

FSH β links photoperiodic signalling to seasonal reproduction in Japanese quail

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Abstract

Endogenous programs in avian reproductive seasonal life history transitions have been known for over 30 years. Attempts to identify the neural and molecular substrates of photoperiodic time measurement have, to date, focussed on blunt changes in light exposure during a restricted period of photoinducibility. Here we report mediobasal hypothalamus (MBH) and pituitary gland specific transcript expression cascades associated with photoperiod-induced seasonal life history transitions. We identified multiple waves of transcript expression that were not synchronized across brain regions. Follicle-stimulating hormone- β (FSH β) expression increased during the simulated vernal equinox, prior to photoinduced increases in prolactin, thyrotropin-stimulating hormone- β and testicular growth. Analyses showed sustained elevated levels of daily FSH β expression in the pituitary under conditions of the vernal, compared to autumnal equinox, short (<12L) and long (>12L) photoperiods. FSH β expression increased in Quail held in non-stimulatory photoperiod, indicative of an endogenous programmed change in transcription. These data identify that FSH β provides an endogenous program for the photoperiod-dependent external coincidence timing for seasonal transitions in reproduction. FSH β may prime the pituitary for subsequent stimulation by triiodothyronine-mediated changes in tanycytes during the photoinducible phase. Overall, the data indicate a multi-cellular, multi-neural interval timing mechanism resides in the brain to control seasonal life-history transitions in birds.

eLife assessment

This **important** article provides insights into the neural centres in the Japanese quail brain that are associated with photoperiod-induced life-history states. The physiological and transcriptomic analyses of the mediobasal hypothalamus and pituitary gland offer evidence for a coincidence timing mechanism for measuring day length, which is relevant for the field of circannual biology. Despite some shortcomings in data analysis, the study's **convincing** experiments and findings have the potential to captivate the attention of molecular and organismal endocrinologists.

Temperate and equatorial species display endogenous cycles in physiology and behavior that serve to anticipate future seasonal environments (Gwinner, 1986; Wingfield, 2008; Helm & Stevenson, 2014). Even in the absence of seasonal fluctuations in temperature, daylength and food availability, endogenous circannual programs in migration (Gwinner and Dittami, 1990), hibernation (Pengelley and Fisher, 1957), and reproduction (Woodfill et al., 1994; Lincoln et al., 2006) are maintained with remarkable temporal precision. The anatomical and cellular basis of circannual rhythms remain poorly characterized, but current evidence indicates that, in mammals, interval timing may reside in pituitary lactotropes (Lincoln et al., 2006) or thyrotropes (Wood et al., 2020). In most long-lived species (e.g., >2 yrs.), the annual change in daylength, referred to as photoperiod, acts to entrain endogenous circannual calendar cells to time physiological state to seasons (Bradshaw and Holzapfel, 2007). However, our understanding of how the brain integrates photoperiodic cues and endogenous rhythms to define the transitions between seasonal life-history states remains elusive.

In most birds, reptiles, and amphibians, annual changes in daylength are detected by photoreceptors located in the mediobasal hypothalamus (MBH) (Perez et al., 2019). In Japanese quail (*Coturnix japonica*), days that exceed a ‘critical length’ that coincides with a period of photoinducibility (Follett and Sharp, 1969) trigger a molecular cascade that starts with the upregulation of thyrotropin-stimulating hormone beta subunit (*TSHβ*) in the pars tuberalis of the pituitary gland and results in gonadal maturation (Nakao et al., 2008). The current gap in our knowledge is how short days (i.e., <12hr) induce endogenous programs in gonadal growth (Follett and Sharp, 1969) and how prolonged exposure to short days sensitises the brain to respond, at a molecular level, to stimulatory long days. Previous studies in European starlings (*Sturnus vulgaris*) demonstrated that exposure to short days increased Gonadotropin-releasing hormone (*GNRH*) expression in the preoptic area (Stevenson et al., 2009; Stevenson et al., 2012a). As both GnRH and gonadal growth increased in the absence of stimulatory long day lengths, another cellular pathway must underlie the endogenous development of reproductive physiology in birds. We theorized multiple neuroendocrine regions including the preoptic area, mediobasal hypothalamus and pituitary cells are independently involved in the timing of seasonal transitions in physiology. Specifically, we hypothesized that pituitary gonadotropes establish a state of photosensitivity to stimulatory long day photoperiods and thus act as calendar cells that provide endogenous timing of gonadal growth.

Results

To address this problem, we simulated the photoperiodic regulation of seasonal physiology of Japanese quail, using sequential changes of an autumnal decrease, followed by a vernal increase in daylength and measured testes volume, body mass and abdominal fat. As anticipated (Follett and Sharp, 1969; Robinson and Follett, 1982), light phases that exceed the critical day length (i.e., >12hr) resulted in robust gonadal growth (Fig. 1a). Increases in body mass and abdominal fat deposition were delayed until the vernal increase in day lengths reached 10hrs (10v) (Fig. 1b,c). EdgeR analyses, of MBH sequences obtained using Minion, identified 1481 transcripts were differentially expressed ($P < 0.05$) (Table S2), and BioDare2.0 established 398 have rhythmic patterns (Fig. 1e; Table S3). DAVID gene ontology analyses indicated that gonadotropin-releasing hormone receptor and Wnt signalling pathways were consistently identified as the predominant cellular mechanism recruited during each photoperiodic transition (Table S2). Increased proopiomelanocortin (*POMC*) expression coincided with body mass growth and abdominal fat accumulation (Fig. 1f). Thyroid hormone catabolism enzyme deiodinase type-3 (*DIO3*) increased after prolonged exposure to non-stimulatory photoperiods and was only therefore transiently elevated from 8L to 10v (10v; Fig. 1g). Highest levels of vimentin

