Dissociating mechanisms that underlie seasonal and developmental programs for the neuroendocrine control of physiology in birds

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Title: Dissociating mechanisms that underlie seasonal and developmental programs for the neuroendocrine control of physiology in birds

Abbreviated title: Age-dependent photoperiodism in quail

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Abstract

Long-term programmed rheostatic changes in physiology are essential for animal fitness. Hypothalamic nuclei and the pituitary gland govern key developmental and seasonal transitions in reproduction. The aim of this study was to identify the molecular substrates that are common, and unique to developmental and seasonal timing. Adult and juvenile quail were collected from reproductively mature and immature states and key molecular targets examined in the mediobasal hypothalamus (MBH) and pituitary gland. qPCR assays established deiodinase type-2 (DIO2) and type-3 (DIO3) expression in adults changed with photoperiod manipulations. However, DIO2 and DIO3 remain constitutively expressed in juveniles. Pituitary gland transcriptome analyses established 340 transcripts were differentially expressed across seasonal photoperiod programs; and 1189 transcripts displayed age-dependent variation in expression. Prolactin (PRL) and follicle-stimulating hormone subunit beta (FSHβ) are molecular markers of seasonal programs and are significantly upregulated in long photoperiod conditions. Growth hormone expression was significantly upregulated in juvenile quail, regardless of photoperiodic condition. These findings indicate that a level of cell autonomy in the pituitary gland governs seasonal and developmental programs in physiology. Overall, this paper yields novel insights into the molecular mechanisms that govern developmental programs and adult brain plasticity.
Significance statement

Seasonal physiology is pervasive in the animal kingdom. While much is known regarding how the brain perceives annual changes in daylength (also referred to as photoperiod) and dynamics of the neuroendocrine control of seasonal physiology in adult animals, studies in juveniles are limited. Here, we assess genome-wide and targeted transcriptomic changes in the pituitary gland, a key brain region connecting photoreception with physiological plasticity in adult and juvenile Japanese quail. The analyses identified several novel transcripts that are correlated with photoperiod- and developmental programs in seasonal physiology. The findings demonstrate a level of pituitary gland cell specificity for the regulation of both development and reproductive fitness, that is dependent on both age and experienced photoperiod.
Introduction

Seasonal and developmental programs in the neuroendocrine control of reproductive physiology in mammals and birds are well characterised (Stevenson et al 2012, Plant 2015, Stevenson et al., 2017). However, very few studies have directly compared the similarities and differences in how the hypothalamus governs these long-term changes in physiology. Animals in temperate regions experience variable environmental conditions within and across seasons, including changes in daylength, ambient temperature, and availability of food (Sharp 1996, Hau 2001). The annual change in daylength, referred to as photoperiod, is a powerful signal that animals use as a predictive cue to anticipate environmental conditions ideal for breeding (Bradshaw and Holzapfel 2007, Wood and Loudon 2014, Payton et al 2017, Stevenson et al 2022). In most mammals, the nocturnal duration of melatonin secretion from the pineal gland provides a physiological code of photoperiod and drives many molecular, cellular, and morphological changes in the median eminence and pituitary gland (Wood and Loudon 2014, Stevenson et al 2022). Conversely, birds have photoreceptors located deep in the hypothalamus that directly detect light stimulation to drive photoperiod induced changes in seasonal physiology (Perez et al 2019, Liddle et al 2022). Despite these markedly different neuroendocrine control mechanisms, the pituitary gland is a conserved anatomical structure that provides the essential gating mechanism to permit and inhibit communication from the hypothalamus to peripheral tissues (e.g., gonads).

Juvenile animals have a heightened sensitivity to the effects of photoperiod on reproductive physiology. Siberian hamsters raised in long summer-like photoperiods initiate puberty at approximately post-natal day 21 (Spears et al 1990). Transfer of hamsters to short winter-like photoperiods induces a rapid inhibition of gonadal development resulting in gonadal involution in less than 7 days (Prendergast et al 2004). A single sub-cutaneous injection of melatonin to weaned hamsters (i.e., postnatal day18) is sufficient to prevent gonadal growth,
despite being housed in stimulatory long photoperiods (Prendergast et al 2013). Similarly, Japanese quail reared in long stimulatory photoperiods reach sexual maturity by 28-35 days post-hatch (Follett 1976). Birds that are transferred to shorter photoperiods (e.g., 12 light, 12 dark) delay gonadal growth by up to 2 weeks (Follett and Maung 1978) and photoperiods less than 12hr light completely prevent reproductive maturation (Follett and Sharp 1969, Abdelnabi and Ottinger, 2003).

