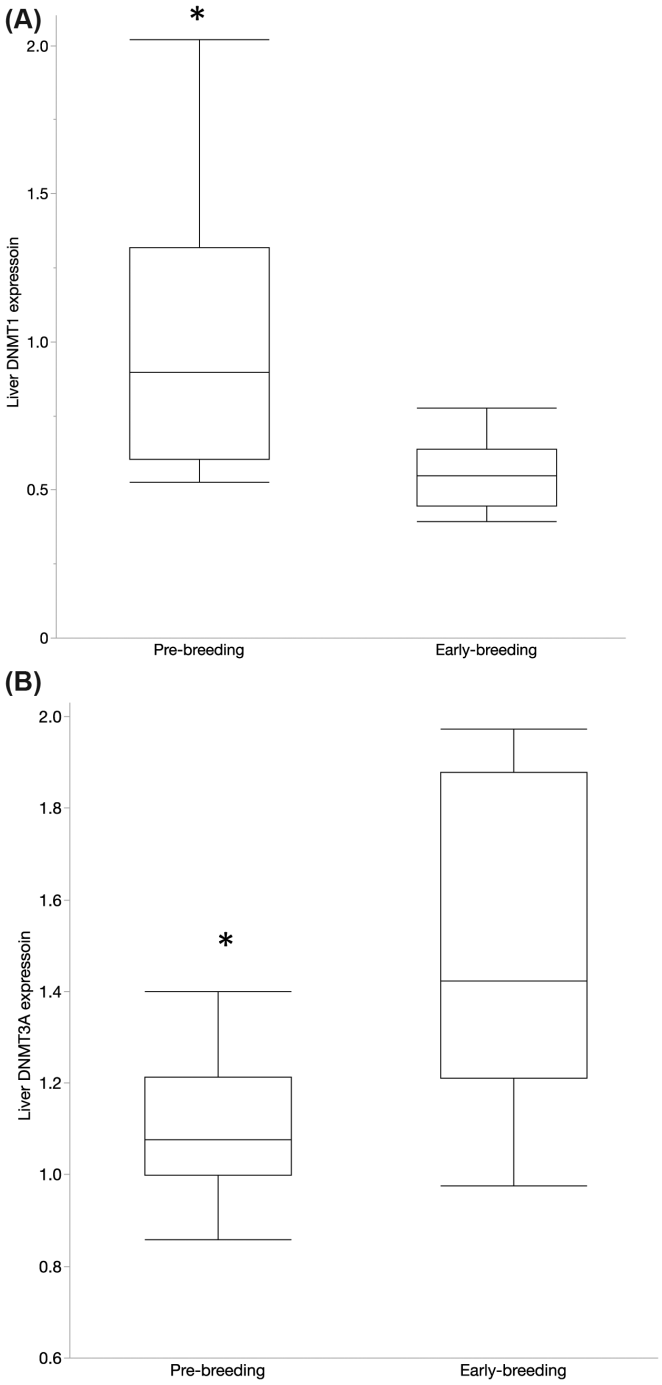

Figure 2. Liver expression of two methyltransferase genes: DNMT1 and DNMT3a. Birds sampled during the pre-breeding period had significantly greater expression of DNMT1 compared with birds sampled during the early-breeding period (A), while liver expression of DNMT3a was greater in birds sampled during the early-breeding compared with the pre-breeding period (B). In the boxplots the median is indicated by the center line within the box, with the 25th and 75th quartiles forming the bottom and top of the box; the whiskers extend 1.5 times the inter-quartile range with outliers; *indicates groups that differ significantly, $p < 0.05$.

early-breeding season when the earliest females had started to lay in the population, compared with expression observed in females sampled several weeks prior to laying in the population (pre-breeding). Interestingly, we also observed a positive relationship between liver VTG2 expression and follicle size in females sampled during the early-breeding period; i.e. females with greater VTG2 expression had larger F1 follicles (Supporting information); all individuals sampled during the earlier pre-breeding period had undeveloped small white follicles. We have also reported previously that these