immunoreactivity in the median eminence coincided with the peak in *DIO3* expression (**Fig. 1h** [↗](#)) suggesting the localized removal of active thyroid hormone is limited to a short phase and occurred prior to stimulatory photoperiods (**Fig. 1i** [↗](#)). The photoperiod-induced change in vimentin was anatomically localized to the median eminence, as the area of immunoreactivity in the dorsal 3rd V endymal layer did not change with seasonal transitions in reproduction (**Fig. S1**). Next, we used MinION to sequence transcripts in the pituitary gland across the seasonal transitions in reproduction. EdgeR analyses identified 3090 transcripts were differentially expressed in the pituitary gland (**Table S1**). DAVID gene ontology analyses identified that gonadotropin-releasing hormone receptor and epidermal growth factor pathways are consistently observed across photoperiodic transitions (**Table S4**). BioDare2.0 established that 130 transcripts were rhythmically expressed (**Fig. 1j** [↗](#); **Table S5**). A remarkable 96.5% (384/398) of MBH transcripts show a photoperiod-induced spiked patterned and only 12/14 remaining transcripts displayed sine waveforms. Conversely, 100% of pituitary transcripts conformed to sine (124/130) or cosine (6/130) waveforms. The predominant rhythmic patterns of expression likely reflect endogenous long-term molecular programs, characteristic of calendar cell function. *GNRH* expression in the preoptic area was temporarily decreased during the 8L short photoperiod indicating another nucleus specific cellular timer is present in this brain region (**Fig. S1**). Overall, the pituitary showed a distinct transcriptomic profile compared to the MBH suggesting independence in the representation of seasonal photoperiodic timing. The vernal photoperiodic transition from 8L to 12v resulted in a significant increase in *FSHβ* (**Fig. 1k** [↗](#)) without a change in tanycyte restructuring (**Fig. 1h** [↗](#)). Subsequent transition to 14v and then 16v resulted in the upregulation of prolactin (*PRL*) (**Fig. 1l** [↗](#)) and thyrotropin-stimulating hormone-β (*TSHβ*), respectively (**Fig. 1m** [↗](#)). These data establish that multiple cells in the pituitary code seasonal photoperiodic time and *FSHβ* shows an endogenous increase in expression prior to reproductively stimulatory photoperiods.

To ascertain common molecular mechanisms involved in the transcriptional regulation of photoperiodically regulated transcripts, transcription factor enrichment analysis was conducted on significant MBH (**Table S6**) and pituitary gland (**Table S7**) transcripts. Association plots show no overlap in DNA binding motifs between MBH and pituitary transcripts (**Fig. S2**) suggesting tissue-specific transcription binding factor regulation. Within the pituitary gland, several common transcription factors, such as the Jun proto-oncogene, Spi1 proto-oncogene and myocyte enhancer factor 2 (*MEF2a*) might be actively involved in the photoperiodic regulation of transcript expression. These findings indicate multiple transcription factors are likely recruited to control tissue-specific, and cell-specific transcript expression and seasonal life history transitions in physiology.

To establish whether increased pituitary *FSHβ* during the vernal 12hr transition reflects constitutively elevated expression or is driven by the sampling ‘time of day’, we collected tissue samples every 3 hours from quail that transitioned to the autumnal equinox (i.e., 12a), the short photoperiod (8L), vernal equinox (12v) and long photoperiod (16L) seasonal phases (**Fig. 2a** [↗](#)). Photoinduced changes in testes volume confirmed seasonal reproductive condition (**Fig. 2b** [↗](#)). Circadian clock genes aryl hydrocarbon receptor nuclear translocator like (*ARNTL1*) and period 3 (*PER3*) exhibit robust anti-phase daily waveforms in expression, in the pituitary gland (**Fig. 2c,d** [↗](#)) (**Table S8**). Only *ARNTL1*, but not *PER3* nor *DIO2*, had a rhythmic waveform in the MBH (**Table S8**; **Fig. S3**). Consistent with the previous study, *FSHβ* expression was higher at the vernal equinox compared to all other photoperiod groups (**Fig. 2e** [↗](#)). *PRL* expression was higher in long photoperiod (16L) compared to the two equinox and short photoperiod (8L) (**Fig. 2f** [↗](#)). *FSHβ* did not display a daily rhythm (**Table S8**), which is likely due to the absence of D-box and E-box motifs in the *FSHβ* promoter (**Fig. 2g** [↗](#)). *FSHβ* promoter does contain many DNA motifs that are targeted by several transcription factors that are responsive to hormonal and nutrient pathways indicating multiple upstream regulators are recruited to drive transcription. These data support the conjecture that a long-term programmed increase in *FSHβ* occurs under vernal non-stimulatory photoperiod, and it is not driven by short-term daily photic cues.