Only a few studies have examined how photoperiod manipulations early in development impact the neuroendocrine regulation of reproductive physiology. In adult birds and mammals, the expression of deiodinase enzymes, type-2 (DIO2) and type-3 (DIO3) are molecular signatures of photoperiodic state (Nakane and Yoshimura 2014, Rani and Kumar 2014). Long photoperiods increase DIO2 expression which catalyses the conversion of inactive thyroxine (T4) into active triiodothyronine (T3), short photoperiods initiate the reversal by stimulating DIO3 expression and reducing hypothalamic triiodothyronine (Yoshimura et al 2003, Barrett et al 2007). In juvenile Siberian hamsters, transfer to short photoperiods or sub-cutaneous injections of melatonin rapidly increase DIO3 expression in the mediobasal hypothalamus (Prendergast et al 2013, Sáenz de Miera et al 2017). Whether similar rapid changes occur in juvenile birds is currently unknown. Resolving whether DIO3, or DIO2 display an augmented sensitivity to photoperiodic cues early in development is important to develop our understanding of conserved neuroendocrine pathways that underlie photoperiod, and developmental programming of seasonal physiology.

Seasonal and reproductive maturation requires the release of gonadotropin-releasing hormone (GNRH) into the hypophyseal portal system, allowing for transport into the pituitary gland where GNRH acts on gonadotrophs to stimulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Stevenson et al 2012). Therefore, the pituitary gland can be viewed as the final mediator between seasonal and developmental programming of
seasonal physiology. The objectives of this study were twofold. First, the expression profiles of DIO2 and DIO3 in the mediobasal hypothalamus (MBH) tissue were examined in adult and juvenile Japanese quail held in long- or short-photoperiods. The findings indicate neither DIO2 nor DIO3 show a similar pattern in adults compared to juveniles suggesting a clear dissociation between developmental programming and seasonal photoperiodic regulation of deiodinase expression. The second objective was to identify seasonal and developmental programmed changes in the pituitary gland. Oxford Nanopore RNA sequencing was conducted to produce a pituitary transcriptome and enable a comprehensive overview of age- and light-dependent molecular changes. The data described herein suggest cellular specificity in how the pituitary gland controls long-term programs in physiology.

**Materials and Methods**

**Animals and ethical permissions**

Japanese quail were purchased from Moonridge Farms, Exeter. 5-week-old male quail (n=12) were housed on a 16L:8D (L = light, D = dark) light schedule. All birds were provided Farmgate layers pellets and tap water *ad libitum*. 2-day-old Japanese quail eggs (n=40) were placed in an incubator (Brinsea OVA-Easy Advance 380, Temp = 37.5 Celsius, Relative Humidity = 50-60%) until hatching (16-18 days). Hatched chicks (n=8 males, n=3 females) were moved to quail pens provided with heat lamps (37.5 Celsius) and provided blended Heygates Superstarter Crumb and tap water *ad libitum*. Animal research was conducted according to the ARRIVE guidelines and Home Office approved Project and Establishment licenses. All animal procedures were performed in accordance with the relevant establishment’s animal care committee regulations.

**Experimental design**
5-week-old birds were acclimated to a long photoperiod for 2 weeks. Then birds were placed on non-stimulatory photoperiods to induce gonadal involution and collected in short days (8L) after 8 weeks. A subset of the short-day birds (n=6) was humanely killed using cervical dislocation followed by decapitation. Another group (n=6) were placed on stimulatory photoperiods to induce gonadal growth and collected in long photoperiod after 8 weeks. Newly hatched juveniles were acclimated to a long photoperiod for 5 days. Birds were divided into 2 groups pseudo-randomly assigned in either a long (16L, n=6) or short photoperiod (8L, n=5) for 5 days. The 16L juvenile group contained 4 males and 2 females, whereas the 8L juvenile group contained 4 males and 1 female. On post-hatch day 10, chicks were killed by cervical dislocation followed by decapitation. For all birds, brain and pituitary tissues were frozen on dry ice and stored at -70 Celsius. During collections, the pituitary stalk was severed, leaving the pars tuberalis attached to the mediobasal hypothalamus (MBH). Pituitary tissue including the pars distalis, retained for both qPCR analyses and sequencing, was dissected from the sella turcica. Testes length was measured using callipers to the nearest 0.01mm. Ovary length of the 3 female chicks was measured to confirm photoperiod state. Birds were assigned a fat score from 0-5 based on a scale established in white-crowned sparrows (Wingfield and Farner 1978).

**Whole brain dissections**

To isolate the MBH/hypothalamic regions, a brain matrix was used within a cryostat chamber. Previously published anatomical coordinates acted as guidance for dissection (Nakao et al 2008, Stevenson et al 2012). Brains were positioned ventral side up and oriented with the caudal direction facing upward. The MBH was dissected using brain matrix. First a 3mm coronal section from the optic chiasm in a posterior direction was performed. Then 2mm lateral cuts and a 2mm dorsal cut were conducted to isolate the MBH. This dissection
protocol reliably isolates the MBH in Japanese quail (Majumdar et al., 2023). Brains were then returned to -70 Celsius.

