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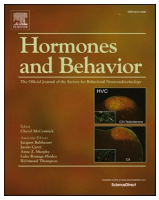

Pre-natal exposure to glucocorticoids causes changes in developmental circadian clock gene expression and post-natal behaviour in the Japanese quail

Jessica Harvey-Carroll [a,b,*], Tyler J. Stevenson [c], Luc F. Bussière [b], Karen A. Spencer [a]

[a] School of Psychology and Neuroscience, University of St Andrews, Scotland
[b] Department of Biological and Environmental Sciences & Gothenburg Global Biodiversity Centre, University of Gothenburg, Sweden
[c] Institute of Biodiversity, Animal Health & Comparative Medicine, University of Glasgow, United Kingdom of Great Britain and Northern Ireland

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ABSTRACT

The embryonic environment is critical in shaping developmental trajectories and consequently post-natal phenotypes. Exposure to elevated stress hormones during this developmental stage is known to alter a variety of post-natal phenotypic traits, and it has been suggested that pre-natal stress can have long term effects on the circadian rhythm of glucocorticoid hormone production. Despite the importance of the circadian system, the potential impact of developmental glucocorticoid exposure on circadian clock genes, has not yet been fully explored. Here, we showed that pre-natal exposure to corticosterone (CORT, a key glucocorticoid) resulted in a significant upregulation of two key hypothalamic circadian clock genes during the embryonic period in the Japanese quail (*Coturnix japonica*). Altered expression was still present 10 days into post-natal life for both genes, but then disappeared by post-natal day 28. At post-natal day 28, however, diel rhythms of eating and resting were influenced by exposure to pre-natal CORT. Males exposed to pre-natal CORT featured an earlier acrophase, alongside spending a higher proportion of time feeding. Females exposed to pre-natal CORT featured a less pronounced shift in acrophase and spent less time eating. Both males and females exposed to pre-natal CORT spent less time inactive during the day. Pre-natal CORT males appeared to feature a delay in peak activity levels. Our novel data suggest that these circadian clock genes and aspects of diurnal behaviours are highly susceptible to glucocorticoid disruption during embryonic development, and these effects are persistent across developmental stages, at least into early post-natal life.

1. Introduction

Embryonic development is a dynamic process that can give rise to multiple post-natal phenotypes. The environment within which the embryo develops, combined with gene interactions are the major drivers of these phenotypic responses (Kapoor et al., 2006; Maccari and Morley-Fletcher, 2007). One major neuroendocrine axis that links an individual to the environment, even during development, is the Hypothalamic-Pituitary-Adrenal (HPA) or stress axis (Matthews and McGowan, 2019). The importance of this system is highlighted in the ability of the organism to respond to environmental challenges appropriately, maximising survival. The HPA axis produces glucocorticoids (GC's), such as corticosterone (CORT), acting on a range of target tissues and physiological systems, resulting in a co-ordinated stress response (Cockrem, 2007; Romero and Gormally, 2019; Wingfield, 1994). Exposure to elevated GC's during pre-natal development has been shown to have pleiotropic effects on subsequent phenotypes, with individuals often showing persistent effects across life stages in a range of physiological and behavioural traits (Henriksen et al., 2011; McGowan and Matthews, 2018; Spencer, 2017; Spencer et al., 2009). One system that has been less explored in terms of the effects of developmental stress is the mechanism underlying the circadian rhythm.

Circadian rhythms are critical for survival, regulating the coordination of behaviours in response to environmental cues (Hastings et al., 2018). The circadian system serves to ensure internal continuous rhythms are phased at appropriate times for the environment (Cassone, 2014; Spoelstra et al., 2016). This is best exemplified by the changes observed in reproductive organ functioning and size as photoperiods

* Corresponding author at: School of Psychology and Neuroscience, University of St Andrews, Scotland.
E-mail address: jessica.harveycarroll@gmail.com (J. Harvey-Carroll).

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vary across seasons (Follett and Sharp, 1969; Nakao et al., 2008).

The circadian system consists of connected clocks present in nearly all tissues throughout the body. Central clocks, such as that found in the suprachiasmatic nucleus, are capable of entraining periphery clocks which are organised hierarchy and are capable of cross-modulation (Carneiro and Araujo, 2012; Stokkan et al., 2001; Zhang et al., 2021). Entrainment of peripheral clocks results in synchronous internal rhythms (such as metabolic rate, DNA transcription) being expressed concurrently to the environmental rhythms (Reppert and Weaver, 2002). This ability to synchronize rhythms is mediated by a feedback network of genes, which cycles over the course of approximately 24 h. The core mechanism of the molecular clock can be divided into activators and repressors which form transcriptional and translational feedback loops resulting in a cyclic expression of genes and encoded proteins (Partch et al., 2014). Activators dimerise following translation in the cytoplasm and re-enter the nucleus as proteins. The dimers then initiate transcription of repressors. Translated repressors then re-enter the nucleus and inhibit their own transcription through interference with activator dimers. Numerous other gene networks interact and mediate the core network. The core gene network interacts with auxiliary loops to then control gene expression of downstream targets (Cassone, 2014; Cox and Takahashi, 2019; Takahashi, 2016). Amongst the main genes controlling the central circadian network are *Bmal* (Brain and Muscle Arnt-like proteins) and *Per* (Period) family genes. These exist in multiple paralogs, with the most prominent being *Bmal1* and *Per2*. *Bmal1* acts as an activator, stimulating the expression of *Per2* proteins. *Per2* acts as a repressor, in turn promoting Rev-erbs to repress *Bmal1*, and resulting in a 24-h cyclic expression of the genes. This rhythmic onset can be entrained by a variety of stimuli such as light and food (Duong et al., 2011; Shi et al., 2010).

There is significant evidence for direct interaction between this gene system and the stress axis (Astiz and Oster, 2018; Yun et al., 2020). From a systems level it is known that GC's play an important role in entraining the circadian system. GC's themselves feature circadian fluctuations in expression, and are generally highest immediately prior to awakening, and then gradually decrease until the next period of awakening (Oster et al., 2016; Pruessner et al., 1997). Furthermore, chronic and sub-acute post-natal physical and social stress have been demonstrated to cause a phase advance in PER2 adrenal and pituitary clock rhythms of adult mice, alongside tissue specific peripheral circadian clock shifts. Despite the profound effect on clock gene expression, no overt differences in overall activity levels were found (Razzoli et al., 2014; Tahara et al., 2015; Yamamoto et al., 2005).

Evidence of clock-HPA interactions at a molecular level is rapidly increasing. GC directly interact with circadian clock genes. Core circadian clock genes, primarily PER paralogs, feature a GC response element (GRE) in the promotor region, facilitating direct GR induced expression of circadian genes (Cheon et al., 2013; So et al., 2009; Yamamoto et al., 2005). Additionally through binding to circadian clock dimers, GR's have been found capable of altering downstream circadian gene expression, for example, GR's can suppress transcription of Rev-erbα through binding to BMAL-CLOCK complexes. The GR-BMAL-CLOCK complexes bind to *E*-Boxes in the promotor of the Rev-erbα gene, reducing expression. This has been proposed as a mechanism of GC phase-resetting of the clock (Murayama et al., 2019). In addition to circadian clock genes being modulated by the HPA system, the HPA axis can, in turn, be modulated via interactions with circadian clock genes. The repressors CRY1/2 are capable of preventing GC dependent activation of GR, indicating a general role of CRY1/2 in inhibition of steroid hormone synthesis (Lamia et al., 2011). Furthermore an activator, CLOCK, has been demonstrated to suppress binding of GR to DNA recognition sequences, reducing GC-responsive gene expression (Charmandari et al., 2011; Nader et al., 2009). The use of GC's for both the circadian and HPA system are thought to be beneficial for functional coordination of the two systems (Spencer et al., 2018).

Whilst most work has been carried out in adult animals exploring these interactions, there is also evidence for an important role for the HPA axis in mammalian circadian clock development. For example in rodents, following chronic maternal restraint stress male offspring displayed a phase advance of their active period (Morley-Fletcher et al., 2019). In addition to diurnal activity patterns, exposure to pre-natal stress in rodents caused an advanced onset of the circadian CORT rhythm in post-natal life (Koehl et al., 1997, 1999). More recently, further rodent work on pre-natal stress has shown that the expression of several circadian clock genes may also be altered into post-natal life (Morley-Fletcher et al., 2019). As with in utero development, embryos *in ovo* can be exposed to increased levels of CORT following chronic stress of the mother (Henriksen et al., 2011). Transgenerational effects of CORT on the circadian system have been found in Japanese quail. Exposure to pre-natal CORT has been found sufficient to alter the development of offspring circadian genes (Harvey-Carroll et al., 2023). Taken together with the mammalian studies, these results suggest that the circadian clock system is highly susceptible to stress disruption during pre-natal development, and that stress could program persistent changes in how that system functions. However, we still have no data which links pre-natal GC exposure to circadian gene expression during the latter half of embryonic development and early post-natal life to ecologically relevant behaviour in an oviparous species.

In this study we investigated the effects of exposure to physiologically relevant levels of CORT, a key hormone involved in stress, on the embryonic development, and post-natal expression of circadian clock genes using a well-established comparative model of circadian neurobiology, the Japanese quail *(Coturnix japonica)* (Underwood and Siopes, 1984). We also investigated changes to early post-natal diurnal rhythms following pre-natal CORT exposure.

2. Methods

All procedures and housing of animals complied with the local ethics committee at the University of St. Andrews and in accordance with the Animals (Scientific Procedures) Act 1986 ASPA regulations under PIL IE1CF3B75 held by JLH and PPL 70/8159 and PAF9F705D held by KAS. For Experiment 2, ethical approval from School of Psychology & Neuroscience Ethics Committee was obtained (SEC approval code 185).

2.1. Pre-natal clock gene expression timepoints

163 fertile Japanese quail eggs (supplier Moonridge farm, Exeter, UK) were incubated in batches (Supplementary Table 1) (Ova-Easy 190A, Brinsea Products Ltd., UK) in complete darkness (with material covering the glass incubator front to prevent external light interference) whilst on a rotating platform at 37.4 °C with 60 % humidity. Fertility was confirmed at day 5 of incubation via an egg candling torch. Hens with elevated plasma corticosterone (CORT) levels deposit increased levels of CORT in eggs during formation (Henriksen et al., 2011). Fertile eggs were injected at day 5 at the apex under sterile conditions with 10 μl of 850 ng/ml CORT prepared in sterile peanut oil (Sigma Aldrich, Poole, UK). Metabolism of maternally deposited CORT has been demonstrated to occur at approximately day 6 of development (Vassallo et al., 2014). The total dose administered was 8.5 ng, as per a previous study. This concentration increases egg yolk concentration to within 1.8 SD of a physiologically relevant dose (Zimmer et al., 2013). Control eggs were injected with 10 l sterile peanut oil. Needle punctures were sealed (Germolene New Skin, UK), eggs were labelled with their treatment, and returned to the incubator within 30 min of being removed. Hypothalamus tissue was collected from incubating eggs at three prenatal developmental stages at three different times (Supplementary Table 2); embryonic day (e) 11 (CORT $n = 36$, control $n = 28$), e14 (CORT n = 28, control $n = 27$), e17 (CORT n = 27, control $n = 25$). Embryos were kept in the dark until immediately prior to decapitation. Hatching would have occurred at day 18.