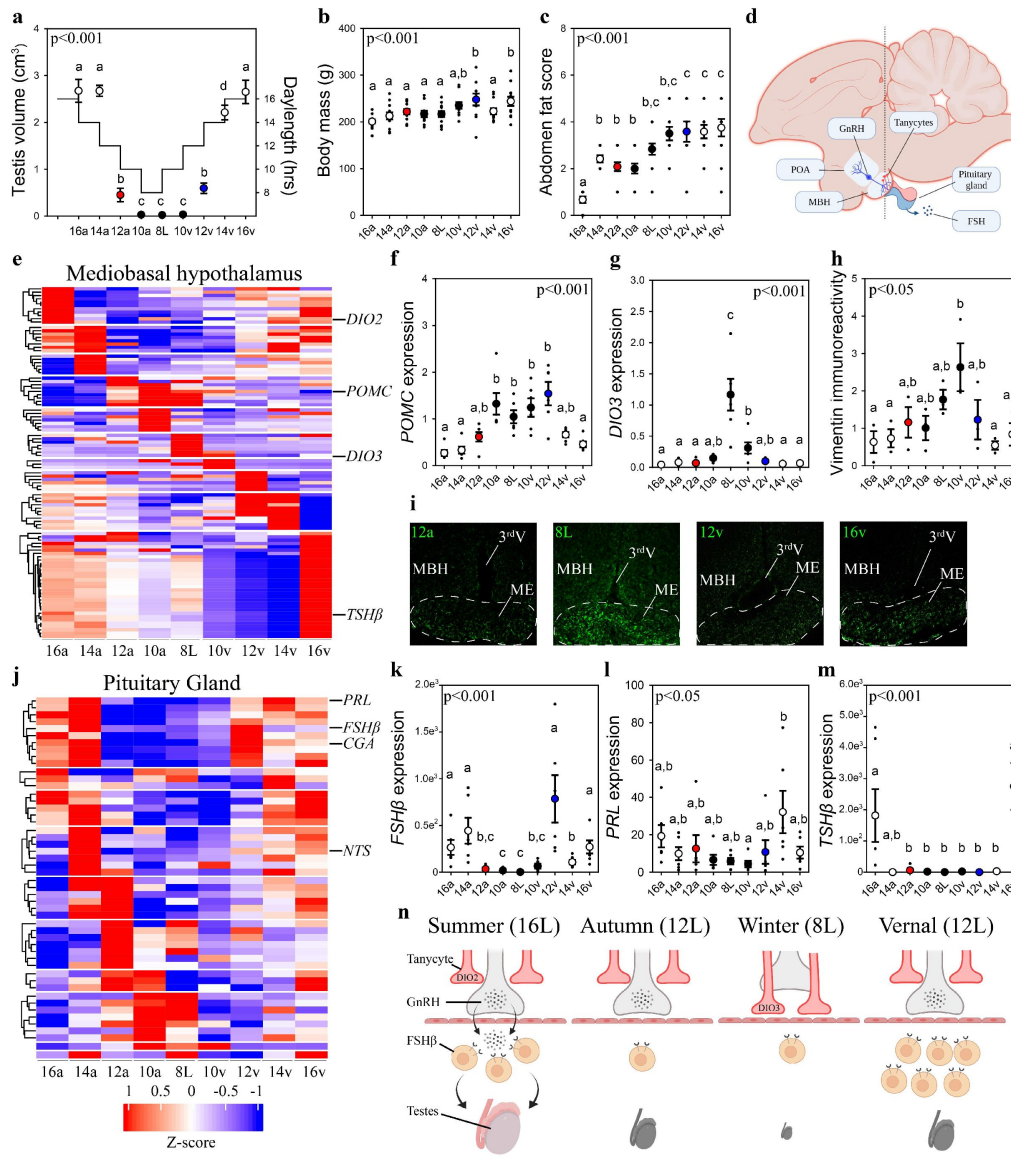


Fig 1.

Vernal increase in pituitary *FSHβ* precedes molecular switches in MBH and testes growth.

a, Schematic representation of the simulated annual rhythm in photoperiod. Quail were collected in 16hr light, 8hr dark photoperiod and then every 2 weeks the photoperiod was decreased by 2hrs to 14hr, 12hr, 10hr and then a 8L short photoperiod. Photoperiod was then increased to mimic the vernal transition and birds were collected at 10hr, 12hr, 14hr and 16hr light photoperiods. Testis volume confirmed critical day length (i.e. >12hr) induced growth. **b**, Body mass and **c**, abdominal fat deposition increased until the autumnal equinox (12a), and then increased during the vernal photoperiod transitions. **d**, Diagram highlighting hypothalamic preoptic area (POA), mediobasal hypothalamus (MBH) and pituitary gland. Tanycytes in the MBH gate GnRH release into the pituitary. **e**, Heat-map of RNA-seq of MBH punches identifies distinct waves of transcripts as quail transition across photoperiodic conditions. **f-g**, qPCR assays for proopiomelanocortin (*POMC*) and deiodinase type-3 (*DIO3*) confirmed restricted activation during 10v-8L and 8L-10v phases, respectively. **h-i**, vimentin immunoreactivity in the median eminence (ME) shows tanycyte morphology growth is limited to 10a, 8L, and 10v photoperiods. **j**, Heat-map illustrating photoperiodic transitions in pituitary transcripts. **k-m**, confirmed that *FSHβ* is elevated under non-stimulatory photoperiods followed by increased prolactin (*PRL*) in 14v and thyrotropin stimulating hormone-β (*TSHβ*) in 16v. **n**, diagram summarizing that long photoperiods increase *GnRH* synthesis and release into the pituitary gland to stimulate *FSHβ* and induce testis growth. Transition to autumnal equinox phases results in reduced *FSHβ* expression and regressed testis. Prolonged exposure to short photoperiods inhibits *GnRH* expression, triggers tanycyte extension, maintains low *FSHβ* and regressed testis. Vernal transitions in photoperiod to the equinox results in resumption of *GnRH* and elevated *FSHβ* expression without testis growth. Data are mean $\pm$ SEM, and residual dot plot. **a-c, f,g,k,m** One-way ANOVA with Bonferroni corrected Tukey's test for multiple comparisons. **h, i** One way ANOVA with Tukey tests for significant pairwise comparison. Letters denote significant difference between photoperiod phase. Raw data available in Table S1.

To identify if *FSHβ* expression is driven by an endogenously programmed mechanism or in response to the gradual increase in light, adult quail were exposed to the 8L, 10v or 12v light schedules or kept in 8L for an additional 4 weeks (8Lext). The 8Lext treatment permitted confirmation whether *FSHβ* expression would increase in that photoperiod, and therefore reflect an internal timing mechanism. *FSHβ* expression increased 19-fold after 4 additional weeks of 8L suggesting that endogenous drivers initiate transcription despite no change in daylength ($P < 0.05$) (**Fig. 3a**). But the dominant stimulator of *FSHβ* expression was the transition to 10v and 12v photoperiod, which both expressed significantly increased levels compared to 8L ($P < 0.001$). As *Opn5* was identified in the pituitary transcriptome (Table S1), we then assessed its transcript expression across the photoperiod treatments. We discovered that *Opn5* expression patterns paralleled *FSHβ* suggesting the potential for direct light detection by *Opn5* and subsequent regulation of *FSHβ* expression. Based on *FSHβ* promoter analyses (**Fig. 2g**), we examined the expression of myocyte enhancer factor 2 (*MEF2a*) expression as a potential upstream regulator of *FSHβ* expression. *MEF2a* remained relatively constant suggesting this transcription factor binding protein is not the primary driver of *FSHβ* expression (**Fig. S4**). Similarly, *DNMT3a* expression did not change across photoperiod treatments (**Fig. S4**) suggesting that epigenetic modifications (i.e., DNA methylation) may not provide the endogenous programmed change leading to constitutive *FSHβ* expression. The precise molecular change upstream from *FSHβ* transcription remains to be identified. In the MBH, *DIO3* displayed a rapid reduction in expression after transfer to 10v and was found to be significantly reduced in 12v photoperiod ($P < 0.001$) (**Fig. 3c**). There was a significant difference in MBH *DIO2* expression (**Fig. S4**), but this observation was driven by a decrease in 8ext. Interestingly, *GNRH* in the preoptic area was found to significantly increase after extended exposure to short photoperiods (**Fig. 3D**). These data indicate that endogenous switches in *FSHβ*, and possibly *GNRH* expression, in response to short photoperiods may reflect multiple independent cellular timers that establish a physiological state of photosensitivity.