**RNA extraction and cDNA synthesis**

For all samples, RNA was extracted using TRIzol reagent following manufacturers protocol. RNA was then purified using RNEasy MinElute cleanup kit (QIAGEN). RNA quality and purity were measured with Nanodrop ND-100 (NanoDrop Technologies). Both pituitary gland and hypothalamic RNA retained for cDNA synthesis and qPCR analyses, however some pituitary gland RNA was also retained for direct use in RNA sequencing. cDNA was synthesised from TRIzol-extracted RNA using a reaction mixture containing 4μl ~50ng/μl total RNA (~200 ng total), 2μl 5X first strand buffer (Thermofisher Scientific), 1μl DTT (10mM), 0.2μl 20mM Random Primers (Promega), 0.2μl 20mM dNTP mix (Thermofisher Scientific), 0.26μl RNasin® Ribonuclease Inhibitor (Promega), 0.26μl Superscript III reverse transcriptase (Thermofisher Scientific), and 2.08μl RNAse free water. The reaction mixture was incubated at 42 Celsius for 1 minute followed by 50 Celsius for 1 hour. cDNA mixtures were diluted in LOTE buffer (3mM Tris-HCL (Thermofisher Scientific) and 0.2 mM EDTA(Sigma)) before storage at -20 Celsius.

**q-PCR procedure**

Transcription levels of target genes were quantified using SYBR Green Real-Time PCR master mix (Thermofisher Scientific) and specific primer sequences for amplification (Table 1). cDNA samples were amplified using an Agilent Stratagene Mx3000p under the following conditions: 1) denaturing: 95 Celsius for 10 minutes; 2) cycling: 45 times through a 95 Celsius denature for 30 seconds, an annealing temperature for 1 minute (primer specific) and an extension of 72 Celsius for 1 minute. A melting curve assay after the qPCR amplification consisted of increasing from 55 Celsius, held for 30 seconds, to 95 Celsius. PCR Miner was
used to quantify cycling times, reaction efficiency, and sample variability (Zhao and Fernald 2005). β-Actin was chosen as the reference gene for quantification of targeted transcript expression. The standard Delta-Delta Ct method was used to produce fold change in gene expression (2^-ΔΔCt), with the adult 8L group acting as the reference for the calculation of the ΔΔ value.

**Oxford Nanopore RNA-sequencing procedure**

TRIzol-extracted RNA was sequenced using a GridION (Oxford Nanopore Technologies) after processing using the protocol outlined in the Oxford Nanopore Technologies PCR-cDNA sequencing barcoding kit (SQK-PCB109). Raw Fast5 reads were base called and demultiplexed using the guppy base caller before removing adapters from reads and filtering long reads (>25bp) using Porechop and Filtlong respectively. The parameters for each sequencing assay included running at a voltage of -180mV for 72 hours. Gene transcripts were aligned to a reference *Coturnix japonica* genome (https://www.ncbi.nlm.nih.gov/datasets genome/GCF_001577835.1/) before using Salmon to quantify transcript expression levels. EdgeR was used to identify differentially expressed genes based on their significance value (P<0.01). For analyses comparing all groups, the adult 8L group was chosen as the reference for comparison.

**Gene ontology**

Functional annotation of transcripts identified by Oxford Nanopore RNA-sequencing was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, Sherman et al 2022). Significantly differentially expressed transcripts across both photoperiod and age were categorised using the functional annotation chart tool.

**Statistical analyses and plots**
All raw data are provided in extended data (Table 2-1). 2-way ANOVA was conducted on testes length, fat score, and hypothalamic DI02 and DI03 expression. EdgeR was used to determine statistically significant differentially expressed transcripts in the pituitary gland (Robinson et al 2010, McCarthy et al 2012, Chen et al 2016). For the testes length and fat score measures, the 3 juvenile females were omitted from the analyses. All plots were created in RStudio (R Core Team 2021, RStudio Team 2022) using the ggplot2 package and figures created using Adobe Illustrator. For all statistical analyses, a p-value of <0.05 (and a false discovery rate, FDR<0.2, where applicable) was defined as the statistical cut-off for significance. This FDR significance threshold was determined based on the knowledge that FSHβ is significantly upregulated in long day adults.

Results

Short photoperiods induce gonadal and adipose involution in adult and juvenile quail

Two-way ANOVA identified that short photoperiod significantly reduced testes length in adult and juvenile quail (Fig.1A, Table 2). The accompanying table includes F- and P-statistics for all two-way ANOVA analyses in both figures 1 and 3. Adult quail had significantly larger testes compared to juveniles. There was a significant interaction suggesting photoperiod effects were larger in adults compared to juvenile birds. Two females in long photoperiod had large ovaries (0.49cm and 0.57cm) and the single female in short photoperiod had a regressed ovary (0.13cm), however it was not possible to collect data regarding follicular development. Short photoperiod also reduced adipose tissue indicated by lower fat scores compared to birds in long photoperiod (Fig.1B). Juvenile quail had lower fat scores compared to adult birds. There was no significant photoperiod by development interaction. All 3 juvenile females, omitted from the fat score analyses, had a fat score of 3.