2.2. Post-natal clock gene expression timepoints

For post-natal day 10 timepoints, 33 different Japanese quail eggs were incubated as above. At day 5 of incubation fertility was checked and eggs were injected with identical treatments to Experiment 1 (control $n = 19$, CORT $n = 14$). 4 days Prior to hatching, the eggs from the two treatments were placed in separate labelled hatchers and egg turning was ceased (Janoel24 incubator, 80 % humidity). Hatched chicks remained in the hatcher until feathers were dry, a period of approximately 24 h. Chicks (control n = 19, CORT $n = 14$) were then moved into treatment specific pens (floor area $1m^2$) with ad libitum food (minced Turkey crumb, BOCM, UK), water and an electric contact brooder (Comfort chicks and hatchers, UK). Chicks were kept on a 12:12 light cycle at all times. Chicks were sacrificed via intraperitoneal injection of Pentobarbital at post-natal day 10 at either Zeitgeber time (ZT) 1 (1 h after lights on, CORT $n = 6$, Control $n = 10$) or ZT10 (2 h before lights off, CORT = 8, control $n = 9$). The hypothalamus was visually identified and dissected from the main brain and then flash frozen immediately on dry ice and stored at -80 °C until further analysis.

For Post-natal day 28, 43 different fertile Japanese quail eggs were incubated in an identical manner. Eggs were injected at day 5 as above (control $n = 16$, CORT $n = 27$). Prior to hatching, eggs were split across 4 rooms with staggered 12:12 light cycles. The hatchers were covered to ensure no light exposure. Light cycles in each room were staggered by 1 h to allow dissection for all timepoints to occur within one hour. Hatched chicks (ZT1 control $n = 7$, CORT n = 14, ZT10 control n = 9, CORT $n = 13$) remained in the hatcher until feathers were dry, a period of approximately 24 h. Chicks were moved across to treatment specific pens (floor area 1 m2) in batches, to ensure CORT and control chicks spent equal amounts of time in the hatcher. Chicks were provided with ad libitum food (minced Turkey crumb, BOCM, UK), water and an electric contact brooder (Comfort chicks and hatchers, UK). Chicks were kept on a 12:12 light cycle at all times. At three days old, chicks were moved into larger pens. The pens were designed to allow video recording of all individuals so chicks were kept in a smaller area under licence (W 50 cm x L 123 cm H 48 cm). Chicks were provided with ad libitum food (chick crumb, Dodson & Horrell Ltd., UK) and water. For AM rooms chicks were kept at a density of 3 (n = 2) and 4 ($n = 11$ pens) individuals. The different densities were due to unequal sample sizes. For PM rooms chicks were divided evenly between pens, with 4 chicks in each. One electric contact brooders were provided for each pen, alongside ad libitum food and water. At post-natal day 28, chicks were sacrificed at ZT1 and ZT10 as above and the hypothalamus was immediately removed and flash frozen.

2.3. RNA extraction and qPCR

Hypothalamic RNA was extracted via Absolutely RNA miniprep kit (Agilent, UK) as per manufacturer's instructions. RNA concentrations for all samples were analysed on a QuBit 2.0 fluorometer using RNA HS Assay Kit (Thermofisher, UK). A subset of 57 samples were analysed for integrity using a 2100 BioAnalyzer system, RNA 6000 nanokit (Agilent, UK). The Average RIN value was 7.8. 79 % of samples had a RIN of 7 or above. The lowest RIN was 1, and the highest RIN was 10.

25 ng of cDNA was then synthesised (Nanoscript2 reverse transcription kits, primer design, UK). qPCR analysis was then performed using the synthesised cDNA. Specific primers were designed and validated (PrimerDesign .ltd, UK), amplifying single products only for *Bmal*1- the major positive regulator of the clock system and *Per2* (Table 1).

2.4. Behavioural recordings

To record behaviour of individual quail, infrared reflective tape (IFM electronic Reflective Tape, RS components, UK) was cut into individual shapes and applied to the chicks' back feathers using nail polish. Browning Spec Ops Advantage camera traps (NatureSpy, UK) were mounted directly above pens. Camera attachment was checked, and batteries were changed once per day. The quail were recorded for 2 consecutive days, from post-natal day 22 to post-natal day 24. 5 min of video footage were watched every 30 min for 48 consecutive hours (CORT $n = 15$, Control n = 15). The amount of time (in seconds) spent performing predetermined behaviours (Supplementary Table 3) was recorded for each quail. It was presumed if there was no video at a specific timepoint the quail were inactive (or all under the hen) and failed to trigger the camera trap. The absence of a video within the bin was classed as 'inactive'. Time spent inactive and under the hen were combined for analysis.

On three occasions during the 48 h the room lights turned off once the camera had been triggered and was filming. The infrared lights cannot become active after the camera trap has been triggered and filming has already begun. Because of this, on the three occasions the infrared lights were not active during recording, quail could not be seen. The seconds which IR lights were inactive were excluded from analysis. The camera attachments for one control cage ($n = 4$) slipped 42 h into recording. A section of the cage was obscured so this data (6 h) was excluded as the trigger setting on the camera was not a reliable indicator of activity. An hour was excluded for husbandry disturbances. Each disturbance in the rooms lasted between 20 and 30 mins. 30 min was left for the quail to resume normal behaviour. The final datasets after excluding husbandry checks and camera checks were 41, 38, 41 and 41.5 h for rooms 1–4 respectively.

2.5. Data analysis

All statistical analyses were conducted in R (v. 4.3.1) (R Core Team, 2023). Data and script is available at https://github.com/jharv3y/Pre-natal-exposure-to-glucocorticoids.

2.6. QPCR analysis

Two individual linear models were conducted to assess the stability

Table 1
Validated primer sequences used for qPCR analysis.

Gene	Function	Forward Primer	Reverse Primer	Amplicon melting temperature (Tm)	Product length	Distance from 3′ end of sequence
Bmal1	Major positive regulator of the clock system	GTACGTTTCTCGACATGCAATAGA	AGGTGTCCTATATCATCTTGATGGAA	71.9 °C	138	1433
Per2	Major positive negative of the clock system	CTGGCAAACCTGAAAGTGTTGTA	CTCCACTTGGACCATCTTCTATCA	72.1 °C	123	2545
β-Actin	Housekeeping	CAGCAAGCAGGAGTATGATGAA	AAGGGTGTGGGTGTTGGTAA	73.4 °C	87	181

β-Actin was used as the endogenous reference gene due to being previously identified as the most appropriate endogenous gene via GeNorm (Zimmer and Spencer, 2014). All qPCR runs were conducted in duplicate, with a 20 µl total reaction volume as per manufactures instructions (Precision plus Mastermix, PrimerDesign). All standard curve efficiencies were above 96 %, and $r^2 > 0.9$. ΔCT was calculated as $\Delta CT = 2^{-(\bar{x}\ Gene\ of\ interest - \bar{x}\ housekeeping)}$.

of β-Actin for the pre-natal and post-natal samples respectively. For pre-natal hypothalamic samples, β-actin was found to be significantly affected by age $F_{(1,142)=}15.71$, $p < 0.001$. A significant three way interaction driven by age was also identified ($F_{(4,142)} = 5.28$, $p < 0.001$). For post-natal hypothalamic samples, β-actin expression was found to be significantly affected by post-natal age ($F_{(1, 63)} = 186.769$, $P < 0.001$). A significant three way interaction between condition, time and age driven by age was also identified ($F_{(1,63)} = 9.858$, $p = 0.003$). Due to β-Actin being unstably expressed across age, separate age specific models were conducted for analysis. For both datasets, gene expression data was log-transformed to fulfil the assumptions of a linear model.

To compare relative gene expression of pre-natal samples, regression models (ANOVA) were run for gene expression for each embryonic age. Batch was also included to control for batch effects (Gene~condition+time + batch + time * condition). The models were designed to test for main effects of independent variables (time and condition) on the dependant variable (gene expression). Interaction effects between independent variables were also included. If significant effects were found, Tukey multiple comparisons of means with a 95 % confidence was run as a post hoc test.

For post-natal samples, regression models were again run for gene expression for each age. Batch was also included to control for batch effects (Gene~condition+ZT + batch + ZT * condition). Sex included within the model for this age (Gene~condition+ZT + batch + sex + ZT * condition * sex). The models were designed to test for main effects of independent variables (time, condition and sex) on the dependant variable (gene expression). Interactions between independent variables were also included. If significant effects were found, Tukey multiple comparisons of means with a 95 % confidence was run as a post hoc test.

For both pre-natal and post-natal models effect sizes were calculated using the R package "lsr" (Navarro, 2015). Effect sizes are reported as eta-squared (η2).

2.7. Diurnal behaviour analysis

To investigate diurnal patterns of behaviour, generalized additive mixed effects (GAMM) models were conducted using the R package "mgcv"(Wood, 2011). GAMM's are ideal for modelling circadian patterns in behaviour as they allow relationships in complex non-linear data to be assessed by fitting flexible non-linear smooth functions (Pedersen et al., 2019; Zuur et al., 2009). Behaviour was categorised as feeding (foraging, eating, drinking) and resting (stationary, under hen, camera trap not triggered). We fitted cyclic cubic regression splines to ensure that intercepts at 0 and 24 h aligned, in keeping with the circular nature of the data, and adjusted the knots in the spline to avoid over- or underfitting. GAMM's were built using the binomial distribution with logit link and conducted for time spent 'feeding' and 'resting' behaviour (as opposed to all other categories) respectively. To assess if pre-natal CORT treatment altered the diurnal behaviour rhythm, time spent performing behaviour (in seconds) changed across 24 h was modelled as a function of treatment, sex and an interaction between treatment and sex. Individual and housing group were added as random effects. Five models were run for each behaviour to systematically investigate which variables should be modelled. The most parsimonious model was then selected using AIC values (Supplementary Table 4.)