Discussion

The data reported herein demonstrate that that multiple independent interval timers are required for the control of seasonal life-history transitions in reproductive physiology (**Fig. 3e**). The two cell types primarily implicated are thyrotropes and gonadotropes. Short winter days initiate a gradual increase in *FSHβ* expression in the pars distalis and maintain low *TSHβ* in the pars tuberalis. As photoperiods increase there is a steady elevation in *FSHβ* expression, however, the release of FSH is prevented as daylengths are below the critical threshold (i.e., 12L:12D) (Gwinner, 1986). Stimulatory daylengths longer than 12hr trigger thyrotropes to increase *TSHβ* leading to a cascade of molecular events in the MBH that permit GnRH to stimulate gonadotropes to release *FSHβ* and initiate gonadal development (Nakao et al., 2008; Yoshimura et al., 2003; Yamamura et al., 2004). Note that other pituitary cell types, somatotropes and corticotropes do not appear to show any molecular switches across the photoperiodic phases. These findings uncover a two-component mechanism for the cellular basis of the external coincidence model for the avian photoperiodic response (**Fig. S5**). The endogenously programmed increase in *FSHβ* establishes a state of photosensitivity to stimulatory day length, and *TSHβ* thyrotropes in the pars tuberalis monitor daylength and when light stimulation occurs during a period of photoinducibility, initiate gonadal development. The two-component model is exciting as it accommodates evidence for endogenous growth of quail gonads in the absence of photostimulation (Follett and Sharp, 1969). Moreover, a TSH-independent programmed change in *FSHβ* expression addresses how seasonal rhythms in tropical, non-photoperiod birds can be regulated (Gwinner and Dittami, 1990).

The high-dimensionality and high-frequency analyses of seasonal transition in physiology used in this study, also uncovered that photoperiod differentially regulates two endocrine systems: reproduction and energy balance. *POMC* has well described roles in the neuroendocrine regulation of food intake and body mass (Yeo and Heisler, 2012). Our findings reveal that photoperiod induced seasonal transitions in reproduction and body mass are not parallel. Based

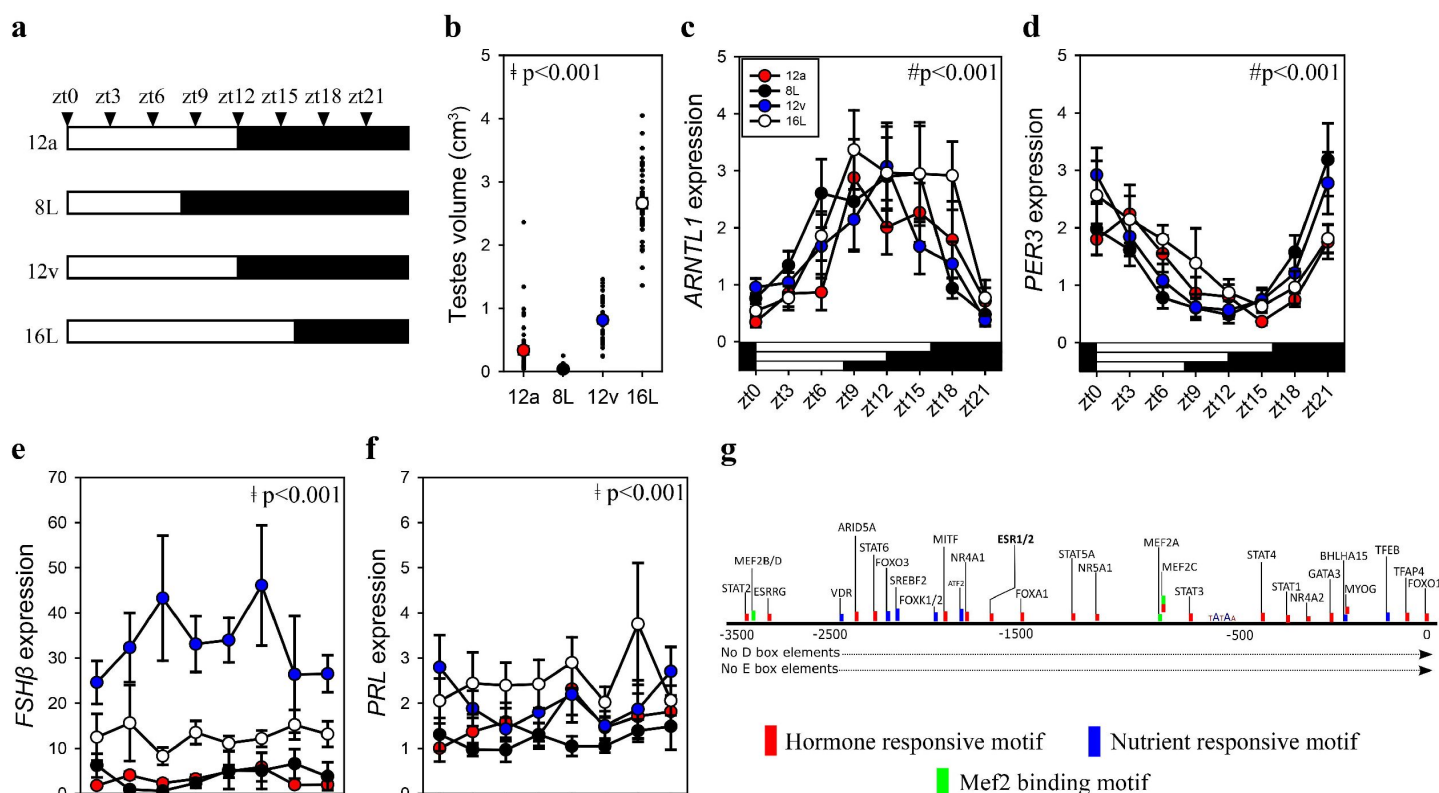


Figure 2.

***FSHβ* is constitutively expressed during the vernal equinox.**

a, schematic representation of four photoperiod treatment groups with arrows to indicate the daily sampling time. **b**, testes volume remained in a regressed non-functional state in autumnal equinox (12a), short photoperiod (8L) and vernal equinox (12v). Photoperiods that exceeded the critical day length (i.e., > 12hr) induced testes growth. **c**, Pituitary circadian clock gene *ARNTL1* maintained daily rhythmic expression waveforms across all photoperiods ($P < 0.001$), no significant difference between photoperiod treatment ($P = 0.42$). **d**, *PER3* displayed a daily waveform across 12a, 8L, 12v and 16L groups ($P < 0.001$) and was anti-phase compared to *ARNTL1*. No significance difference between photoperiod treatments ($P = 0.31$). **e**, *FSHβ* expression was significantly higher in 12v compared to long photoperiods (16L; $P < 0.001$), autumnal equinox (12a; $P < 0.001$) and short photoperiod (8L; $P < 0.001$), but was not rhythmic ($P = 0.66$). **f**, Similarly, *PRL* was high in 16L compared to 12a ($P < 0.001$), and 8L ($P < 0.001$), there was no significant daily rhythm ($P = 0.52$). **g**, *FSHβ* promoter was devoid of circadian gene binding D-box and E-box motifs but contains a series of hormone and nutrient responsive motifs. **b-f** Two-way ANOVA followed by Tukey's pairwise tests, rhythmic analyses were conducted using GraphPad Prism. ‡ indicates significant photoperiod treatment effect; # denotes significant time of day effect. Data are mean $\pm$ SEM, and residual dot plot (a-d). Rhythmic analyses are presented in Table S7 and raw data are available in Table S1.