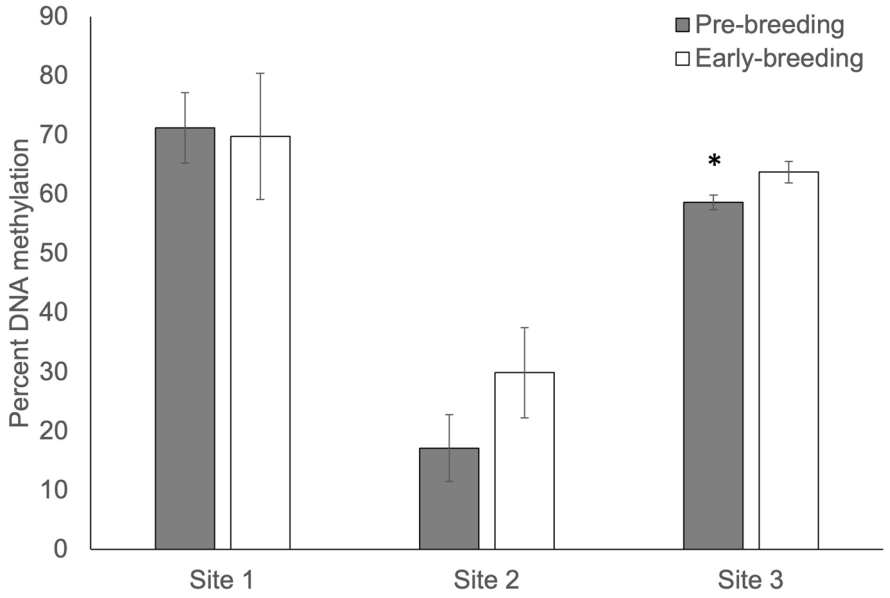

Figure 3. Percent DNA methylation in liver tissue of each of three CpG sites located 900–990 bp upstream from the start codon of VTG2 from individuals sampled during the pre-breeding or during the early-breeding period. There were no differences in DNA methylation at CpG site 1 or site 2, however there was a significant increase in DNA methylation at site 3 in the birds sampled during the early-breeding compared with those sampled during the pre-breeding period. Bars are means ± SEMs. *indicates groups that differ significantly, $p < 0.05$.

same early-breeding females had higher circulating levels of very-low-density lipoprotein (VLDL), another key yolk-precursor, compared with females sampled several weeks prior (Needham et al. 2019). Together, these findings demonstrate significant changes in the liver over this fairly short timeframe (~ 3 weeks), with increases in products for yolk-development as females transition into follicular maturation necessary for breeding. It may be possible that these changes in liver production of yolk-precursor proteins are facilitated by epigenetic modifications enabling changes in expression of genes relevant for production of these proteins.

Changes in DNA methyltransferase expression

We observed changes in liver DNMT1 and DNMT3a expression during the transition from pre-breeding to the early-breeding period. DNA methyltransferase genes have been observed to vary in response to seasonal cues such as photoperiod in other animals (below), and have been suggested to play an important role in regulating seasonal changes in physiology and behavior. For example, knockdown of DNMT1 and DNMT3 altered the ability of Nasonia wasps to use photoperiod as a cue for appropriate timing of diapause induction (Pegoraro et al. 2016).

We observed greater expression of DNMT3a, an enzyme capable of de-novo DNA methylation, in the liver of females closer to breeding compared with females further from breeding. In a mammalian model of seasonal breeding, the Siberian hamster, DNMT3a expression was found to vary in reproductive tissues in response to photoperiod manipulations. Researchers not only documented greater expression of DNMT3a in testes and uterine tissue in hamsters held in reproductively inhibitory short-days, but that an injection of estrogen and progesterone in ovariectomized females reduced expression of this transcript, suggesting that expression of this gene may be responsive to reproductive steroid hormones produced by the ovary (Lynch et al. 2016). Interestingly, the pattern of expression of DNMT3a in the hypothalamus displayed the opposite pattern of expression to the ovary, with greater expression in this area of the brain in females held under reproductively stimulatory long days and reduced expression under inhibitory short days (Stevenson 2017, Tolla and Stevenson 2020). In a non-breeding seasonal context (i.e. during migration), DNMT3a also varied within the brain between spring and autumn in a songbird (Sharma et al. 2018). This suggests that while both seasonally breeding hamsters and songbirds alter expression of this gene in a seasonal context, the patterns of expression and response to photoperiod (or a photoperiod-related signal) varies by tissue type.