3. Results

3.1. Characterizing the effects of corticosterone exposure on hypothalamic circadian clock genes expression throughout embryonic development

3.1.1. Embryonic day 11

In the earliest embryonic stage studied, E11, a significant interaction of time and treatment was identified for both *Bmal1* ($F_{(2,50)} = 3.27$, $P = 0.046$, $\eta2 = 0.068$) and *Per2* ($F_{(2,50)} = 4.8$, $P = 0.012$, $\eta2 = 0.155$). Post hoc analysis revealed increasing expression of *Bmal1* between sampling timepoints for CORT exposed embryos only (9 am-2 PM $P = 0.0037$, 9 am-5 PM $P < 0.0001$). Post hoc analysis for *Per2* expression showed a significant increase in expression levels from 9 AM to 5 PM ($P = 0.024$) for CORT exposed embryos only (Fig. 1). *Bmal1* expression was found to be altered between timepoints ($F_{(2,50)} = 19.6$, $P < 0.0001$, $\eta2 = 0.002$). Post hoc analysis revealed a significant increase of *Bmal1* expression from 9 AM to 2 PM ($P = 0.0004$) and an overall increase between 9 AM and 5 PM ($P < 0.0001$). No main effect of time on hypothalamic *Per2* expression was identified at this stage ($F_{(2,50)} = 1.27$, $P = 0.29$, $\eta2 = 0.003$) (Fig. 2). No main effect of treatment was found for hypothalamic expression of either circadian clock genes (*Bmal1*: $F_{(1,50)} = 0.44$, $P = 0.51$, $\eta2 = 0.051$; *Per2*: $F_{(1,50)} = 0.002$, $P = 0.97$, $\eta2 = 0.056$, Fig. 3).

3.1.2. Embryonic day 14

At E14 *Per2* featured a significant interaction between time and condition ($F_{(2,48)} = 6.5$,$P = 0.0032$, $\eta2 = 0.193$, Fig. 1). Post hoc analysis revealed a significant increase of *Per2* expression in control embryos only, between timepoints (9 am-2 PM $P = 0.017$; 9 am-5 PM $P = 0.0029$ (Fig. 3)). No significant interactions were identified for *Bmal1* ($F_{(2,48)} = 1.83$,$P = 0.17$, $\eta2 = 0.051$). No significant effects of time (Fig. 2) or condition (Fig. 3) were identified for either hypothalamic circadian clock gene (Time, *Bmal1*: $F_{(2,48)} = 2.77$, $P = 0.073$, $\eta2 = 0.061$; *Per2*: $F_{(2,48)} = 2.89$, $P = 0.065$, $\eta2 = 0.273$; Condition, *Bmal1:* $F_{(1,48)} = 0.036$, $P = 0.85$, $\eta2 = 0.035$; *Per2:* $F_{(1,48)} = 0.003$, $P = 0.96$, $\eta2 = 0.130$).

3.1.3. Embryonic day 17

A significant interaction between condition and time was identified for both hypothalamic circadian genes (*Bmal1:* $F_{(2,43)} = 30.85$, $P < 0.0001$, $\eta2 = 0.437$; *Per2:* $F_{(2,42)} = 6.59$, $P = 0.0032$, $\eta2 = 0.122$, Fig. 1). *Bmal1* expression was found to be highest at 5 PM in CORT exposed embryos only (9 am-5 pm: $P < 0.0001$, 2 pm-5 PM: $P < 0.0001$). A significant increase at 5 PM in CORT embryos was also identified compared to controls ($P < 0.0001$). *Per2* expression in CORT embryos was significantly increased from 9 AM to 2 PM ($P = 0.0034$) and increased 9 AM to 5 PM ($P < 0.0001$). Again, a significant increase was found at 5 PM for CORT embryos compared to controls ($P = 0.002$).

A significant effect of time was identified for both hypothalamic circadian clock genes at E17 (*Bmal1*: $F_{(2,43)} = 12.28$, $P < 0.0001$, $\eta2 = 0.023$; *Per2*: $F_{(2,42)} = 13.93$,$P < 0.0001$, $\eta2 = 0.014$. Fig. 2). *Bmal1* expression was found to be significantly increased at 5 PM compared to 9 AM ($P < 0.0001$) and 2 PM ($P = 0.0044$). *Per2* expression was significantly increased at 5 PM compared to 9 AM only ($P < 0.0001$). Condition significantly affected expression of both circadian clock genes (*Bmal1*: $F_{(1,43)} = 10.49$, $P = 0.0023$, $\eta2 = 0.016$; *Per2*: $F_{(1,42)} = 11.2$, $P = 0.0012$, $\eta2 = 0.001$. Fig. 3). Expression of both *Bmal1* and *Per2* were significantly increased in CORT exposed embryos than controls.

3.2. Characterizing the effects of pre-natal corticosterone on post-natal circadian clock gene expression

3.2.1. Post-natal day 10

At post-natal day 10 a significant effect of time was identified for expression of both circadian clock genes (*Bmal1*: $F_{(1,29)} = 12.63$, $P = 0.0013$, $\eta2 = 0.160$; *Per2*: $F_{(1,29)} = 26.98$, $P < 0.0001$, $\eta2 = 0.276$. Fig. 2). *Bmal1* expression was significantly increased at ZT10 compared to ZT1 whilst *Per2* expression was significantly decreased at ZT10. Treatment was also found to effect expression of both circadian clock genes. A significantly reduced expression of both genes was identified (*Bmal1*: $F_{(1,29)} = 7.705$, $P = 0.0095$, $\eta2 = 0.082$; *Per2*: $F_{(1,29)} = 5.84$, $P = 0.022$, $\eta2 = 0.070$. Fig. 3). No significant interactions were identified between time and condition for either gene (*Bmal1*: $F_{(1,29)} = 0.02$, $P = 0.89$, $\eta2 = 0.0004$; *Per2*: $F_{(1,29)} = 0.34$, $P = 0.56$, $\eta2 = 0.006$, Fig. 1).

3.2.2. Post-natal day 28

At post-natal day 28 a significant increase of *Bmal1* expression was

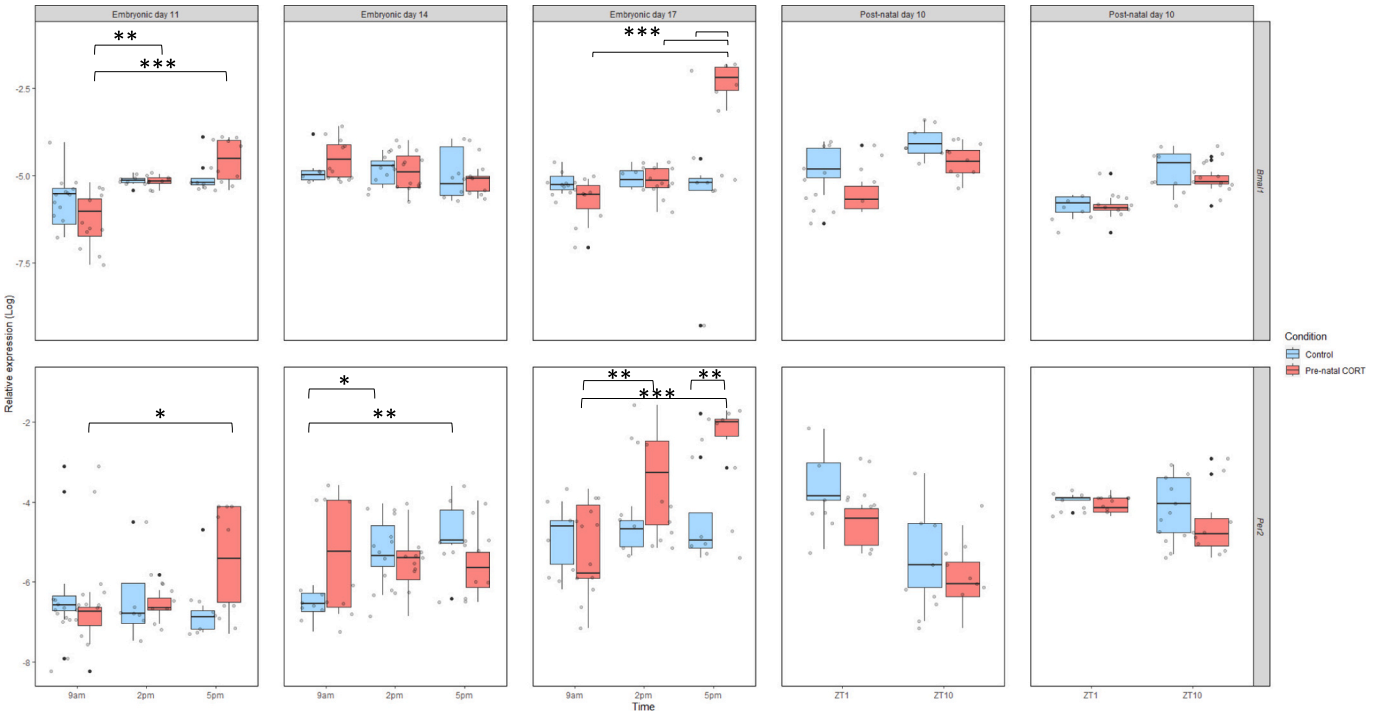

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Fig. 1. Treatment and time off day interactions across embryonic development and early post-natal life for *Bmal1* and *Per2* expression at sampling timepoints. Data is log transformed. Boxplots depict median relative expression levels and the 25th and 75th percentiles. Whiskers are 1.5× the interquartile range, data points outside this range are marked as outliers (circles). Raw Delta CT values are shown. ***indicate significant difference ($P < 0.001$), ** indicate significant differences ($P < 0.01$) and * indicates significant differences ($P < 0.05$).