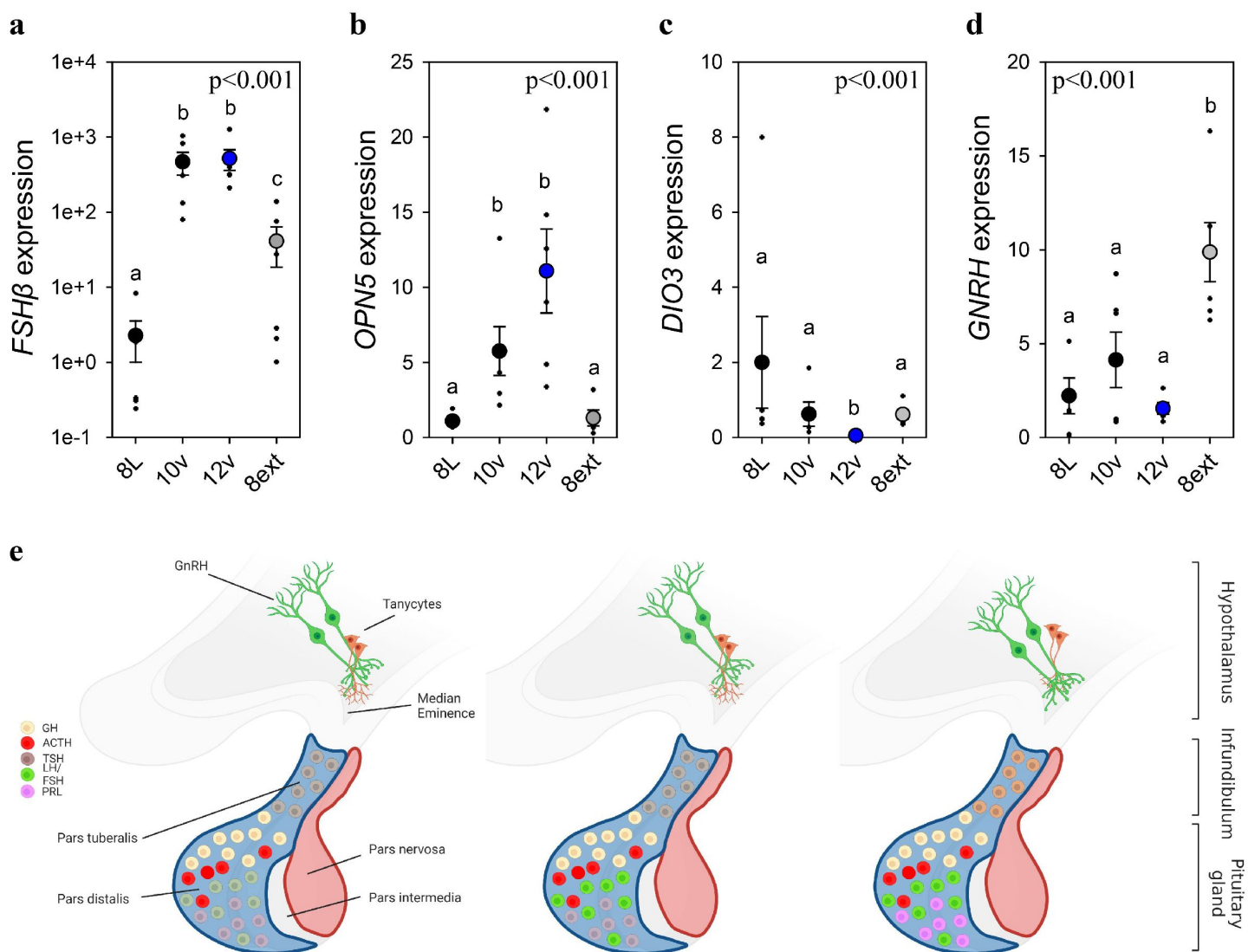


Figure 3.

Endogenous and light-induced *FSHβ* expression.

a, *FSHβ* expression increased during the photoinduced transition from 8L to 10v, and 12v. *FSHβ* also showed a smaller, yet significant increase in expression after prolonged exposure to 8L. Y-axis is presented in log-scale due to the significant increase in *FSHβ* expression in 10v and 12v. **b**, *OPN5* was detected in the pituitary gland and showed a significant increase in expression in the transition to 10v and 12v similar to *FSHβ* expression. **c**, *DIO3* was significantly reduced in 12v quail compared to all other treatment groups. **d**, *GNRH* expression remained constant during the transition from 8L to 12v. However, continued exposure to 8L was observed to increase *GNRH* expression. Data are mean $\pm$ SEM, and residual dot plot (a-d). One-way ANOVA with Tukey's test for multiple comparisons. Letters denote significant difference between photoperiod phase. Raw data available in Table S1. **e**, Schematic representation of the endogenous and light-dependent increase in pituitary cell types during the transition from 8L to stimulatory 16v light treatments. Increased color indicates increased transcript expression.

on these results we propose that a separate light-dependent mechanism independently regulates reproduction and energy balance via the *GNRH* and *POMC* neuronal systems, respectively. This has significant implications for understanding species-specific seasonal transitions in life-histories. The comprehensive transcriptome dataset generated in this study will facilitate ecological studies that seek to uncover the molecular substrates which environmental cues, such as temperature, impact phenological timing and mistiming in birds (Visser and Gienapp, 2019 [↗](#)).

Materials and Methods

Data and code availability statement

All raw data are available in Extended data Table 1. R code used in Study 1 is freely available and indicated in the methods reference list. The full R code used is available upon request.

Animals

All Japanese quail were provided by Moonridge Farms, Exeter United Kingdom (moonridgefarms.co.uk) (<http://moonridgefarms.co.uk/> [↗](#)). Chicks were raised under constant light and constant heat lamp conditions. From 5 weeks of age, birds were held in the Poultry facilities at the University of Glasgow, Cochno Farm for the duration of the photoperiodic experiments. Food (50:50 mix of Johnston and Jeff, quail mix & Farm Gate Layers, poultry layers supplemented with grit) and tap water was provided ad libitum. All procedures were in accordance with the National Centre for the Replacement, Refinement and Reduction of Animals in Research ARRIVE guidelines (<https://www.nc3rs.org.uk/revision-arrive-guidelines> [↗](#)). All procedures were approved by the Animal Welfare and Ethics Review Board at the University of Glasgow and conducted under the Home Office Project Licence PP5701950.