Opposite to the pattern observed for DNMT3a, expression of DNMT1, which maintains methylation of DNA through cell division (Schübeler 2015), was significantly reduced in liver of birds closer to breeding during the early-breeding sampling period versus females that were sampled during the pre-breeding period, several weeks before the earliest females laid in the population. Hypothalamic DNMT1 in Siberian hamsters is reduced under reproductively inhibitory short-days in the hypothalamus (Stevenson and Prendergast 2013), suggesting that this enzyme may play an important role in photoperiod-induced seasonal changes. Our results appear to be the first in vertebrates showing rapid changes in DNMT1 associated with a seasonal transition in tissue outside of the brain.

The methyltransferase gene DNMT3b did not amplify in our RNA extracts from liver. Prior to processing our liver

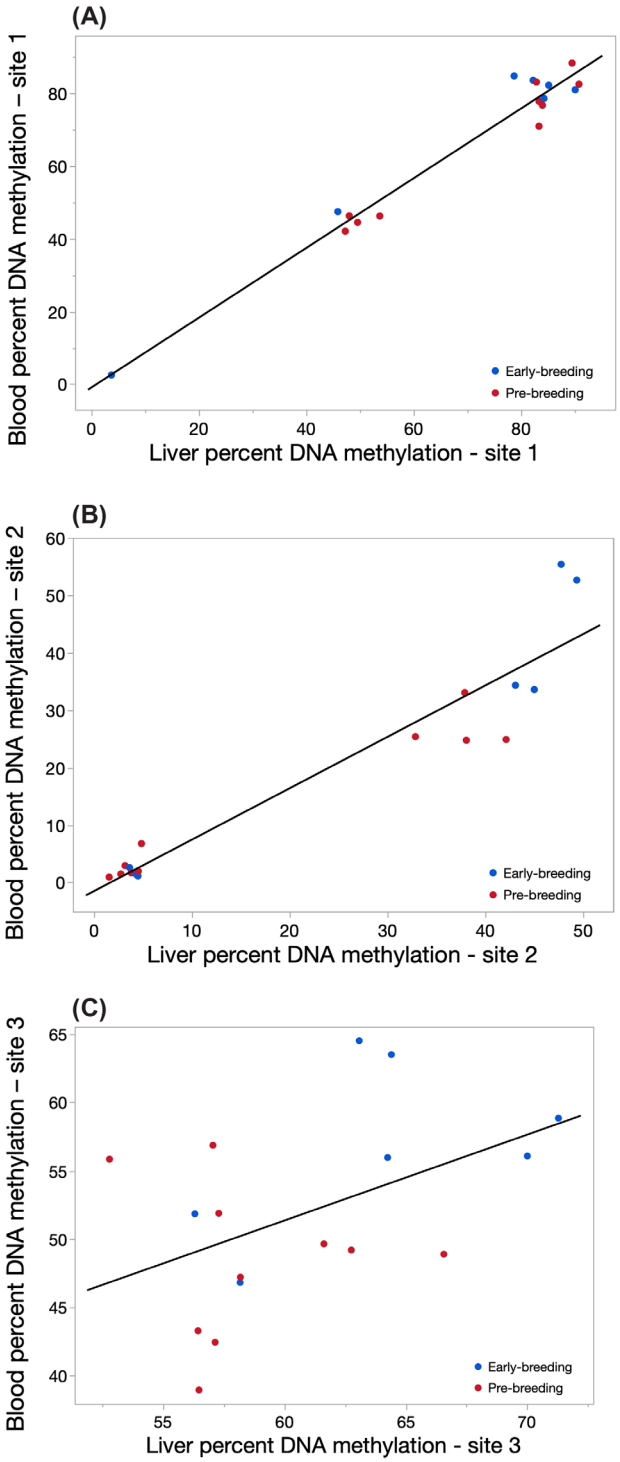

Figure 4. Correlation between liver and blood the percent DNA methylation at each of the three CpG sites located 900–990 bp upstream from the start codon of VTG2. Individuals sampled during the pre-breeding season are in blue and individuals sampled during early-breeding are in red. DNA methylation at the first (A) and second (B) CpG site in blood and liver tissue were statistically correlated, displaying a strong positive correlation. The third (C) CpG site also showed a positive relationship between DNA methylation of blood and liver tissue, however this relationship was not significant.

samples, we tested primers on DNA extracted from red blood cells to verify primer specificity. DNMT3b and other DNMT gene products are known to be subject to alternative splicing that vary by tissue (Lin et al. 2000, Xu et al. 2002). Thus, a splice variant of this gene product may be one reason that the primers that we designed amplified this product in our blood-derived samples, but not in our liver samples.

Modification of site-specific DNA methylation

The changes in DNMT expression profiles across the transition into breeding suggest the strong potential for epigenetic modifications in the liver relevant to the transition into the breeding stage. We investigated for potential changes in methylation in the promoter region of VTG2, whose gene product vitellogenin is necessary for proper yolking of developing follicles. We identified three CpG sites in this region 900–990 basepairs upstream from the start codon. Contrary to our prediction, we observed an increase in methylation at one CpG site (site 3) in the promoter region and no differences at two other CpG sites (sites 1 and 2). A post-hoc power analysis revealed that we may have been underpowered to detect a potential difference between sampling periods at liver site 2; the power analysis suggested we would need a sample size of 38 in each sampling period to detect a difference at site 2. However, the power analysis