	Bmal:		*Per2:*	
Sample sizes;	Pre-natal Control	Pre-natal CORT	Pre-natal Control	Pre-natal CORT
Embryonic day 11				
9am	N=12	N=12	N=12	N=12
2pm	N=8	N=8	N=8	N=8
5pm	N=8	N=8	N=8	N=8
Embryonic day 14				
9am	N=8	N=8	N=8	N=8
2pm	N=11	N=12	N=11	N=12
5pm	N=8	N=8	N=8	N=8
Embryonic day 17				
9am	N=9	N=11	N=9	N=10
2pm	N=8	N=8	N=8	N=8
5pm	N=8	N=6	N=8	N=6
Post-natal day 10				
ZT1	N=10	N=6	N=10	N=6
ZT10	N=9	N=8	N=9	N=8
Post-natal day 28				
ZT1	N=8	N=9	N=8	N=9
ZT10	N=9	N=12	N=9	N=12

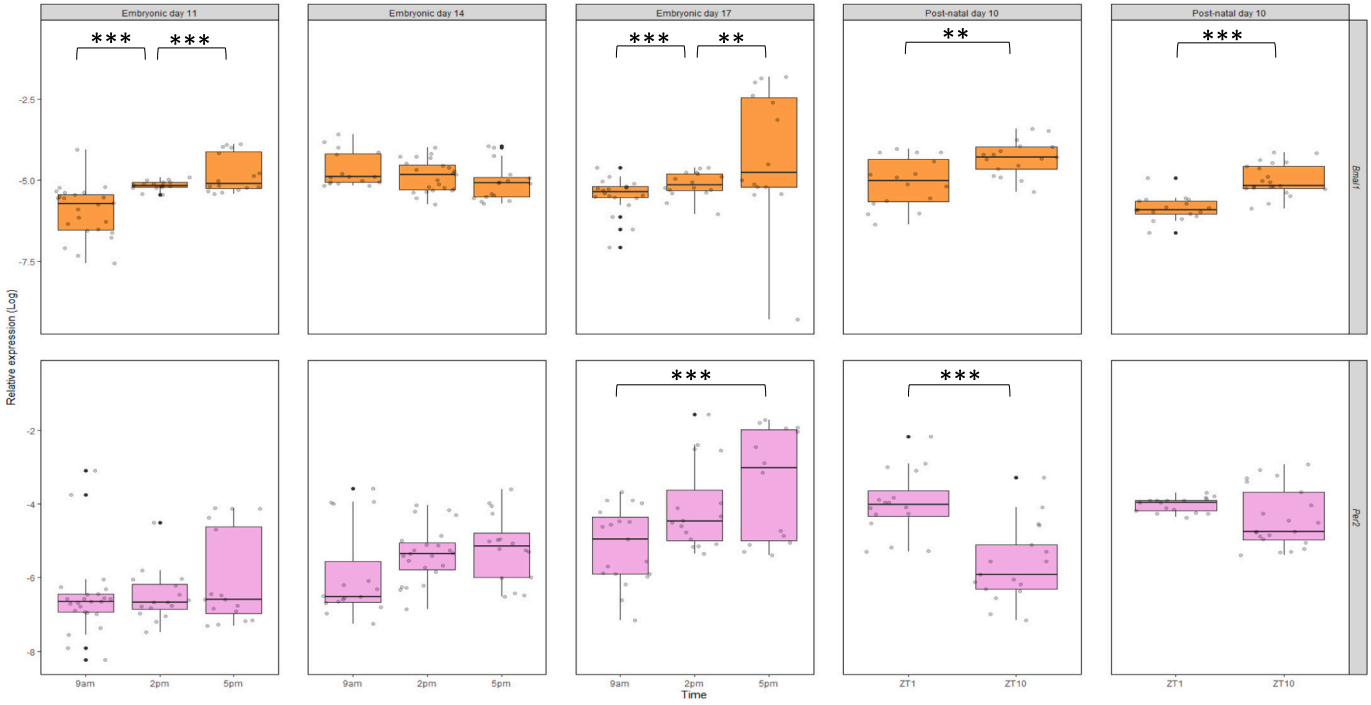
identified at ZT10 ($F_{(1,30)} = 39.16$, $P < 0.0001$, $\eta2 = 0.223$. Fig. 2). *Per2* expression was not found to alter between timepoints ($F_{(1,30)} = 3.15$, $P = 0.086$, $\eta2 = 0.016$). Sex did not affect hypothalamic clock gene levels (*Bmal1*: $F_{(1,30)} = 0.81$, $P = 0.38$, $\eta2 = 0.018$; *Per2*:$F_{(1,30)} = 0.38$,$P = 0.54$, $\eta2 = 0.003$). No effect of treatment was found for either gene (*Bmal1*: $F_{(1,30)} = 1.63$, $P = 0.21$, $\eta2 = 0.001$; *Per2*: $F_{(1,30)} = 2.23$,$P = 0.15$, $\eta2 = 0.0003$, Fig. 3). Additionally, no significant interactions were identified (Fig. 1, Table 2).

3.3. Characterizing the effects of pre-natal corticosterone exposure on post-natal behaviour

3.3.1. Eating rhythmicity

Our generalized additive models produced divergent circadian patterns for different subsets, and these patterns did not depend heavily on our choice of spline fitting method or the number of knots used. In the text below we present summaries and visualizations for models fit using REML and having 8 knots (eating) and 10 knots (resting) because those models were not unduly affected by low numbers of atypical observations during poorly sampled periods.

Eating rhythmicity appeared to feature an alteration following exposure to pre-natal CORT between sexes (Fig. 4A). Significant smooth terms were identified for treatment and sex interactions (Table 3). Males exposed to pre-natal stress spent more time eating than controls. The acrophase of eating rhythmicity appeared to be left shifted, featuring an early onset. A second peak in eating is also seen, corresponding to approximately the same ZT as the control acrophase. Female quail displayed less pronounced differences in eating rhythmicity, however the amplitude for eating was markedly lower.



(caption on next page)

Fig. 2. Time of day effects across embryonic development and early post-natal life for *Bmal1* and *Per2* expression. Data is log transformed. Boxplots depict median relative expression levels and the 25th and 75th percentiles. Whiskers are 1.5× the interquartile range, data points outside this range are marked as outliers (circles). Raw Delta CT values are shown. ***indicate significant difference ($P < 0.001$), ** indicate significant differences ($P < 0.01$) and * indicates significant differences ($P < 0.05$).

	Bmal:	*Per2:*
Sample sizes;		
Embryonic day 11		
9am	N=24	N=24
2pm	N=16	N=16
5pm	N=16	N=16
Embryonic day 14		
9am	N=24	N=24
2pm	N=16	N=16
5pm	N=16	N=16
Embryonic day 17		
9am	N=20	N=19
2pm	N=16	N=16
5pm	N=14	N=14
Post-natal day 10		
ZT1	N=16	N=16
ZT10	N=17	N=17
Post-natal day 28		
ZT1	N=17	N=17
ZT10	N=21	N=21

3.4. Resting rhythmicity

A significant interaction between treatment and time was again identified for quail of both sexes exposed to pre-natal CORT (Table 4, Fig. 4b). Males exposed to pre-natal CORT spent considerably less time resting in the light phase, when compared to controls, and featured a pronounced right shift (delay) in the resting acrophase during the light phase. Again, the difference was less apparent between females, however females exposed to pre-natal CORT spent less time resting than controls. This was particularly apparent during the early hours of the light phase.

4. Discussion

This paper presents the first characterization of the development of two main hypothalamic circadian clock genes following exposure to pre-natal CORT at physiologically relevant levels. We present evidence showing exposure to pre-natal GC's is sufficient to alter circadian clock gene expression throughout pre-natal and early post-natal development. The changes in circadian clock gene expression induced by pre-natal CORT did not however persist to day 27 of post-natal life. Despite this, diurnal rhythmicity in feeding and resting behaviour appeared to be affected for both male and female Japanese quail exposed to pre-natal CORT at this time. This is the first evidence that pre-natal CORT exposure alters eating rhythmicity and intensity in later post-natal life prior to sexual maturity. The absence of alterations to hypothalamic circadian clock gene expression at post-natal day 28 following pre-natal CORT exposure suggests wider circadian networks may be responsible for driving modifications in diurnal behaviour.

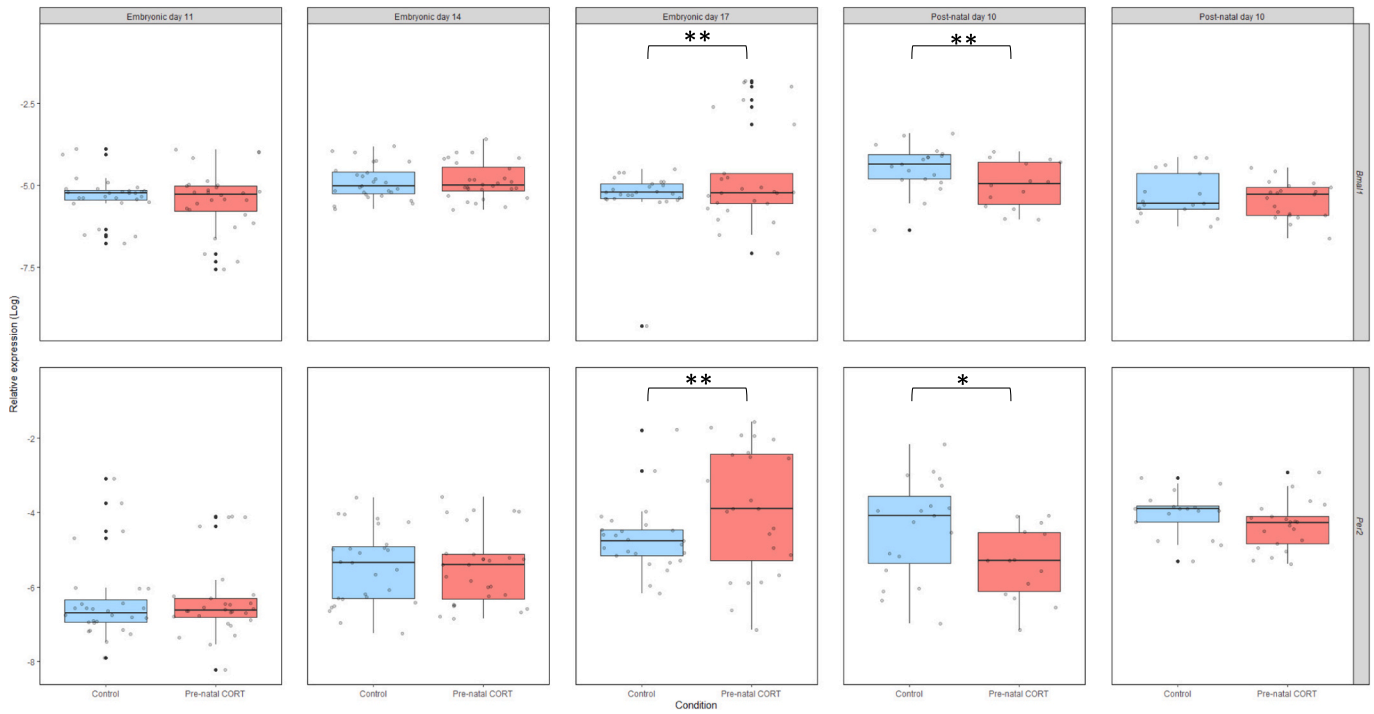


Fig. 3. Treatment effects across embryonic development and early post-natal life for *Bmal1* and *Per2* expression. Data is log transformed. Boxplots depict median relative expression levels and the 25th and 75th percentiles. Whiskers are 1.5× the interquartile range, data points outside this range are marked as outliers (circles). Raw Delta CT values are shown. ***indicate significant difference ($P < 0.001$), ** indicate significant differences ($P < 0.01$) and * indicates significant differences ($P < 0.05$).