Age-dependent photoperiodic changes in hypothalamic deiodinase expression
Two-way ANOVA identified a significant photoperiod by age interaction for hypothalamic

$DIO2$ expression (Fig.1C, Table 2). Overall, there was no significant main effect of
photoperiod on $DIO2$ expression. There was a significant effect of age on $DIO2$ expression.
There was a significant interaction for hypothalamic $DIO3$ expression (Fig.1D). There was
also a significant main effect of photoperiod and age. These finding suggest that $DIO2$ and
$DIO3$ is highly sensitive to photoperiodic state in adult quail with significantly higher levels
in long photoperiod and short photoperiod, respectively. Surprisingly, $DIO2$ was observed to
be significantly elevated in short photoperiods in juvenile birds. $DIO3$ expression levels were
similar in both long- and short-photoperiod conditions.

Photoperiod or developmental induced changes in transcript expression in the pituitary
gland

Our sequencing assays resulted in transcriptomes being produced with an average N50 value
of approximately 1kb, and an average depth of 4.53. edgeR analyses identified 206 transcripts
that were differentially expressed between long and short photoperiod treatments in adults
(Fig.2A; Extended Data Table 2-1). 126 transcripts were upregulated and 80 were
downregulated in long compared to short photoperiod. As anticipated, $FSH\beta$, $PRL$, and
neurotensin ($NTS$) were found to be differentially expressed between long and short
photoperiods in adults. 184 transcripts were differentially expressed between long and short
photoperiod treatments in juveniles (Fig.2B). 40 transcripts were upregulated and 144 were
downregulated in long compared to short photoperiod. $HAPLN1$ was found to be
differentially expressed between long and short photoperiods in juveniles. 685 transcripts
were differentially expressed between 16L adults and 16L juveniles (Fig.2C). 175 transcripts
were upregulated and 510 were downregulated. $GH$ was found to be differentially expressed
between 16L adults and 16L juveniles. 678 transcripts were differentially expressed between 8L adults and 8L juveniles (Fig. 2D). 422 transcripts were upregulated and 156 were downregulated. Again, GH was identified as a differentially expressed transcripts between 8L adults and 8L juveniles. edgeR identified 331 transcripts were differentially expressed across the four treatment groups (Fig. 3A). A heatmap of the top 50 most significantly differentially expressed genes comparing across all 4 groups (adult and juvenile 8L and 16L) was plotted, including FSHβ and GH (Fig. 3A). PRL, FSHβ, GH, OPN5, NTS, MEF2A, and MEF2D were selected to plot the counts per million for each treatment group (Extended Data Fig. 3-1 A-G).

Relevant statistics have been provided in the appropriate figure legend.

qPCR analyses confirm transcript expression identified in transcriptome analyses

In order to confirm the differential expression, qPCR analyses were performed on select neuroendocrine transcripts of interest including: FSHβ, LHβ, PRL, GH, and OPN5. There was a significant main effect of photoperiod (Fig. 3B, Table 2) and age for FSHβ expression. There was no significant interaction on FSHβ expression. These data support the conjecture that FSHβ expression is elevated in long photoperiods and is higher in adults. There was a significant photoperiod by age interaction on LHβ expression (Fig. 3C). There were significant main effects for photoperiod and age on LHβ expression. There was a significant main effect of photoperiod on PRL expression (Fig. 3D) and age. There was no significant photoperiod by age interaction on PRL expression. There was a significant photoperiod by age interaction on GH expression (F1,16=5.67, p<0.05; Fig. 3E). There was a significant main effect of age but not photoperiod on GH expression. There was a significant main effect of photoperiod on OPN5 expression (Fig. 3F) and age. There was no significant photoperiod by age interaction. Overall, transcript analyses using qPCR assay were consistent with the counts per million determined by transcriptome sequencing.
In order to determine the general functions of significantly differentially expressed transcripts of interest across photoperiod and age, DAVID functional annotation was used (Table 2-2 and Table 2-3, respectively). For these analyses, a p-value of p<0.05 was used as the significance cut-off. In the comparison between adults and juveniles, among the functional groups with highest significance included those involved in the extracellular matrix (ECM), ECM-receptor interaction, secretion, and focal adhesion. The photoperiod comparison again showed these functional groups as significant, but also showed significant transcripts involved in symport, behaviour, neuroactive ligand-receptor interaction, and transcription regulation.

**Discussion**

This study demonstrates that somatotrophs significantly increase GH expression during development regardless of the prevailing photoperiod condition. Conversely, lactotrophs and gonadotrophs are primarily sensitive to photoperiod condition with increased FSHβ and LHβ expression in long days. Only LHβ expression was dependent on both photoperiod and developmental condition suggesting this transcript has seasonal- and age-dependent regulation. Despite both adults and juveniles having smaller testes in short photoperiods, there are several transcripts that show developmental and photoperiod dependent expression. For example, juvenile quail had 143/144 upregulated in short photoperiod that were not significantly differentially expressed in adults. Moreover, there were 39/40 transcripts downregulated in juvenile quail without any significant differential expression in adults. Surprisingly, the well characterized DIO2 and DIO3 photoperiodic changes in expression were only identified in adult birds. qPCR analyses confirmed the gene count data identified by RNA-sequencing and provided strong internal replication. Overall, these findings indicate
a suite of photoperiod-induced molecular changes in the pituitary gland that are age-
dependent and a plethora of targets that could help uncover how juveniles have a heightened
sensitivity to photoperiod cues compared to adult birds.