suggested that the lack of difference between treatments at site 1 are likely real; we would have needed a sample size of greater than 4500 per sampling period to detect a difference at site 1. While the increase in methylation at site 3 was unexpected, this increase in methylation could be the result of increased expression of DNMT3a observed during this sampling period; DNMT3a is capable of de novo DNA methylation. However, while there was a significant increase in methylation at CpG site 3, the biological significance of this should be more clearly tested, as the actual difference between both groups in percent methylation at this site was low. Unexpectedly, both VTG2 expression and DNA methylation at site 3 in this upstream region from VTG2 were elevated, opposite of what we had predicted if DNA methylation inhibits transcription. However, we did not observe a statistically significant relationship between DNA methylation at this site (or the other two CpG sites) and VTG2 expression. While the region we targeted was based off previous work in poultry that observed changes in DNA methylation associated with age-related changes in reproduction in poultry (Saluz et al. 1986, Gupta et al. 2006), this may not be a critical region regulated directly or indirectly by estrogen in this, or other songbirds. Indeed, we do not observe canonical estrogen response elements in this region. We did, however, identify another region 527 bp downstream from the site that we sampled for pyrosequencing that does in fact contain an estrogen response element (ERE) half-site motif (368 bp from start codon). However, no CpG sites were observed close to this ERE.

Although we only targeted VTG2, a gene needed for yolk production in oviparous females, there are likely other important targets in the liver for epigenetic modification. In addition to VTG proteins, the developing follicle also

incorporates $VLDL_y$, a liver product that is also upregulated in response to estrogen (Vézina et al. 2003). Production of VLDLs, including triglycerides, a key component of VLDL, are regulated by a suite of genes (Borén et al. 2022) that could be targets of future investigations. VTG2 as well as other genes necessary for formation of yolk-target lipoproteins are estrogen responsive. Future work should investigate possible changes in methylation of the ESR1, the estrogen-receptor alpha gene, or potential methylation of the estrogen-receptor DNA response elements. Indeed, a human study investigating genome-wide changes in methylation in white blood cells across puberty found that the majority of differentially methylated regions across this period of reproductive development were associated with nearby estrogen response elements (EREs;Thompson et al. 2018). Thus, it may be fruitful for future studies to identify and investigate CpG sites in gene regulatory regions that are in close proximity to EREs.