Study 1 – Photoperiod-induced transition in seasonal life history states

Male quail (N=108) were housed in a summer-like long-day (LD) photoperiod (16L:8D). To mimic the autumnal decline and subsequent vernal increase in the annual photoperiodic cycle, birds were exposure to a sequential change in day length from 16L, to 14L, to 12L, to 10L, to 8L, then back to 10L, to 12L, to 14L and lastly 16L (**Fig. 1a** [↗](#)). Each photoperiod treatment lasted for 2 weeks to minimize the impact of photoperiodic history effects (Stevenson et al., 2012a [↗](#)). At the end of each photoperiodic treatment a subset of quail (n=12) was pseudo randomly selected for tissue collection. Birds were killed by cervical dislocation followed by jugular cut. A jugular blood sample was collected in 50ul heparin (Workhardt, UK) and stored at -20°C. Brain and pituitary stalk were rapidly dissected, frozen on powdered dry ice and stored at -80°C. Testes were dissected and weighed to the nearest 0.001g using the Sartorius microbalance (Sartorius, Germany). The length and width of the testes were measured using callipers and volumes were calculated using the equation for a spheroid ($\frac{4}{3} \times 3.14 \times [L/2] \times [W/2]^2$) (King et al., 1997 [↗](#)). Body mass was measured using an Ohaus microscale to the nearest 0.1g. Fat score was assessed using the common scale developed by Wingfield and Farner (1978) [↗](#). The scale range is 0 to 5, which 0 is no visible fat and 5 is bulging fat bodies that exceed the abdominal fat pads.

Study 2 – Daily rhythms in molecular profiles during solstices and equinoxes

To investigate the daily molecular representation of summer-like and winter-like solstices and the autumnal and vernal equinoxes, Japanese quail (N=188) were subjected to the same photoperiodic treatments described in Study 1. A subset of quail was pseudo randomly selected after the autumnal 12L:12D (n=47), short day 8L:16D (n=48), vernal 12L:12D (n=46) and long day 16L:8D (n=47) treatment conditions. For each of the four photoperiodic treatment conditions, five-six

birds were collected shortly after lights on (Zeitgeber time (zt) 0), and then every three hours for twenty-four-hour period. This resulted in a high frequency daily sampling period that included zt0, zt3, zt6, zt9, zt12, zt15, zt18, and zt21. Brain, pituitary gland, and liver was extracted and stored at -80°C . Testes mass was determined as described above.

Study 3 – Endogenous programming of FSH β in the quail pituitary gland

Male quail (N=24) were housed in LD photoperiod for 2 days, and then day length was decreased to 12hr for one day, and then 8L for six weeks. A subset of quail was killed by Home Office approved Schedule 1 methods and established the photoregressed 8L group (n=6). To examine endogenous increase in FSH β expression, a subset of birds (n=6) was maintained in SD photoperiods for four more weeks (8Lext). The other twelve birds were transitioned to the 10L:14D (10v) light treatment for 2 weeks and another subset of birds were collected (n=6). The last subset of birds was transitioned to 12L:12D (12v) for two weeks and were then killed (n=6). For all birds, the brain and pituitary glands were dissected and immediately frozen in dry ice and then placed at -80°C . This experimental design provided the ability to examine endogenous changes in pituitary cell function via the 8Lext group, and the photoinduced increase in photosensitivity (i.e., 10v and 12v).

Hypothalamic and pituitary dissection

During brain extraction the pituitary stalk was severed. The procedure leaves pituitary gland components of the pars intermedia, pars distalis and pars nervosa resting in the sphenoid bone. To isolate the anterior hypothalamus/preoptic area and the mediobasal hypothalamus, we used a brain matrix and coordinates based on previously published anatomical locations ([Stevenson et al., 2012b](#); [Nakao et al., 2008](#), respectively). Brains were placed ventral surface in an upward direction. For the anterior hypothalamus/preoptic area a 2mm diameter from 2mm brain slice was collected. The rostral edge of the optic nerve was identified and then a 1mm cut in the rostral and 1mm cut in the caudal direction was performed. Brain slices were checked to confirm the presence of the tractus septomesencephalicus in the rostral section and the decussation supraoptica dorsalis in the caudal section. These anatomical regions reliably capture the GnRH neuronal population in birds ([Stevenson and Ball, 2009](#)). The MBH was isolated from a 2mm punch from a 3mm brain slice that spanned the decussation supraoptica dorsalis to the nervus oculomotorius ([Nakao et al., 2008](#)).

Ribonucleic acid extraction and qPCR

RNA was extracted from anterior hypothalamus/preoptic area, mediobasal hypothalamus and pituitary gland tissue using QIAGEN RNeasy Plus Mini Kit (Manchester, UK). Nucleic acid concentration and 260/280/230 values were determined by spectrophotometry (Nanodrop, ThermoScientific). cDNA was synthesized from 100ng RNA using SuperScript III (Invitrogen), and samples stored at -20°C until quantitative PCR (qPCR) was performed. qPCRs were conducted using SYBR Green Real-time PCR master mix with 5ul cDNA and 10ul SYBR and primer mix. PCR primer sequences and annealing temperatures are described in Extended Data Table S10. qPCRs for mRNA expression in tissue were performed using Stratagene MX3000. qPCR conditions were an initial denature step at 95°C for 10 min. Then, 40 cycles of a denature at 95°C for 30 seconds, a primer specific annealing temperature for 30 seconds, and then an extension phase at 72°C for 30 seconds. The qPCR reaction was terminated at 95°C for one-minute. A melt curve assay was included to confirm specificity of the reactions. The efficiency and cycle thresholds for each reaction was calculated using PCR Miner ([Zhao and Fernald, 2005](#)). All samples were assessed based on the Minimum Information for Publication of Quantitative Real-Time PCR guidelines ([Bustin et al., 2009](#)).

Minion transcriptome sequencing

RNA for transcriptome sequencing was extracted using QIAGEN RNeasy Plus Mini Kit (Manchester, UK). RNA concentration and 260/280/230 values were determined by Nanodrop spectrophotometer. RNA was synthesized into cDNA using Oxford Nanopore Direct cDNA Native Barcoding (SQK-DCS109 and EXP-NBD104) and followed the manufacturers protocol. A total of 6 Spot-ON Flow cells (R9 version FLO-MIN106D) were used for each tissue. A single quail was randomly selected from each treatment group so that a single Flow cell had $n=9$ samples giving a total of $N=56$ quail for mediobasal hypothalamus and pituitary stalk transcriptome sequencing. Transcriptome sequencing was conducted using MinION Mk1B (MN26760, Oxford Nanopore Technologies).