		Bmal:	*Per2:*
Sample sizes;			
Embryonic day 11			
	Control	N=28	N=28
	Pre-natal CORT	N=28	N=28
Embryonic day 14			
	Control	N=27	N=27
	Pre-natal CORT	N=28	N=28
Embryonic day 17			
	Control	N=25	N=25
	Pre-natal CORT	N=25	N=24
Post-natal day 10			
	Control	N=19	N=19
	Pre-natal CORT	N=14	N=14
Post-natal day 28			
	Control	N=17	N=17
	Pre-natal CORT	N=21	N=21

Table 2
Interactions for hypothalamic clock gene expression at post-natal day 28.

Interaction	Result
Time and condition	*Bmal1*:$F_{(1,30)} = 1.17$, P = 0.29, η2 = 0.046;*Per2*: $F_{(1,30)} = 0.932$, $P = 0.34$, η2 = 0.002
Time and sex	*Bmal1*:$F_{(1,30)} = 0.88$, $P = 0.36$, η2 = 0.031; *Per2*: $F_{(1,30)} = 0.215$, $P = 0.65$, η2 = 0.007
Condition and sex	*Bmal1*:$F_{(1,30)} = 0.93$, P = 0.34, η2 = 0.0006; *Per2*: $F_{(1,30)} = 0.27$, $P = 0.61$, η2 = 0.0009
Time, condition and sex	*Bmal1*:$F_{(1,30)} = 1.71$, $P = 0.20$, η2 = 0.022;*Per2*: $F_{(1,30)} = 0.08$, $P = 0.78$, η2 = 0.0021

4.1. Development of the clock system

We present novel data on hypothalamic circadian clock ontogeny within quail chick development. At E11, the earliest developmental stage investigated, *Bmal1* expression was found to significantly increase from 9 AM to 5 pm. *Per2* expression however, was found to be constant between timepoints. The increase of *Bmal1* corroborates with post-natal studies which have demonstrated *Bmal1* to feature peak expression in the relative evening (Helfer et al., 2006). No significant effect of time on circadian gene expression was found at E14. The day before hatching, *Bmal1 expression* was again found to significantly increase between timepoints. Interestingly *Per2* was found to significantly increase across the sampling intervals, mirroring *Bmal1* expression. The altered expression between timepoints reflects the dynamic nature of circadian gene expression, however more sampling timepoints are required to explore cycling. In the mature vertebrate circadian system *Per2* decreases across the day as *Bmal1* increases (Cassone, 2014; Takahashi, 2016). Immature circadian clock system genes do not oscillate in an anti-phase pattern during early development (Honma, 2020; Nagy et al., 2009; Vallone et al., 2007). In chicken (*Gallus gallus)* embryonic day 18 and 19, activators (*Bmal* and *Clock)* and repressors (*Per* and *Cry)* have been found to cycle independently in pineal glands (Herichová et al., 2008; Nagy et al., 2009). However, the developmental time that cycling of circadian clock genes begins appears to be later in peripheral tissues

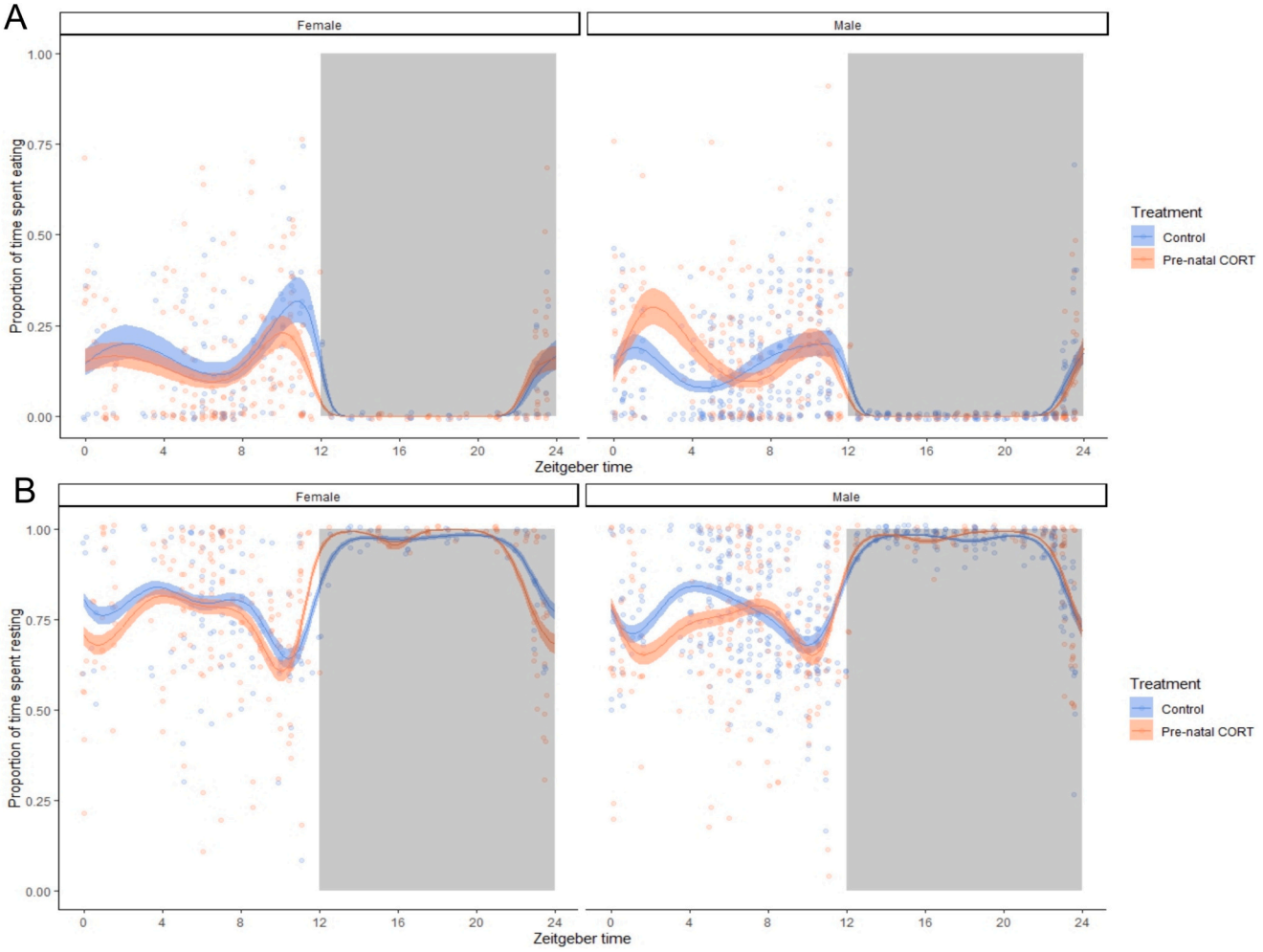


Fig. 4. The best fitting GAMM's for rhythmicity in time spent eating (A) and resting (B) across a 24 h period. Between ZT12 to ZT24 was the dark phase (represented by dark grey). Model is plotted without random effects. A. Male and female quails exposed to pre-natal CORT displayed different changes to rhythmicity of eating behaviours. B. Male and female quails exposed to pre-natal CORT displayed different changes to rhythmicity of eating behaviours.
Sample sizes:

	Males	Females
Pre-natal CORT	N=8	N=7
Pre-natal Control	N=11	N=4

Table 3
Results of the best-fitting GAMM for time spent eating.

Approximate significance of smooth terms:					
Interactions	eDF	Ref DF	Chi squared	*p*-Value	Significance
Zeitgeber time * Female Control	5.98	6.00	3451	<0.0001	***
Zeitgeber time * Female CORT	5.98	6.00	2932	<0.0001	***
Zeitgeber time * Male Control	5.98	6.00	2753	<0.0001	***
Zeitgeber time * Male CORT	5.97	6.00	6255	<0.0001	***
cage	4.20	7.00	3885	0.24	
individual	21.58	29.00	1423	0.23	

Table 4
Results of the best-fitting GAMM for time spent resting. *** indicate significant difference (P < 0.001), ** indicate significant differences (P < 0.01) and * indicates significant differences (P < 0.05).

Interactions	eDF	Ref DF	Chi squared	p-Value	Significance
Zeitgeber time * Female Control	7.88	8.00	14,327.00	<0.0001	***
Zeitgeber time * Female CORT	7.95	8.00	35,147.00	<0.0001	***
Zeitgeber time * Male Control	7.96	8.00	20,014.00	<0.0001	***
Zeitgeber time * Male CORT	7.95	8.00	24,743.00	<0.0001	***
Cage	5.02	7.00	9068.00	0.0014	*
Individual	23.31	29.00	2158.00	0.0411	*

(liver and heart) compared to the pineal gland (Zeman et al., 2009). When kept in light/dark conditions chicken embryos have been demonstrated to have cyclic expression of *Per2* starting at embryonic day 16 in the SCN, and 18 in the pineal gland. Amplitude and rhythmicity of *Per2* expression increases until the end of embryonic development (Okabayashi et al., 2003). By embryonic day 19 clear *Per2* rhythmicity is found in the pineal gland (Herichová et al., 2008). If chicken eggs are kept in complete darkness no rhythmic *Per2* expression has been identified during embryonic development (Okabayashi et al., 2003). It appears however if eggs are taken from LD and changed to DD *Per2* rhythms continue but with a reduced amplitude (Herichová et al., 2008). Taken together these studies indicate the reliance of light to initiate rhythmic *Per2* transcription within embryonic development. In this paper, eggs were incubated in complete darkness to control for unpredictable light exposure, as no paradigm currently exists for incubating Japanese quail eggs in an ecologically relevant manner. Our results, demonstrated that expression of *Per2* within embryonic hypothalamus at day 17 changes between sampling timepoints, despite being kept in constant darkness. This may suggest that that endogenous cycling of hypothalamic *Per2* is present, regardless of light exposure prior to hatching and despite the immature feedback loop between activators and repressors.

We identified seemingly normal anti-phase expression of both clock genes at post-natal day 10 (Abraham et al., 2009; Honma, 2018; Reppert and Weaver, 2002). In chicken, cycling of the core pineal circadian genes have been identified at post-natal day 4, and post-natal day 14 in the hypothalamus (Herichová et al., 2008; Jiang et al., 2017). Furthermore, the rate at which different rhythms mature varies. Despite having a functioning melatonin rhythm by the end of embryonic life the CORT rhythm is not fully developed in chicken until 17 weeks post hatching (Webb and Mashaly, 1985; Zeman and Herichová, 2011). At post-natal day 28 we found altered expression between timepoints in only *Bmal1*. This lack of alteration to *Per2* expression between timepoints may indicate that the hypothalamic circadian system is still developing during the first month of post-natal life. Alternatively this may be an artifact of the limited time samples. Furthermore, no sex differences were identified in circadian gene expression at post-natal day 28. This may be due to sexual immaturity as Japanese quail do not approach maturity until 35–45 days post-hatching (Narinc et al., 2013). To our knowledge this is the first paper to investigate hypothalamic circadian gene expression at these developmental ages in birds. Our results suggest that the hypothalamic circadian system is still developing in early post-natal life, and a fully functional auto-inhibitory feedback loop is not present until at least early post-natal life in a precocial organism. The immaturity of the system during early post-natal life may serve as an adaptive trait, allowing the circadian system to synchronize with the external environment over a longer timeframe.