Potential seasonal cues altering DNA methylation

While it is possible that these epigenetic modifications are a direct result of ovarian steroid signaling (e.g. estrogens; Lynch et al. 2016), they may also be responsive to seasonally relevant environmental signals like photoperiod, a topic that we explore further below. Expression of DNMT enzymes have been observed to be responsive to photoperiod in several tissues including the brain, ovary and testis of the seasonally breeding Siberian hamster (Stevenson and Prendergast 2013, Lynch et al. 2016, Stevenson 2017). Photoperiod-induced seasonal transitions into migration have also been associated with DNMT expression changes in the brain of birds (Sharma et al. 2018). Our birds were sampled in the wild under naturally changing photoperiods, and daylength increased by ~ 50 min between the pre-breeding and early-breeding sample collection periods. Thus, similar to photoperiod effects on changes in DNMT expression in mammalian reproductive tissues, photoperiod signals may also influence expression of these genes in the liver, an organ necessary for successful reproduction in oviparous vertebrates like birds.

The hormone melatonin acts as a signal of daylength to distant tissues. A study of great tit females implanted with melatonin prior to the breeding season observed delayed laying (Greives et al. 2012). The liver produces yolk precursor proteins necessary for follicular maturation, and avian liver tissue expresses abundant melatonin receptors (Jones et al. 2012) suggesting a possible role for melatonin's actions on liver leading to delayed laying, despite a general consensus based on studies of males that melatonin does not play a role in photoperiodic seasonal breeding in birds (Dawson 2002). Melatonin has been shown to alter DNA methylation in a non-seasonal context in mammals (Davoodvandi et al. 2022) and has also been implicated in mammalian photoperiodic changes in DNMT expression in different tissues (Stevenson and Prendergast 2013, Lynch et al. 2016). Thus, it is possible that melatonin induced changes in expression of DNMTs influences yolk precursor production, though the exact mechanisms for melatonin-induced delay has yet to be clarified. Future work will be needed to clarify the potential role melatonin may play in avian photoperiodic change in DNMT expression.

Temperature may also regulate activity of these enzymes. Hypothalamic DNMT3b expression was different under cool and warm temperatures in redhead buntings *Emberiza bruniceps* transferred to a long photoperiod for one week, but this temperature effect was gone after 2.5 weeks at long photoperiod (Trivedi et al. 2019). In our study, temperature did increase between these sampling periods, with daily highs increasing more dramatically than the daily lows measured in the nearby town of Lead, SD (pre-breeding max = 5.0°C, early-breeding max = 18.0°C; pre-breeding min = −1.1°C, early-breeding min = −0.4°C) (NOAA Climate Data Online). Future work will be needed to better understand if and how the environmental changes such as photoperiod and temperature influence epigenetic processes.

The quantity, type, or timing of availability of food may be additional cues that varied between our sampling periods. Food is a supplemental cue that may act to fine tune the timing of final ovarian maturation and laying within a given photoperiodic window (Dawson 2008). In zebra finches, the timing of food availability altered liver DNMT1 and DNMT3b expression (Mishra et al. 2020). Urban breeding great tits have a significantly different genome wide liver methylation pattern compared with rural counterparts, and anthropogenic food resources were identified as one of the primary drivers of these differences (Watson et al. 2021). Other cues, such as social cues like male song have also been related to variation in seasonal clutch initiation (Perfito et al. 2015), though it is not clear if these types of cues influence liver epigenetic modifications and/or directly regulate production of lipids needed for follicular maturation.