**Long days facilitate gonadal growth in adults and juveniles**

Both adults and juveniles had larger testes in a long photoperiod as opposed to a short
photoperiod, consistent with previous publications (Robinson and Follett 1982). Follett and
Farner have previously shown that prolonged exposure of juvenile quail from hatching to
long photoperiods can cause increased testes growth (Follett and Farner 1966). The change in
juveniles could be due to two potential mechanisms. First, similar to adults, the photoperiod
induces light-dependent changes in gonadal growth. Alternatively, the second mechanism
could be due to developmental programs. In hamsters, early exposure to photoperiod or
melatonin can delay gonadal growth (Prendergast et al 2013, Sáenz de Miera et al 2017). In
the current study, it is possible that short photoperiod exposure delayed the developmental
program (or puberty) in juvenile quails. This conjecture also applies to the photoperiod-
induced changes in fat score. Future analyses of the MBH and pituitary are required to
establish the mechanisms upstream of the pituitary that influence photoperiodic and
developmental programs in reproduction physiology.

**Adult neuroendocrine transcription in the MBH is typical for a long day response**

Adult MBH *DIO2* and *DIO3* was increased and decreased in 16L compared to 8L
respectively, as expected (Yasuo et al 2005, 2006). We found that juvenile *DIO2* expression
increased during short days and that *DIO3* expression did not significantly differ between
long and short photoperiods. Given the role of *DIO2* and *DIO3* in the neuroendocrine signal
transduction cascade that links photoreception with reproductive physiology (Nakane and
Yoshimura 2010), the dynamics of *DIO2* are surprising. These data appear to suggest that
other molecular neuroendocrine changes drive photoperiodic sensitivity in juvenile quail. In juvenile hamsters, $DIO2$ has similar levels in long- and short-photoperiod conditions (Prendergast et al. 2013). $DIO2$ expression, leading to hypothalamic increase in T3, is thought to establish reproductive maturation in mammals. $DIO2$ has a wide role in developmental and has established roles for fisheye metamorphosis and in T3-dependent amphibian metamorphosis (Duarte-Guterman et al. 2010, Itoh et al. 2010). These data indicate that juvenile photoperiodic responsiveness might not be driven by tanycytes rewriting consistent with adult responses. An alternative proposition is other neuronal circuits associated with developmental control of the GnRH system are involved. The stimulation of pituitary cells by GnRH to secrete $LH\beta$ and $FSH\beta$ is partly regulated by the secretion of melatonin in sheep (Misztal et al. 2002). Gamma-amino butyric acid (GABA) also regulate GnRH neurones independent of the neuroendocrine response to changing photoperiod (Bentley et al. 2006). Hence, these findings support an alternative means by which pituitary $LH\beta$ secretion may be governed outside of the $DIO2/DIO3$ involved neuroendocrine cascade.

**Photoperiod- and age-dependent changes in pituitary gland transcriptomes**

Our volcano plot comparing differential expression of adults in long and short photoperiodic conditions revealed a total of 206 differentially expressed transcripts. 126 transcripts were upregulated in a long photoperiod and 80 were downregulated. Among the differentially expressed transcripts were $PRL$, $FSH\beta$, and $NTS$. Long photoperiod increases in $PRL$ expression, associated with seasonal timing, are common within the literature (Goldsmith and Hall 1980, Sharp 2005). Similarly, $FSH\beta$ content increases during long photoperiod conditions (Follett and Maung 1978, Urbanski and Follett 1982, Nicholls et al. 1983) where increased FSH in the pituitary gland precede increases in plasma concentration. Given its role in acting directly on gonads to induce growth, it is not surprising that $FSH\beta$ levels should be high in stimulatory long day photoperiods. $NTS$ has a suggested role in regulating $LH$
secretion (Rostène and Alexander 1997). Yamada and Mikami describe the distribution of luteinising hormone-releasing hormone (LHRH) as coinciding with dense populations of NTS when using comparable data between ducks and quail (Yamada and Mikami 1981). Although LHβ is absent from the reference *Coturnix japonica* reference genome, and therefore was not identified through RNA-sequencing, LHβ is known to increase in long photoperiod conditions (Follett et al 1975, Nicholls et al 1983). Hence, an accompanying increase in NTS expression would be expected.

Our volcano plot comparing differential expression across age in 16L birds revealed a total of 685 differentially expressed transcripts. 175 transcripts were upregulated in juveniles and 510 were downregulated. Critically, among the differentially expressed transcripts was GH, whose expression is known to be downregulated in adults (Schew et al 1996). GH expression is therefore an excellent marker of age, supporting the significance of the remaining age-dependent differentially expressed transcripts presented here. Similar results were observed in our volcano plot comparing differential expression across age in 8L birds. Here, 678 transcripts were differentially expressed, with 422 being upregulated and 156 being downregulated in juveniles. Again, GH was one transcript whose upregulation in juveniles was apparent.