4.2. The effects of pre-natal CORT on circadian clock gene expression

Interactions were identified between time and pre-natal CORT exposure. Both condition and time affected *Bmal1* embryonic expression at E11 and E17, with CORT exposure increasing *Bmal1* across time of day. Control embryos did not feature time of day effects. Condition and time of day significantly affected *Per2* expression at each embryonic age investigated. Similarly to *Bmal1* expression, CORT exposure increased *Per2* expression across time of day for both E11 and E17. Our findings, when considered in conjunction with evidence from mammals of GR induced expression of clock genes (Cheon et al., 2013; So et al., 2009; Yamamoto et al., 2005) and the ability of GC to phase-reset the circadian clock (Murayama et al., 2019) suggests that the CORT may be able to promote increases in circadian gene expression during development across time of day. This may serve as a level of preparedness for the offspring, potentially increasing development speed of the system in preparation for a challenging post-natal life. The ability of GC to seemingly promote upregulation of *Bmal1* and *Per2* expression also demonstrates that factors other than light are capable of altering embryonic circadian clock gene expression, highlighting a new mechanism of maternal effects and transgenerational developmental control.

Pre-natal CORT exposure appears to affect clock gene expression immediately before hatching (E17) and early post-natal life (day 10). CORT exposure elicited an overall increase of expression of circadian clock genes at E17. Contrastingly CORT exposure caused a significant decrease of clock gene expression at post-natal day 10. Again, this may be related to aiding integration of a matching or unmatching environment signalled by the mother (Monaghan, 2007), acting as a circadian critical period. No effects of pre-natal CORT were identified at post-natal day 28. As pre-natal CORT effects are only seen immediately prior to hatching and in early postnatal life, it may indicate that the circadian system is 'prepared' for a stressful early life environment (Harvey-Carroll et al., 2023). If the environment is not stressful the system may revert back to an 'unstressed' state, as seen at post-natal day 28.

Maternally deposited CORT is known to be metabolised by developing embryos. Embryos are exposed to the highest levels of maternal CORT during early development. Metabolism begins to appear at day 6 of development and by day 15, 80 % of CORT is metabolised. If concentrations of CORT within the yolk are elevated (recapitulating maternal stress), the embryo is exposed to the highest levels before day 9 of development, after which CORT levels are equivalent due to metabolism, regardless of initial dose (Vassallo et al., 2014, 2019). The ontogeny of endogenous GC production in Japanese quail has yet to be described, however in chicken *(Gallus domesticus)* production begins at approximately day 8 of development (Jenkins and Porter, 2004). This has led to the proposition that exposure to maternal CORT prior to day 9 may contribute towards the initial HPA setting (and in turn endogenous CORT production) in developing Japanese quail (Vassallo et al., 2019). Given this information, it is unlikely that the treatment effects seen in day 11 are remnants of injected CORT and are instead the result of altered biology from increased CORT levels within development.

This paper identified an overall decrease of both circadian clock genes during early post-natal life following pre-natal CORT exposure. This agrees with findings from post-natal stress studies, suggesting that GC are the main mode of action to elicit circadian changes. Chronic post-natal stress in rodents can reduce *Per2* expression within the SCN in addition to both the adrenal and pituitary (Jiang et al., 2011; Logan et al., 2015; Razzoli et al., 2014). A decrease in *Bmal1* gene amplitude has also been found in male mice (Logan et al., 2015). A more recent study investigating pre-natal stress in mice found *Per2* and *Bmal1* expression to be decreased in adults at ZT22 compared to ZT10 (Yun et al., 2020).

4.3. Effects of pre-natal CORT exposure on early post-natal diurnal rhythms

Despite differences in circadian clock gene expression not persisting to post-natal day 28, we identified that a single exposure to pre-natal CORT was sufficient to alter the diurnal rhythm of both eating and resting. This suggests that the hypothalamic circadian core clock network alone is not responsible for observed alterations to behaviours, and that it is likely a complex interaction between peripheral and central clocks, alongside a wider hormonal control (Kulkarni et al., 2023; Qian et al., 2019).

For eating rhythmicity, the acrophase in males appeared earlier, with a higher amplitude than controls. Female quail exposed to pre-natal CORT displayed a decreased acrophase amplitude in eating, however this was less apparent than the differences seen in males. The increased time spent eating corroborates rodent studies which have found mice exposed to pre-natal stress were found to have a greater caloric intake than controls. Male mice were also found to have a reduced caloric efficiency than female counterparts (Pankevich et al., 2009). Additionally down-regulation of genes involved in metabolism led to the suggestion of impaired glucose metabolism in mice exposed to pre-natal stress (Morley-Fletcher et al., 2019). Offspring from rats exposed to chronic phase shifts of photoperiods have been shown to have metabolic alterations (such as increased adiposity, hyperleptinaemia and hyperinsulinaemia) demonstrating the wide ranging potential of a pre-natal (circadian) stressor to alter offspring health (Varcoe et al., 2011).

The increased feeding found in this paper may be a result of altered metabolism from altered levels of glucocorticoids in pre-natal CORT animals (la Fleur, 2006; McGowan and Matthews, 2018; Perez-Leighton et al., 2023). Furthermore, it may be possible that the increased eating seen in individuals exposed to pre-natal stress is due compensatory growth triggered by maternal stress (Metcalfe and Monaghan, 2001). Male Japanese quail exposed to pre-natal CORT have been found to have a reduced growth rate between post-natal days two and six (Hayward et al., 2006). An initial period of retarded growth prior to accelerated growth (possibly reflected by the increased eating in male quails seen in this study) is common within compensatory growth (Metcalfe and Monaghan, 2001).

Increased activity levels are a generally accepted trait in male individuals exposed to pre-natal stress (Kiryanova et al., 2017; Son et al., 2007). Some discrepancies exist, depending on the type of pre-natal stress received, for example male rats exposed to pre-natal hypoxia (a model of early-life stress) were less active than controls (Joseph et al., 2002). Historically only male rodents have been used for such studies. However more recently, sex differences have been investigated, with a study demonstrating pre-natally stressed females being more inactive than controls (Morley-Fletcher et al., 2019). We found exposure to pre-natal glucocorticoids was sufficient to recapitulate these findings in Japanese quail- with animals exposed to pre-natal CORT spending less time resting. The exposure to pre-natal CORT had the most pronounced effect in male rhythmicity. Males exposed to pre-natal CORT consistently spent less time resting during the light phase than controls and appear to feature a phase delay in the light phase acrophase, when compared to controls. This corroborates findings in pre-natally stressed mice conducted by Morley-Fletcher et al. (2019). Males exposed to pre-natal stress were identified to be more active than controls during the respective active phase. A phase advance in activity was also detected, however in this paper we identify a slight phase delay in male activity. We also found females exposed to pre-natal CORT spent less time resting than controls. This is the opposite to findings by Morley-Fletcher et al. (2019), in which female mice exposed to pre-natal stress were less active compared to controls (Morley-Fletcher et al., 2019). The differences seen between results may be due to the different ecological niche's occupied by the study organisms used.

For both behaviours, we identified a more pronounced alteration following pre-natal CORT exposure in male quail, however no sex differences were identified in hypothalamic circadian clock genes. This may be due to the male bias in the animals used. Alternatively, the marked differences found in males exposed to pre-natal CORT indicate males may be capable of more behavioural flexibility than females. This may be due to less constrained energy budget (Marn et al., 2022). The behavioural flexibility seen in males may act as an adaptive advantage, as males are in direct intraspecific competition for mating. Females however, are predominantly in indirect competition (Hagelin, 2002), therefore it may be more advantageous for males than females to feature behavioural flexibility.

There is little research on the effects of pre-natal stress and post-natal endogenous hormone rhythmicity. To our knowledge, it is only the endogenous post-natal CORT rhythm that has been investigated following pre-natal stress in rodents. It may be possible that behavioural alterations identified in this study are a consequence of an altered post-natal CORT rhythm. Investigation of rodents exposed to pre-natal stress have demonstrated strong evidence of a phase advance in the post-natal CORT rhythm of both male and female offspring (Koehl et al., 1997, 1999). To our knowledge no studies have investigated the effects of pre-natal stress on the post-natal CORT rhythm in other species, therefore further conclusions cannot be drawn. One study in Japanese quail found no sex differences in baseline plasma CORT levels following exposure to pre-natal CORT, however little inferences can be made about alterations to the endogenous rhythmicity (Hayward et al., 2006).

To examine the full extent of pre-natal CORT exposure to circadian behaviour, rather than diurnal rhythmicity, future experiments should investigate the 'free-running' eating and resting rhythmicity under DD conditions. Furthermore, additional sampling timepoints, alongside inclusion of the pineal and retina should be considered to assess in depth the impact of pre-natal CORT on circadian gene rhythmicity. In this paper, the anatomical specificity within the hypothalamus has been compromised for quantification with qPCR. Future studies should use in-situ methodology to investigate the heterogeneity of altered circadian clock gene expression following pre-natal CORT within the hypothalamus. Additionally the effects of pre-natal CORT on post-natal hormones should be investigated to further the understanding of transgenerational stress-physiology interactions.

Overall we present data demonstrating exposure to pre-natal CORT alone is sufficient to alter the ontogeny of circadian clock genes, which persists into early pre-natal life. The differences are then lost by post-natal day 28, however alterations in rhythmicity in diurnal behaviours persists.

CRediT authorship contribution statement

Jessica Harvey-Carroll: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tyler J. Stevenson:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Luc F. Bussière:** Writing – review & editing, Visualization, Formal analysis. **Karen A. Spencer:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

There are no conflicts of interest or competing interests.

Data availability

Data and code used for analysis is available at doi:10.17630/d1664b42-f24d-4d03-b9f5-74997bcfc10b.

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Ethics approval

All procedures and housing of animals complied with the local ethics committee at the University of St. Andrews and in accordance with the Animals (Scientific Procedures) Act 1986 ASPA regulations under PIL IE1CF3B75 held by JLH and PPL 70/8159 held by KAS. SEC approval code 185 from School of Psychology & Neuroscience Ethics Committee was obtained.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2024.105562.