Sequencing was performed by MinKNOW version 20.10.3 and Core 4.1.2. The parameters for each sequencing assay were kept to 48hrs, -180mV voltage and fast5 files saved in a single folder for downstream bioinformatic analyses.

Transcriptome Analyses

The transcriptome data analysis pipeline is outlined in Extended Data Figure S6. All bioinformatic steps were conducted using R Studio and run in a Conda environment. First, fast5 files were demultiplex and basecalled by Guppy 4.2.1. Then Porechop v0.2.4 was conducted to remove adapters from reads followed by Filtlong v0.2.0 to filter long reads with minimum 25 bases and mean q weight of 9. Transcripts were aligned to the Japanese quail reference genome and transcriptome using Minimap2 v2.17 (Li, 2018 [\[1\]](#)). Transcript expression levels were determined using Salmon v0.14.2 and EdgeR v3.24.3 for normalisation and differential expression (Patro et al., 2017 [\[2\]](#)). DAVID was conducted to identify functional pathways active during the transitions across photoperiodic states (Extended Data Table 2 and Table 4) (Dennis, 2003 [\[3\]](#)).

BioDare2.0 analyses of significant differentially expressed genes

To identify seasonal rhythmic expression of transcripts, we selected differentially expressed genes identified by EdgeR ($P < 0.05$). Data were analysed using non-linear regression for rhythmicity using the online resource BioDare 2.0 (Zielinski et al., 2014) (biodare2.ed.ac.uk). The empirical JTK_CYCLE method was used for detection of rhythmicity and the classic BD2 waveform set was used for comparison testing. The type of transcript rhythmicity was confirmed as (e.g., sine/cos/arcsine) or non-rhythmic (spike) expression. Rhythmicity was determined by a Benjamini-Hochberg controlled p -value (BH corrected $p < 0.1$). Data for heatmaps were clustered using PAM clustering from the cluster package (29). Heatmaps were created using the Complexheatmaps package (Gu et al., 2016 [\[4\]](#)). Heat maps generated from statistically significant transcripts are presented in Fig1 [\[5\]](#) and Extended Data Table 3 and Table 5.

Enrichment factor identification in promoters of differentially expressed genes

To explore potential upstream molecular pathways involved in the regulation of differentially expressed genes, we conducted a transcription factor analyses. The aim was to identify which transcription factor or factors are responsible for observed changes in gene expression and whether, if any overlap occurs across tissues. Transcription factor enrichment analysis was achieved using ChIP-X Enrichment Analysis 3 (ChEA3) (Keenan et al., 2019 [\[6\]](#)). We used a conservative approach and only used transcripts detected as significant by BioDare 2.0 analysis (BH corrected $p < 0.05$). Enriched transcription factors were ranked using the ENCODE database and presented in Extended Data Table 6 and Table 7.

Daily waveform analyses of transcript expression

To establish rhythmicity in daily waveform expression of MBH and pituitary transcripts, we conduct cosinor analyses (Cornelissen, 2014 [↗](#)). $2\text{-}\Delta\Delta$ Cycling time (Ct) values obtained for the genes investigated in Study 2 were subjected to cosinor analyses based on unimodal cosinor regression [$y = A + (B\cos(2\pi(x - C)/24))$], where A, B, and C denote the mean level (mesor), amplitude, and acrophase of the rhythm, respectively. The significance of regression analysis determined at $p < 0.05$ was calculated using the number of samples, R² values, and numbers of predictors (mesor, amplitude, and acrophase) (Singh et al., 2013 [↗](#)). Data are compiled in Extended Data Table 8.

Bioinformatic analyses of FSH β promoter

To identify potential links between transcription factors identified using bioinformatic tests, and transcriptome data, we examined binding motifs in the FSH β promoter. The upstream promoter sequence of 3500 bp (−3500 to 0) of Japanese quail FSH β was obtained from Ensemble (<http://www.ensembl.org/index.html> [↗](#)). This promoter sequence was analysed by CiiDER transcription binding factor (TBFs) analysis tool (Gearing et al., 2019 [↗](#)) against JASPER core database. DNA motifs in the promoter were unique for 470 transcription binding factors. The top 80 transcription binding factors were then subjected to PANTHER gene ontology enrichment analyses (Mi et al., 2013 [↗](#)). Several pathways were discovered and included hormone responsive, epigenetic and responsive to nutrients (Extended Data Table 9).

Immunocytochemistry and histological analyses

Snap frozen brains from experiment 1 were sectioned at 20 μ m using a cryostat (CM1850, Leica). The tissues sections were collected in supercharged slides (631-0108, VWR) and stored in −80°C till processed for staining. We used mouse monoclonal antibody (OAAEE00561, Aviva systems) raised against the Human vimentin gene (NCBI Reference Sequence: NP_003371.2). The antibody has been shown to specifically bind with vimentin expressed in avian cells (<https://www.avivasysbio.com/vim-antibody-oaee00561.html> [↗](#)). Human and quail vimentin peptide show high similarity of 86.96% similarity. We used Goat Anti-mouse Alexa Flour488 (A11001, Invitrogen) for the secondary antibody. As negative control procedures, we performed omission of primary antibody and omission of secondary antibody.

Immunocytochemistry was performed using the standard immunofluorescence protocol (Majumdar et al., 2015 [↗](#)) with minor modifications. Briefly, sections (brain) in slides were first enclosed in margin using ImmEdge pen (H-4000, Vector Labs). The sections were then first post fixed in 10% neutral buffered formalin (5735, ThermoScientific) for 4 hours. After fixing, the sections were washed (3 times; 5 min each) with TBS (phosphate buffer saline with 0.2% triton). Then they were blocked in 20% Bovine serum albumin (BSA) in TBS for 1 hour at room temperature (RT). Subsequently the blocking solution was removed by pipetting and the sections were incubated with primary antibody (1:300 dilution) for 2 hours at RT and finally overnight at 4°C. The next day, sections were first washed with TBS (3 times; 5 min each) and then incubated with secondary antibody (1:200 dilution) for 2 hours at RT. Finally, the sections were again washed with TBS (3 times; 5 min each) and mounted in Fluoromount-G mounting media with DAPI (004959-52, Invitrogen). The dried slides were visualized using Leica DM4000B fluorescence microscope equipped with Leica DFC310 FX camera. Leica Application Suite (LAS) software was used for image acquisition. All the images were taken at constant exposure for the FITC channel at 10X and 20X magnification.