**Function pathways modified during photoperiod and developmental programming**

A functional annotation analysis across photoperiodic conditions suggests an influence of seasonal time on transcripts involved with the brain and nervous system, as neuroactive ligand-receptor interaction scored highly in significance. In both neuroactive ligand-receptor interaction and secretion, *PRL* was identified as a key gene of interest. *FSHβ* was also implicated in neuroactive ligand-receptor interaction. Therefore, this analysis confirms the
photoperiodic differential expression of these important transcripts that we have focussed on in this manuscript.

The extracellular matrix (ECM)-receptor interaction was identified as the most significant category in our comparison of photoperiodic gene functions. The ECM-receptor interaction is known to play an important role in tissue and organ morphogenesis, including in adipogenesis (San et al. 2021). Furthermore, a recent study on the gonadal development of male geese has implicated significant enrichment of the ECM-receptor interaction pathway by differential gene expression in the pituitary gland (Tang et al. 2022). Therefore, the ECM-receptor interaction pathway is a likely area for the identification of photoperiodically-significant transcripts. For example, integrin alpha 3 (ITGA3) showed significant differential expression within the ECM-receptor interaction pathway and is associated with neural migration. Within this pathway we also identified chondroadherin (CHAD), which has a role in mediating adhesion of chondrocytes; and COL2A1, for the production of the pro-alpha1 chain of type-II collagen protein, suggesting that cartilage synthesis is an important physiological change occurring across photoperiodic time.

DAVID functional annotation analyses confirmed that many transcripts, particularly those implicated in growth and development, were differentially expressed across age groups. Transcripts involved in the ECM matrix and ECM receptor interaction were among the most significant in the functional annotation analysis. The ECM matrix is critical for practically all tissue morphogenesis (Gullberg and Ekblom 2003), hence why transcripts implicated in its expression are differentially expressed during the rapidly developing juvenile life stage. Furthermore, the TGF-ß signalling pathway was implicated as significant in this analysis. This pathway is thought to be involved in the testicular development of broiler roosters under long (16L:8D) photoperiod conditions (Sun et al. 2020) and functions similarly in quail (Otake and Park 2016).
Similar to the photoperiod comparison, many named transcripts, implicated in the ECM-receptor interaction pathway, were identified in this comparison across age, including CHAD and COL1A2. Bone morphogenetic proteins 4, 5, and 6 (BMP4, 5, and 6) were implicated in secretion and differentially expressed between adults and juveniles. It is suggested that BMPs may have a role in the multiple craniofacial bone growth of birds; BMP4 is critical for beak development and is expressed in multiple craniofacial bones of Huiyang bearded chickens, and is therefore likely an important transcript of interest when examining age differences in growth and development of Japanese quail (Hong et al 2019).

Overall, it is apparent that these functional analyses provide a valuable resource for the identification of novel transcripts associated with both age and photoperiodism. Other groups in the field have used this common method of functional annotation similarly, in order to identify salient areas for future targeted research, for example in migratory black-headed buntings (Sharma et al 2018) and Japanese quail (Marasco et al 2016).

**Targeted qPCRs replicate findings obtained through transcriptome analyses**

qPCRs for PRL, FSHβ, LHβ, and GH confirm data generated using Oxford Nanopore RNA-sequencing. PRL is a well-known marker of long photoperiod in birds (Yasuo et al 2004, Sharp 2005). qPCR analyses indicate that PRL expression is significantly high in the adult 16L group compared to the 8L group. Surprisingly, there was photoperiodic difference in juveniles. In juveniles, the lack of PRL change supports the conjecture that light exposure modifies a developmental program similar to the lack of changes observed in the MBH DIO2/DIO3 system. Similarly, FSHβ was increased in adults exposed to long photoperiods, consistent with previous reports (Yasuo et al 2006). However, there was no change observed in the juvenile pituitary gland. FSHβ causes gonadal cell proliferation and the inhibition of FSHβ in quail results in the regression of testes size (Brown and Follett 1977). The lack of a
change in $FSH\beta$ in the juveniles suggests that short photoperiod delayed testicular growth. We propose that $PRL$ and $FSH\beta$ are molecular markers of photoperiodic programs in adult Japanese quail.  

$LH\beta$ expression, however, was found to increase in both adult and juvenile 16L conditions (Follett et al 1975, Nicholls et al 1983). It is interesting that $LH\beta$ expression is increased in juvenile housed in long photoperiod and indicates that the testicular growth observed was a result of the direct action of $LH\beta$ on the testes. These data indicate that $LH\beta$ expression is under the control of photoperiodic programs in both adult and juvenile quail. It is currently unclear how gonadotrophs that express $LH\beta$ and $FSH\beta$ are differentially regulated by photoperiod and developmental programs, respectively. $OPN5$ expression mirrored $LH\beta$ in both adult and juvenile pituitary glands, suggesting a potential molecular mechanism that links light and $LH\beta$ expression, independent of $FSH\beta$ expression.