References

Abraham, U., Albrecht, U., Brandstätter, R., 2009. Hypothalamic Circadian Organization in Birds. II. Clock Gene Expression, 20, pp. 657–669. https://doi.org/10.1081/CBI-120022414.

Astiz, M., Oster, H., 2018. Perinatal programming of circadian clock-stress crosstalk. Neural Plast. 2018, 1–12. https://doi.org/10.1155/2018/5689165.

Carneiro, B.T.S., Araujo, J.F., 2012. Food entrainment: major and recent findings. Front. Behav. Neurosci. 6, 83. https://doi.org/10.3389/fnbeh.2012.00083.

Cassone, V.M., 2014. Avian circadian organization: a chorus of clocks. Front. Neuroendocrinol. 35, 76–88. https://doi.org/10.1016/J.YFRNE.2013.10.002.

Charmandari, E., Chrousos, G.P., Lambrou, G.I., Pavlaki, A., Koide, H., Ng, S.S.M., Kino, T., 2011. Peripheral CLOCK regulates target-tissue glucocorticoid receptor transcriptional activity in a circadian fashion in man. PLoS One 6, e25612. https://doi.org/10.1371/journal.pone.0025612.

Cheon, S., Park, N., Cho, S., Kim, K., 2013. Glucocorticoid-mediated Period2 induction delays the phase of circadian rhythm. Nucleic Acids Res. 41, 6161–6174. https://doi.org/10.1093/nar/gkt307.

Cockrem, J.F., 2007. Stress, corticosterone responses and avian personalities. J. Ornithol. 148, 169–178. https://doi.org/10.1007/s10336-007-0175-8.

Cox, K.H., Takahashi, J.S., 2019. Circadian clock genes and the transcriptional architecture of the clock mechanism. J. Mol. Endocrinol. 63, R93–R102. https://doi.org/10.1530/JME-19-0153.

Duong, H.A., Robles, M.S., Knutti, D., Weitz, C.J., Lee, C., Kim, T.-K., Takahashi, J.S., 2011. A molecular mechanism for circadian clock negative feedback. Science 332, 1436–1439. https://doi.org/10.1126/science.1196766.

la Fleur, S.E., 2006. The effects of glucocorticoids on feeding behavior in rats. Physiol. Behav. 89, 110–114.

Follett, B.K., Sharp, P.J., 1969. Circadian rhythmicity in photoperiodically induced gonadotrophin release and gonadal growth in the quail. Nature 223, 968–971. https://doi.org/10.1038/223968b0.

Hagelin, J.C., 2002. The kinds of traits involved in male—male competition: a comparison of plumage, behavior, and body size in quail. Behav. Ecol. 13 (1), 32–41.

Harvey-Carroll, J., Stevenson, T.J., Spencer, K.A., 2023. Maternal developmental history alters transfer of circadian clock genes to offspring in Japanese quail (Coturnix japonica). J. Comp. Physiol. A. 1–15.

Hastings, M.H., Maywood, E.S., Brancaccio, M., 2018. Generation of circadian rhythms in the suprachiasmatic nucleus. Nat. Rev. Neurosci. 19, 453–469. https://doi.org/10.1038/s41583-018-0026-z.

Hayward, L.S., Richardson, J.B., Grogan, M.N., Wingfield, J.C., 2006. Sex differences in the organizational effects of corticosterone in the egg yolk of quail. Gen. Comp. Endocrinol. 146, 144–148. https://doi.org/10.1016/j.ygcen.2005.10.016.

Helfer, G., Fidler, A.E., Vallone, D., Foulkes, N.S., Brandstaetter, R., 2006. Molecular analysis of clock gene expression in the avian brain. Chronobiol. Int. 23, 113–127. https://doi.org/10.1080/07420520500521871.

Henriksen, R., Rettenbacher, S., Groothuis, T.G.G.G., 2011. Prenatal stress in birds: pathways, effects, function and perspectives. Neurosci. Biobehav. Rev. 35, 1484–1501. https://doi.org/10.1016/J.NEUBIOREV.2011.04.010.

Herichová, I., Monošíková, J., Zeman, M., 2008. Ontogeny of melatonin, Per2 and E4bp4 light responsiveness in the chicken embryonic pineal gland. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 149, 44–50. https://doi.org/10.1016/J.CBPA.2007.10.006.

Honma, S., 2018. The mammalian circadian system: a hierarchical multi-oscillator structure for generating circadian rhythm. J. Physiol. Sci. 68 (3), 207–219. https://doi.org/10.1007/S12576-018-0597-5.

Honma, S., 2020. Development of the mammalian circadian clock. Eur. J. Neurosci. 51, 182–193.

Jenkins, S.A., Porter, T.E., 2004. Ontogeny of the hypothalamo–pituitary–adrenocortical axis in the chicken embryo: a review. Domest. Anim. Endocrinol. 26, 267–275. https://doi.org/10.1016/j.domaniend.2004.01.001.

Jiang, N., Wang, Z., Cao, J., Dong, Y., Chen, Y., 2017. Effect of monochromatic light on circadian rhythmic expression of clock genes in the hypothalamus of chick. J. Photochem. Photobiol. B Biol. 173, 476–484.

Jiang, W.-G., Li, S.-X., Zhou, S.-J., Sun, Y., Shi, J., Lu, L., 2011. Chronic unpredictable stress induces a reversible change of PER2 rhythm in the suprachiasmatic nucleus. Brain Res. 1399, 25–32. https://doi.org/10.1016/J.BRAINRES.2011.05.001.

Joseph, V., Mamet, J., Lee, F., Dalmaz, Y., Van Reeth, O., 2002. Prenatal hypoxia impairs circadian synchronisation and response of the biological clock to light in adult rats. J. Physiol. 543, 387–395.

Kapoor, A., Dunn, E., Kostaki, A., Andrews, M.H., Matthews, S.G., 2006. Fetal programming of hypothalamo-pituitary-adrenal function: prenatal stress and glucocorticoids. J. Physiol. 572, 31–44. https://doi.org/10.1113/jphysiol.2006.105254.

Kiryanova, V., Smith, V.M., Dyck, R.H., Antle, M.C., 2017. Circadian behavior of adult mice exposed to stress and fluoxetine during development. Psychopharmacology 234, 793–804. https://doi.org/10.1007/s00213-016-4515-3.

Koehl, M., Barbazanges, A., Le Moal, M., Maccari, S., 1997. Prenatal stress induces a phase advance of circadian corticosterone rhythm in adult rats which is prevented by postnatal stress. Brain Res. 759, 317–320. https://doi.org/10.1016/S0006-8993(97)00394-6.

Koehl, M., Darnaudery, M., Dulluc, J., Van Reeth, O., Le Moal, M., Maccari, S., 1999. Prenatal stress alters circadian activity of hypothalamo–pituitary–adrenal axis and hippocampal corticosteroid receptors in adult rats of both gender. J. Neurobiol. 40, 302–315. https://doi.org/10.1002/(SICI)1097-4695(19990905)40:3<302::AID-NEU3>3.0.CO;2-7.

Kulkarni, S.S., Singh, O., Zigman, J.M., 2023. The intersection between ghrelin, metabolism and circadian rhythms. Nat. Rev. Endocrinol. 1–11.

Lamia, K.A., Papp, S.J., Yu, R.T., Barish, G.D., Uhlenhaut, N.H., Jonker, J.W., Downes, M., Evans, R.M., 2011. Cryptochromes mediate rhythmic repression of the glucocorticoid receptor. Nature 480 (7378), 552–556.

Logan, R.W., Edgar, N., Gillman, A.G., Hoffman, D., Zhu, X., McClung, C.A., 2015. Chronic stress induces brain region-specific alterations of molecular rhythms that correlate with depression-like behavior in mice. Biol. Psychiatry 78, 249–258. https://doi.org/10.1016/j.biopsych.2015.01.011.

Maccari, S., Morley-Fletcher, S., 2007. Effects of prenatal restraint stress on the hypothalamus–pituitary–adrenal axis and related behavioural and neurobiological alterations. Psychoneuroendocrinology 32, S10–S15. https://doi.org/10.1016/J.PSYNEUEN.2007.06.005.

Marn, N., Lika, K., Augustine, S., Goussen, B., Ebeling, M., Heckmann, D., Gergs, A., 2022. Energetic basis for bird ontogeny and egg-laying applied to the bobwhite quail. Conserv. Physiol. 10 (1), coac063.

Matthews, S.G., McGowan, P.O., 2019. Developmental programming of the HPA axis and related behaviours: epigenetic mechanisms. J. Endocrinol. 242, T69–T79.

McGowan, P.O., Matthews, S.G., 2018. Prenatal stress, glucocorticoids, and developmental programming of the stress response. Endocrinology 159, 69–82. https://doi.org/10.1210/en.2017-00896.

Metcalfe, N.B., Monaghan, P., 2001. Compensation for a bad start: grow now, pay later? Trends Ecol. Evol. 16, 254–260. https://doi.org/10.1016/S0169-5347(01)02124-3.

Monaghan, P., 2007. Early growth conditions, phenotypic development and environmental change. Philosophical Transactions of the Royal Society B: Biological Sciences 363, 1635–1645. https://doi.org/10.1098/rstb.2007.0011.

Morley-Fletcher, S., Mairesse, J., Van Camp, G., Reynaert, M.-L., Gatta, E., Marrocco, J., Bouwalerh, H., Nicoletti, F., Maccari, S., 2019. Perinatal stress programs sex differences in the behavioral and molecular chronobiological profile of rats maintained under a 12-h light-dark cycle. Front. Mol. Neurosci. 12, 89. https://doi.org/10.3389/fnmol.2019.00089.