Blood and liver tissue sample comparison

Blood is a tissue that can be non-terminally and repeatedly sampled from the same individual over time, making it an ideal tissue to sample if it also reflects epigenetic status in tissues regulating seasonal breeding. This would enable researchers to relate variation in DNA methylation with clutch initiation and potentially identify critical mechanisms responsible for observed variation in seasonal reproductive timing. Changes in DNA methylation have been observed in the blood related with seasonal timing of reproduction in the great tit (Lindner et al. 2021a), and when investigated across the genome there was a general positive correlation between differences in methylation in liver and blood from paired individuals sampled at different time points relative to onset of breeding (Lindner et al. 2021b). However, there was a substantial number of sites that showed tissue independence. Yet, a study in European starlings *Sturnus vulgaris* investigating correlations of DNA methylation between blood and different regions of the brain did not find correlations between these two tissues (Siller and Rubenstein 2019). Thus, whether the changes in blood samples related with seasonal timing are indicative of changes affecting functionally important genes such as VTG2 in non-blood tissues is unknown. We found that when comparing three CpG sites in the promoter

region of the VTG2 gene, the two sites that did not change across sampling periods in the liver tissue (CpG sites 1 and 2) were strongly correlated between blood and liver, but there was only a marginally significant association between blood and liver methylation at the site that displayed a significant increase in liver CpG methylation between sampling time periods (CpG site 3). These findings suggest that observed strong correlations between blood and liver could reflect CpG sites that do not vary temporally, while sites that do vary temporally might not be correlated across tissues. Alternatively, it should be noted that the range of percent methylation at site 3 is much smaller in both blood and liver than the range of percent methylation at sites 1 and 2. This decreased variation may also make it more difficult to statistically find relationships between these two tissues. Together, these findings, combined with studies in mammals that find tissue-specific methylation patterns (Gama-Sosa et al. 1983, Cui et al. 2020) suggest that it may not always be possible to infer changes in DNA methylation at potentially important loci from a sample from a different tissue (e.g. blood), and extrapolation of methylation found in one tissue on other tissues and tissue function should be avoided without validation.

Conclusions and future directions

The significant changes over the course of ~ 3 weeks in the early spring in liver expression of VTG2, a gene necessary for successful reproduction in birds, as well as significant changes in DNMTs suggest that rapid changes in DNA methylation may play an important role in regulating appropriate timing of reproduction, and that photoperiod may play a role in regulating these changes. Unfortunately, sampling of liver in small wild birds is terminal, making it difficult to draw direct relationships with expression of DNMT, DNA methylation, and clutch initiation. Future experimental work using manipulations known to alter reproductive timing may be one approach to help determine the direct role of DNMT expression and DNA methylation in liver on reproductive timing. This study sampled birds over a period where there were substantial changes in photoperiod. A previous study in wild great tits found that birds implanted with melatonin, a hormone directly related with photoperiod, delayed clutch initiation (Greives et al. 2012). Future would could experimentally examine the effect of melatonin manipulation on DNMT expression and DNA methylation in the liver to more directly potential epigenetic effects on genes critical for yolk production (e.g. production of triglycerides). Future studies should use a whole or reduced-representation genome approach to identify if changes in DNA methylation of other genes with an important role in transitioning the liver function for yolk-precursor production are altered.

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Author contributions

Timothy J. Greives: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (lead); Methodology (equal); Project administration (lead); Writing – original draft (lead); Writing – review and editing (equal). **Joseph Solomon**: Formal analysis (equal); Methodology (equal); Writing – review and editing (equal). **Stefanie Siller Wilks**: Formal analysis (equal); Methodology (equal); Writing – review and editing (equal). **Holland Galante**: Investigation (equal); Methodology (equal); Writing – review and editing (equal); **Katie B. Needham**: Conceptualization (supporting); Data curation (equal); Investigation (equal); Methodology (equal); Writing – review and editing (equal). **Jeff Kittilson**: Formal analysis (supporting); Methodology (equal); Writing – review and editing (equal). **Dustin R. Rubenstein**: Conceptualization (equal); Funding acquisition (supporting); Resources (equal); Writing – review and editing (equal).

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Data availability statement

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.9zw3r22rw (Greives et al. 2025).

Supporting information

The Supporting information associated with this article is available with the online version.

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