A clear example of a molecular marker of developmental programs is $GH$ expression occurring in somatotrophs. In juvenile quail, there was a robust increase in $GH$ expression, independent of photoperiod treatment. Conversely, $GH$ expression was found to be consistent across photoperiod conditions in adults. For adults this is expected, as $GH$ expression is no longer required for growth and development once maximum size has been reached, and hence ceases to be expressed in older birds (Scanes and Lauterio 1984). One limitation of the current experiment is the inability to link $GH$ expression with body mass.

Conclusions and future research

Overall, the findings reported here indicate pituitary gland cell specificity for photoperiodic and developmental programs of reproductive physiology of birds. The data indicate somatotrophs as a cellular basis of developmental programs, lactotrophs as a marker of photoperiod programs, and gonadotrophs as a mix of both programs (Fig.3-2); this conclusion
builds on recently published work indicating a pituitary cell autonomy, separate from changes
driven by the MBH, in seasonal FSHβ expression (Majumdar et al. 2023). Our approach to
correct Oxford Nanopore RNA sequencing and qPCR analyses to investigate the
photoperiodic and developmental responses in quail provide a robust approach to provide a
comprehensive and confirmatory strategy. The data generated suggested a wide range of
potentially interesting transcripts in the comparisons between photoperiod and age, acting as
a resource to guide future research. It is unclear which transcripts and gene ontology pathway
analyses are associated with the pituitary cell types. The next steps are to establish which
functional pathways are associated with photoperiodic and developmental programs at a
cellular resolution. Moreover, many studies have highlighted that photoperiodic cues may be
insufficient for full ovarian development indicating that other supplementary cues are
requires for full reproductive competence (Ball and Ketterson 2008, Tolla and Stevenson
2020). Sex differences in the adult and juvenile seasonal programs responses are therefore a
salient avenue for future research.

Acknowledgements

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using RStudio or BioRender.
References


Rani, S., & Kumar, V. (2014). Photoperiodic regulation of seasonal reproduction in higher vertebrates.


Stevenson, T. J., & Ball, G. F. (2012). Disruption of neuropsin mRNA expression via RNA interference facilitates the photoinduced increase in thyrotropin-stimulating subunit β in birds. European Journal of Neuroscience, 36(6), 2859-2865.


enzyme genes is involved in the photoperiodic gonadal response of Japanese quail. *Endocrinology, 146*(6), 2551-2554.


Figure Legends

**Figure 1:** Long photoperiods induce physiological and hypothalamic neuroendocrine change associated with reproduction in adult and juvenile Japanese quail. A. Measurements of testes length showed a significant interaction between photoperiod and age. B. Fat score ratings, as established by Wingfield and Farner, were overall higher in adults and in 16L photoperiod conditions. C. qPCR analyses show a significant photoperiod by age interaction on DIO2 expression in the mediobasal hypothalamus (MBH). D. A significant photoperiod by age interaction was also found in MBH DIO3 expression. Physiological and transcriptomic data from qPCR were analysed by two-way ANOVA and Tukey’s HSD with an α value of P = 0.05. Letters above each group indicate pairwise comparisons by Tukey’s HSD where appropriate.

**Figure 2:** Volcano plots comparing age and photoperiod revealed a high number of differentially expressed transcripts. Upregulated transcripts (logFC>1) are coloured red and downregulated transcripts (logFC<-1) are coloured blue. A. Significant differentially expressed transcripts across photoperiod treatments in adults (n=206) included FSHβ, PRL, and NTS. B. Significant differentially expressed transcripts across photoperiod treatments in juveniles (n=184) included HAPLN1. C. Significant differentially expressed transcripts across 16L age groups (n=685) included GH. D. Significant differentially expressed transcripts across 8L age groups (n=678) also included GH. A P-value of P<0.01 was deemed significant for all plots.