Murayama, Y., Yahagi, N., Takeuchi, Y., Aita, Y., Mehrazad Saber, Z., Wada, N., Li, E., Piao, X., Sawada, Y., Shikama, A., Masuda, Y., 2019. Glucocorticoid receptor

suppresses gene expression of Rev-erbα (Nr1d1) through interaction with the CLOCK complex. FEBS Letters 593 (4), 423–432.
Nader, N., Chrousos, G.P., Kino, T., 2009. Circadian rhythm transcription factor CLOCK regulates the transcriptional activity of the glucocorticoid receptor by acetylating its hinge region lysine cluster: potential physiological implications. FASEB J. 23, 1572–1583. https://doi.org/10.1096/fj.08-117697.
Nagy, A.D., Kommedal, S., Seomangal, K., Csernus, V.J., 2009. Circadian expression of clock genes clock and Cry1 in the embryonic chicken pineal gland. Trends in Comparative Endocrinology and Neurobiology 1163, 484–487. https://doi.org/10.1111/J.1749-6632.2008.03639.X.
Nakao, N., Ono, H., Yamamura, T., Anraku, T., Takagi, T., Higashi, K., Yasuo, S., Katou, Y., Kageyama, S., Uno, Y., Kasukawa, T., Iigo, M., Sharp, P.J., Iwasawa, A., Suzuki, Y., Sugano, S., Niimi, T., Mizutani, M., Namikawa, T., Ebihara, S., Ueda, H. R., Yoshimura, T., 2008. Thyrotrophin in the pars tuberalis triggers photoperiodic response. Nature 452, 317–322. https://doi.org/10.1038/nature06738.
Narinc, D., Karaman, E., Aksoy, T., Firat, M.Z., 2013. Investigation of nonlinear models to describe long-term egg production in Japanese quail. Poult. Sci. 92, 1676–1682.
Navarro, D., 2015. Learning Statistics With R: A Tutorial for Psychology Students and Other Beginners (version 0.6).. University of New South Wales.
Okabayashi, N., Yasuo, S., Watanabe, M., Namikawa, T., Ebihara, S., Yoshimura, T., 2003. Ontogeny of circadian clock gene expression in the pineal and the suprachiasmatic nucleus of chick embryo. Brain Res. 990, 231–234. https://doi.org/10.1016/S0006-8993(03)03531-5.
Oster, H., Challet, E., Ott, V., Arvat, E., de Kloet, E.R., Dijk, D.-J., Lightman, S., Vgontzas, A., Van Cauter, E., 2016. The functional and clinical significance of the 24-h rhythm of circulating glucocorticoids. Endocr. Rev. 38 https://doi.org/10.1210/er.2015-1080 er.2015-1080.
Pankevich, D.E., Mueller, B.R., Brockel, B., Bale, T.L., 2009. Prenatal stress programming of offspring feeding behavior and energy balance begins early in pregnancy. Physiol. Behav. 98, 94–102.
Partch, C.L., Green, C.B., Takahashi, J.S., 2014. Molecular architecture of the mammalian circadian clock. Trends Cell Biol. 24, 90–99. https://doi.org/10.1016/J.TCB.2013.07.002.
Pedersen, E.J., Miller, D.L., Simpson, G.L., Ross, N., 2019. Hierarchical generalized additive models in ecology: an introduction with mgcv. PeerJ 2019. https://doi.org/10.7717/peerj.6876.
Perez-Leighton, C., Kerr, B., Scherer, P.E., Baudrand, R., Cortés, V., 2023. The interplay between leptin, glucocorticoids, and GLP1 regulates food intake and feeding behaviour. Biol. Rev.
Pruessner, J.C., Wolf, O.T., Hellhammer, D.H., Buske-Kirschbaum, A., Von Auer, K., Jobst, S., Kaspers, F., Kirschbaum, C., 1997. Free cortisol levels after awakening: a reliable biological marker for the assessment of adrenocortical activity. Life Sci. 61, 2539–2549. https://doi.org/10.1016/S0024-3205(97)01008-4.
Qian, J., Morris, C.J., Caputo, R., Garaulet, M., Scheer, F.A.J.L., 2019. Ghrelin is impacted by the endogenous circadian system and by circadian misalignment in humans. Int. J. Obes. 43, 1644–1649.
R Core Team, 2023. R: A Language and Environment for Statistical Computing.
Razzoli, M., Karsten, C., Yoder, J.M., Bartolomucci, A., Engeland, W.C., 2014. Chronic subordination stress phase advances adrenal and anterior pituitary clock gene rhythms. Am. J. Physiol. Regul. Integr. Comp. Physiol. 307, R198–R205. https://doi.org/10.1152/ajpregu.00101.2014.
Reppert, S.M., Weaver, D.R., 2002. Coordination of circadian timing in mammals. Nature 418, 935–941. https://doi.org/10.1038/nature00965.
Romero, L.M., Gormally, B.M.G., 2019. How truly conserved is the “well-conserved” vertebrate stress response? Integr. Comp. Biol. 59, 273–281. https://doi.org/10.1093/icb/icz011.
Shi, S., Hida, A., McGuinness, O.P., Wasserman, D.H., Yamazaki, S., Johnson, C.H., 2010. Circadian clock gene Bmal1 is not essential; functional replacement with its paralog, Bmal2. Curr. Biol. 20 (4), 316–321. https://doi.org/10.1016/j.cub.2009.12.034.
So, A.Y.-L., Bernal, T.U., Pillsbury, M.L., Yamamoto, K.R., Feldman, B.J., 2009. Glucocorticoid regulation of the circadian clock modulates glucose homeostasis. Proc. Natl. Acad. Sci. USA 106, 17582–17587. https://doi.org/10.1073/pnas.0909733106.
Son, G.H., Chung, S., Geum, D., Kang, S.S., Choi, W.S., Kim, K., Choi, S., 2007. Hyperactivity and alteration of the midbrain dopaminergic system in maternally stressed male mice offspring. Biochem. Biophys. Res. Commun. 352, 823–829.
Spencer, K.A., 2017. Developmental stress and social phenotypes: integrating neuroendocrine, behavioural and evolutionary perspectives. Philos. Trans. R. Soc. B 372, 20160242.
Spencer, K.A., Evans, N.P., Monaghan, P., 2009. Postnatal stress in birds: a novel model of glucocorticoid programming of the hypothalamic-pituitary-adrenal Axis. Endocrinology 150, 1931–1934. https://doi.org/10.1210/en.2008-1471.
Spencer, R.L., Chun, L.E., Hartsock, M.J., Woodruff, E.R., 2018. Glucocorticoid hormones are both a major circadian signal and major stress signal: how this shared signal contributes to a dynamic relationship between the circadian and stress systems. Front. Neuroendocrinol. 49, 52–71. https://doi.org/10.1016/j.yfrne.2017.12.005.
Spoelstra, K., Wikelski, M., Daan, S., Loudon, A.S.I., Hau, M., 2016. Natural selection against a circadian clock gene mutation in mice. Proc. Natl. Acad. Sci. USA 113, 686–691. https://doi.org/10.1073/pnas.1516442113.
Stokkan, K.A., Yamazaki, S., Tei, H., Sakaki, Y., Menaker, M., 2001. Entrainment of the circadian clock in the liver by feeding. Science 291, 490–493. https://doi.org/10.1126/science.291.5503.490.
Tahara, Y., Shiraishi, T., Kikuchi, Y., Haraguchi, A., Kuriki, D., Sasaki, H., Motohashi, H., Sakai, T., Shibata, S., 2015. Entrainment of the mouse circadian clock by sub-acute physical and psychological stress. Sci. Rep. 5, 11417. https://doi.org/10.1038/srep11417.
Takahashi, J.S., 2016. Transcriptional architecture of the mammalian circadian clock. Nat. Rev. Genet. 18, 164–179. https://doi.org/10.1038/nrg.2016.150.
Underwood, H., Siopes, T., 1984. Circadian organization in Japanese quali. J. Exp. Zool. 232, 557–566. https://doi.org/10.1002/jez.1402320323.
Vallone, D., Lahiri, K., Dickmeis, T., Foulkes, N.S., 2007. Start the clock! Circadian rhythms and development. Dev. Dyn. 236, 142–155. https://doi.org/10.1002/dvdy.20998.
Varcoe, T.J., Wight, N., Voultsios, A., Salkeld, M.D., Kennaway, D.J., 2011. Chronic phase shifts of the photoperiod throughout pregnancy programs glucose intolerance and insulin resistance in the rat. PLoS One 6, e18504. https://doi.org/10.1371/journal.pone.0018504.
Vassallo, B.G., Paitz, R.T., Fasanello, V.J., Haussmann, M.F., 2014. Glucocorticoid metabolism in the *in ovo* environment modulates exposure to maternal corticosterone in Japanese quail embryos (*Coturnix japonica*). Biol. Lett. 10, 20140502. https://doi.org/10.1098/rsbl.2014.0502.
Vassallo, B.G., Litwa, H.P., Haussmann, M.F., Paitz, R.T., 2019. In ovo metabolism and yolk glucocorticoid concentration interact to influence embryonic glucocorticoid exposure patterns. Gen. Comp. Endocrinol. 272, 57–62. https://doi.org/10.1016/j.ygcen.2018.11.013.
Webb, M.L., Mashaly, M.M., 1985. Maturation of the diurnal rhythm of corticosterone in female domestic fowl. Poult. Sci. 64, 744–750. https://doi.org/10.3382/ps.0640744.
Wingfield, J.C., 1994. Modulation of the adrenocortical response to stress in birds. In: Perspectives in Comparative Endocrinology: XXII Inernational Congress of Comparative Endocrinology, Toronto, Ontario, Canada, pp. 520–528.
Wood, S.N., 2011. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. J. R. Stat. Soc. Ser. B Stat Methodol. 73, 3–36.
Yamamoto, T., Nakahata, Y., Tanaka, M., Yoshida, M., Soma, H., Shinohara, K., Yasuda, A., Mamine, T., Takumi, T., 2005. Acute physical stress elevates mouse period1 mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. J. Biol. Chem. 280, 42036–42043. https://doi.org/10.1074/jbc.M509600200.
Yun, S., Lee, E.J., Choe, H.K., Son, G.H., Kim, K., Chung, S., 2020. Programming effects of maternal stress on the circadian system of adult offspring. Exp. Mol. Med. 52 (3), 473–484. https://doi.org/10.1038/s12276-020-0398-9.
Zeman, M., Herichová, I., 2011. Circadian melatonin production develops faster in birds than in mammals. Gen. Comp. Endocrinol. 172, 23–30. https://doi.org/10.1016/J.YGCEN.2010.12.022.
Zeman, M., Szántóová, K., Herichová, I., 2009. Ontogeny of circadian oscillations in the heart and liver in chicken. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 154, 78–83.
Zhang, Z., Shui, G., Li, M.D., 2021. Time to eat reveals the hierarchy of peripheral clocks. Trends Cell Biol. 31, 869–872. https://doi.org/10.1016/J.TCB.2021.08.003.
Zimmer, C., Spencer, K.A., 2014. Modifications of glucocorticoid receptors mRNA expression in the hypothalamic-pituitary-adrenal axis in response to early-life stress in female Japanese quail. J. Neuroendocrinol. 26, 853–860. https://doi.org/10.1111/jne.12228.
Zimmer, C., Boogert, N.J., Spencer, K.A., 2013. Developmental programming: cumulative effects of increased pre-hatching corticosterone levels and post-hatching unpredictable food availability on physiology and behaviour in adulthood. Horm. Behav. 64, 494–500. https://doi.org/10.1016/j.yhbeh.2013.07.002.
Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A., Smith, G.M., 2009. Mixed Effects Models and Extensions in Ecology With R. Springer, New York.