For analysis and quantification of % area, ImageJ version 1.53j was used. For this, 20X images were first converted into greyscale images (8 bit) and a threshold applied. The threshold was determined using the triangle method on multiple randomly selected images and applied for all the images. The scale of measurement in ImageJ was then set to CM in 300pixels/cm scale. A region of interest in the median eminence and dorsal 3rdV ependymal layer was specified as 300*500

pixels (1*1.67 cm scaled units) and area fraction was measured. At least 3 images from each animal were measured and averaged for each bird. A total of 27 quail were used and distributed evenly across photoperiod treatments (n=3).

Statistical analyses and figure presentation

GLM tests were conducted to test for statistical significance. One-way ANOVA with Bonferroni correction was applied to testes mass, body mass, fat score, vimentin immunoreactivity and qPCR analyses in Study 1. Two-way ANOVAs with photoperiod and zeitgebers main effects were conducted on qPCR analyses in Study 2. One-way ANOVAs were conducted for qPCR data in Study 3. qPCR data were log-transformed if violation of normality was detected. Significance was determined at $P < 0.05$. Figures were generated using Adobe Illustrator and BioRender was used to create images in panels Fig1d,n; Fig3e and Extended Data Figure 6.

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Reviewer #1 (Public Review):

The authors investigated the molecular correlates in potential neural centers in the Japanese quail brain associated with photoperiod-induced life-history states. The authors simulated photoperiod to attain winter and summer-like physiology and samples of neural tissues at spring, and autumn life-history states, daily rhythms in transcripts in solstices and equinox, and lastly studies FSHb transcripts in the pituitary. The experiments are based on a series of changes in photoperiod and gave some interesting results. The experiment did not have a control for no change in photoperiod so it seems possible that endogenous rhythms could be another aspect of seasonal rhythms that lack in this study. The short-day group does not explain the endogenous seasonal response.

The manuscript would benefit from further clarity in synthesizing different sections. Additionally, there are some instances of unclear language and numerous typos throughout the manuscript. A thorough revision is recommended, including addressing sentence structure for improved clarity, reframing sentences where necessary, correcting typos, conducting a grammar check, and enhancing overall writing clarity.

Data analysis needs more clarity particularly how transcriptome data explains different physiological measures across seasonal life-history states. It seems the discussion is built around a few genes that have been studied in other published literature on quail seasonal response. Extending results on the promotor of DEGs and building discussion is an extrapolating discussion on limited evidence and seems redundant.

Last, I wondered if it would be possible to add an ecological context for the frequent change in the photoperiod schedule and not take account of the endogenous annual response. Adding discussion on ecological relevance would make more sense.

Reviewer #2 (Public Review):

This study is carefully designed and well executed, including a comprehensive suite of endpoint measures and large sample sizes that give confidence in the results. I have a few general comments and suggestions that the authors might find helpful.

1. I found it difficult to fully grasp the experimental design, including the length of light treatment in the three different experiments (which appears to extend from 2 weeks

up to 8 weeks). A graphical description of the experimental design along a timeline would be very helpful to the reader. I suggest adding the respective sample sizes to such a graphic, because this information is currently also difficult to keep track of.

2. The authors use a lot of terminology that is second nature to a chronobiologist but may be difficult for the general reader to keep track of. For example, what is the difference between "photoinducibility" and "photosensitivity"? Similarly, "vernal" and "autumnal" should be briefly explained at the outset, or maybe simply say "spring equinox" and "fall equinox."
3. What was the rationale for using only male birds in this study? The authors may want to include a brief discussion on whether the expected results for females might be similar to or different from what they found in males, and why.
4. The authors used the Bonferroni correction method to account for multiple hypothesis testing of measures of testes mass, body mass, fat score, vimentin immunoreactivity and qPCR analyses in Study 1. I don't think Bonferroni is ever appropriate for biological data: these methods assume that all variables are independent of each other, an assumption that is almost never warranted in biology. In fact, the data show clear relationships between these endpoint measures. Alternatively, one might use Benjamini-Hochberg's FDR correction or various methods for calculating the corrected alpha level.
5. The graphical interpretations of the results shown in Figure 1n and Figure 3e, along with the hypothesized working model shown in Figure S5, might best be combined into a single figure that becomes part of the Discussion. As is, I do not think these interpretative graphics (which are well done and super helpful!) are appropriate for the Results section.

Reviewer #3 (Public Review):

It is well known that as seasonal day length increases, molecular cascades in the brain are triggered to ready an individual for reproduction. Some of these changes, however, can begin to occur before the day length threshold is reached, suggesting that short days similarly have the capacity to alter aspects of phenotype. This study seeks to understand the mechanisms by which short days can accomplish this task, which is an interesting and important question in the field of organismal biology and endocrinology.

The set of studies that this manuscript presents is comprehensive and well-controlled. Many of the effects are also strong and thus offer tantalizing hints about the endo-molecular basis by which short days might stimulate major changes in body condition. Another strength is that the authors put together a compelling model for how different facets of an animal's reproductive state come "on line" as day length increases and spring approaches. In this way, I think the authors broadly fulfill their aims.

I do, however, also think that there are a few weaknesses that the authors should consider, or that readers should consider when evaluating this manuscript. First, some of the molecular genetic analyses should be interpreted with greater caution. By bioinformatically showing that certain DNA motifs exist within a gene promoter (e.g., FSHbeta), one is not generating robust evidence that corresponding transcription factors actually regulate the expression of the gene in question. In fact, some may argue that this line of evidence only offers weak support for such a conclusion. I appreciate that actually running the laboratory experiments necessary to generate strong support for these types of conclusions is not trivial, and doing so may even be impossible. I would therefore suggest a clear admission of these limitations in the paper.

Second, I have another issue with the interpretation of data presented in Figure 3. The data show that FSHbeta increases in expression in the 8Lext group, suggesting that endogenous drivers likely act to increase the expression of this gene despite no change in day length. However, more robust effects are reported for FSHbeta expression in the 10v and 12v groups, even compared to the 8Lext group. Doesn't this suggest that both endogenous mechanisms and changes in day length work together to ramp up FSHbeta? The rest of the paper seemed to emphasize endogenous mechanisms and gloss over the fact that such mechanisms likely work additively with other factors. I felt like there was more nuance to these findings than the authors were getting into.

Third, studies 1 - 3 are well controlled; however, I'm left wondering how much of an effect the transitions in day length might have on the underlying molecular processes that mediate changes in body condition. While the changes in day length are themselves ecologically relevant, the transitions between day length states are not. How do we know, for example, that more gradual changes in day length that occur over long timespans do not produce different effects at the levels of the brain and body? This seemed especially relevant for study 3, where animals experience a rather sudden change in day length. I recognize that these experimental methods are well described in the literature, and they have been used by endocrinologists for a long time; nonetheless, I think questions remain.