**Figure 3:** A. A heatmap of the top 50 most significant transcripts in the pituitary gland were plotted in a heatmap. Among the most significant transcripts were FSHβ and GH. B-F. Targeted qPCR analyses were performed in order to investigate the transcription of genes of interest influenced by photoperiod and age. B-D There were significant main effects of both photoperiod and age on FSHβ, LHβ, and PRL expression, as well as an interaction effect between photoperiod and age on LHβ expression. E. There was a significant photoperiod by age interaction on GH expression. F. There was a significant main effect of photoperiod and age on OPN5 expression. For the pituitary sequencing heatmap analysis, a P-value of P<0.01 was deemed significant. Targeted qPCR analyses were performed using two-way ANOVA and Tukey’s HSD with an α value of P=0.05. Letters above each group indicate pairwise comparisons by Tukey’s HSD where appropriate. Additional data relating to these analyses are provided in Extended Data Figures 3-1 and 3-2.
**Table 1**: Targeted qPCR primer sequences and specifications, including forward and reverse primer sequences, annealing temperature, melting temperature, and expected product sizes for DIO2, DIO3, OPN5, LHβ, FSHβ, PRL, and GH.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Forward</th>
<th>Primer Reverse</th>
<th>Annealing Temperature (Celsius)</th>
<th>Melting Temperature (Celsius)</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIO 2</td>
<td>CGCCTACAAGCAGGT CAAAC</td>
<td>CACACTTGCCACCAA CACTCTT</td>
<td>60</td>
<td>82</td>
<td>242</td>
</tr>
<tr>
<td>DIO 3</td>
<td>AGGCTCTCTTCTTC GGGAT</td>
<td>CACACTTGCTAGGC AGCAC</td>
<td>60</td>
<td>83</td>
<td>180</td>
</tr>
<tr>
<td>OP N5</td>
<td>ATGGCATCAGACTGC AACTCC</td>
<td>TAGCACTTGCTAGGC AGCAC</td>
<td>60</td>
<td>84</td>
<td>499</td>
</tr>
<tr>
<td>LH B</td>
<td>TTTACCGCAGCCCTT TGGGT</td>
<td>AGAGCCACGGGTAG GATGACTTT</td>
<td>60</td>
<td>87</td>
<td>125</td>
</tr>
<tr>
<td>FSH B</td>
<td>CTGCGGTGACCATCC TGAATCTTT</td>
<td>GCTTCCATTGTGACT GAAAGGAGCA</td>
<td>62</td>
<td>85</td>
<td>396</td>
</tr>
<tr>
<td>PRL</td>
<td>AATGAAACCCCGACC CTGAG</td>
<td>CCCCTAGTGCAACTT GAGACC</td>
<td>60</td>
<td>79</td>
<td>630</td>
</tr>
<tr>
<td>GH</td>
<td>GCTGCCGAGACATAC AAAGAG</td>
<td>GAGCTGGGATGGTT CTGAG</td>
<td>60</td>
<td>81</td>
<td>109</td>
</tr>
<tr>
<td>B-actin</td>
<td>AATCAAGATCATTGC CCCAC</td>
<td>TAAGACTTGCTGCTGA CACC</td>
<td>60</td>
<td>84</td>
<td>114</td>
</tr>
</tbody>
</table>
**Table 2**: Summary of two-way ANOVA analyses associated with Figures 1 and 3, including F- and P-statistics associated with main effects of photoperiod and age, and their interaction, on testes length, fat score, and the expression of DIO2, DIO3, FSHB, LHB, PRL, GH, and OPN5. Additional data relating to these analyses are provided in Extended Data Tables 2-1, 2-2, and 2-3.

<table>
<thead>
<tr>
<th></th>
<th>Photoperiod</th>
<th>Age</th>
<th>Photoperiod:Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testes Length</td>
<td>F(1,15)=198.46, P=4.69e-10 ***</td>
<td>F(1,15)=112.75, P=2.26e-8 ***</td>
<td>F(1,15)=81.17, P=1.94e-7 ***</td>
</tr>
<tr>
<td>Fat Score</td>
<td>F(1,16)=9.23, P=7.83e-3 **</td>
<td>F(1,16)=6.15, P=2.46e-2 *</td>
<td>F(1,16)=0.00, P=1.00 n.s.</td>
</tr>
<tr>
<td>DIO2</td>
<td>F(1,16)=0.77, P=3.94e-1 n.s.</td>
<td>F(1,16)=6.94, P=1.81e-2 *</td>
<td>F(1,16)=22.12, P=2.4e-4 ***</td>
</tr>
<tr>
<td>DIO3</td>
<td>F(1,17)=10.78, P=4.38e-3 **</td>
<td>F(1,17)=13.40, P=1.94e-3 **</td>
<td>F(1,17)=15.30, P=1.12e-3 **</td>
</tr>
<tr>
<td>FSHB</td>
<td>F(1,17)=17.69, P=5.95e-4 ***</td>
<td>F(1,17)=29.54, P=4.45e-5 ***</td>
<td>F(1,17)=0.83, P=3.75e-1 n.s.</td>
</tr>
<tr>
<td>LHB</td>
<td>F(1,17)=9.47, P=6.83e-3 **</td>
<td>F(1,17)=74.37, P=1.29e-7 ***</td>
<td>F(1,17)=4.68, P=4.51e-2 *</td>
</tr>
<tr>
<td>PRL</td>
<td>F(1,17)=9.27, P=7.34e-3 **</td>
<td>F(1,17)=14.64, P=1.35e-3 **</td>
<td>F(1,17)=1.92, P=1.83e-1 n.s.</td>
</tr>
<tr>
<td>GH</td>
<td>F(1,16)=3.46, P=8.14e-2 n.s.</td>
<td>F(1,16)=21.48, P=2.75e-4 ***</td>
<td>F(1,16)=5.67, P=3.00e-2 *</td>
</tr>
<tr>
<td>OPN5</td>
<td>F(1,16)=17.01, P=7.96e-4 ***</td>
<td>F(1,16)=24.79, P=1.36e-4 ***</td>
<td>F(1,16)=0.05, P=8.21e-1 n.s.</td>
</tr>
</tbody>
</table>

Non-significant P-values are indicated by "n.s.", P-values <0.05 are indicated by ",<0.01 by "**, and <0.001 by "***".