Transcriptional changes in the reproductive axis of parenting pigeons

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Abstract In species that provide parental care, successful rearing of offspring involves a shift from aggressive and sexual behaviors to more caring and nurturing ones. We hypothesize that these shifts in behavior are related to changes in gene expression that are controlled by internal mechanisms rather than by the external cues. We found that gene expression in the hypothalamus is fairly insensitive to the natural transition between egg and nestling stages; however, in the pituitary and gonads, gene expression showed much more plasticity over the course of reproduction. We show evidence that natural transitions in reproductive and parental behavior by mediated internal clock controlling gene expression in the pitary and gonads but that the hypothalamus is very sensitive to external cues that signal a distruption in the natural parental care cycle. This research provides a deeper understanding of the interplay of genes, hormones, and parental care and has important implications for understanding molecular processes that underlie behavior transitions.

Introduction

Reproduction is a critical period in an organism's life cycle. Parents must balance their own needs with the energetic costs and time-constraints required to successfully reproduce and rear their offspring to independence (cite). A complex series of evolutionarily conserved physiological processes underlie behaviors typically associated with reproduction in vertebrates, including courtship, nesting, and parental care. These processes are primarily mediated by the reproductive axis, which consists of the hypothalamus in the brain, the pituitary, and the gonads (i.e., the HPG). Our understanding of the physiological changes that adults navigate as they transition through reproductive states are primarily limited to a number of circulating hormones and a small set of well-known genes (cite). We have yet to come close to conceptualizing the larger symphony of reproductive events that occur to yield reproductive and parental care behaviors.

Using the model of a biparental vertebrate species, the rock dove (Columba livia), our team has previously demonstrated the dynamic nature of gene activity within the reproductive or HPG axis. MacManes et al.(5) reported that HPG gene activity varied by sex, with females exhibiting higher levels of overall gene expression than males. Calisi et al.(1) reported that HPG gene activity varied response to restraint-stress, and Austin et al. identify genes that responds to both corticosterone treatment and restraint stress. While each of these studies advanced our understanding of sex-differences and transcriptional plasticity in the reproducitve axis, none of these studies examined activity of the reproducitve axis of bird that are actively reproducing or caring for offspring. Studying parental care in both sexes of mammalian species is challenging because only the females are able to use lactation to feed their young, and the physiological mechanisms regulating this process are hard to control for. The rock dove is an excellent system for studying reproduction and parental care in both sexes because both males and females feed their young an energy-rich secretion produced from the epithelial lining of their crops, known as crop milk.

In this study, we investigate the activity of the HPG axis over the course of reproduction and parental care by characterizing gene expression as birds transition from non-breeding to nest-building, as they lay and incubate their clutches, and finally as they become parents and raise their chicks. Then, we conducted a series of nest manipulations to ascertain constraints associated with biological timing versus input from external stimuli. We conducted a comprehensive series of analyses examine broad patterns of variation as well as the activity of specific genes in specific tissues. We have also made our data and scripts available so that others can reuse the data to ask different questions or reproduce our results. This research provides a deeper understanding of the interplay of genes, hormones, and parental care and has important implications for understanding molecular processes that underlie behavioral transitions. reviewed in(4) say this, but other say that reviewed in(2).

Results

Data-driven characterization of reproductive and parental stage groups.

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We first examined broad patterns of variation in gene expression in the hypothalamus, pituitary, and gonads of male and female rock doves across a series of timepoints that encapsulate important transitions in reproduction and and parental care (Fig. 1A). t-Distributed Stochastic Neighbor Embedding (t-SNE) is a nonlinear, dimension reduction technique is used to visualize high-dimensional data (van der Maaten and Hinton 2008) and is widely used in in RNA sequencing to identify related and distinct cell types (refs). Differential expespression profiling is useful for pair-wise comparisons of differentially expressed genes (DEGs) with positive log fold change (+LFC) in one group compared to another. Combined, the provide stronger support for similarities and differences between cell-types and treatment groups in multi-dimential datasets. First, the data supports that the hypothalamus, pituitary, and gonads have distinct gene expression profiles as evidenced by little overlap in t-SNE space and an average of 13,000 DEGs between each tissue (Fig. 1B). Second, the data supports that sex-differences are much more promient in the gonads than in the brain (13,000 versus 2,000 DEGs) (Fig. 1C). Because of these widespread tissue and sex-differences, further ananlyses were conducted separately for each tissue and sex (Fig. 1D, Suppl. Fig 1). In all the tissues, the control samples are quiet different from all the other timepoints samples, and only a fraction of the transcriptome is altered during the course of parental care at the timepoints sampled. The most notable differences male and female hpothalamic control samples are very different from on the rest (~7000 DEGs, and only the two nestling care stages (n5 and n9)) show differential expression of more than 1,000 genes. In the pituitary, late inubcation and hatch are quite simlilar to each other and distinct from the earlier incubation stages stages, and temporal changes in gene expression appears to be less variable in males than females. In the female gonads there appear to be more changes in gene expression early one, between nest building and early incuation, but the male gonads show very little variation associated with parental stage.

Hypothesis-driven analysis of candidate parental-care genes.

- Champagne Curley Genes (Fig 2)
- GO genes (Fig 2 Suppl Fig 1)
- Cancer genes (Fig 2 Suppl Fig 2)
- Shiny genes (Fig 2 Suppl Fig 3)

Software for data exploration and hypothesis testing

Data accessibility and reproducibility are key challenges for today's researchers. Using Binder and Shiny, we have provides a means for scientists, physicians, and the general public to explore the data, code, and results from this project at https://raynamharris.shinyapps.io/musicalgenes/. Like Hipposeq (Cembrowski et al elife 2016, 2018), this tools allows quick and easy data exploration of RNAseq data from mutiple cell types that relate to brain function.

Discussion

This RNA-sequencing project was designed to characterize the reproductive axis of bi-parental pigeons across a characteristic reproductive cycle and through the parental main parental stages of egg incubation and parental care using data- and hypotheses driven approaches. We hypothesized that differences in gene expression would be driven by either internal physiology or external stimuli. In the pituitary, we found that the most explanatory source of variation in gene expression was the expression level of prolactin (PRL). Circulating levels of prolactin in the blood in this species are known to rise around day nine and peak on or the day before hatch. Our results confirm this and also identified one hundred genes whose expression is highly correlated with prolactin. Today, this variation, which changes more within a parental stage (e.g. egg incubation or nestling care) suggests that internal physiology drives changes in gene expression that precede changes in external stimuli that lead to behavioral changes (e.g. crop milk or regurgitation). We predicted that either sex steroid peptide hormones would play a role in governing the reproductive transitions, but we did not anticipate discovering a link between PRL expression to genes that are involved with DNA repair and regulation of the cycle. Finally, we aimed to make these data and analyses FAIR (findable, accessible, interoperable, and reproducible), open and reproducible to encourage others to reuse our data to test new hypotheses or explore the data with a different perspective or toolset. The scripts that made the figures and tables are browseable online thanks to RStudio, GitHub and Binder. The data can be visualized and sonified (turned into sound) interactively thanks to RStudio, GitHub, and Shiny.

Materials and Methods

Animal Care

All animal care and use comply with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the University of California, Davis IACUC permit

#18895. Birds were housed at the University of California, Davis in enclosed aviaries with ~8 sexually reproductive adult pairs per aviary. All adults birds are uniquely associated with nests by their unique color band combination. Food and water were provided ad libitum, and nests were monitored daily.

Experimental design and tissue processing

To characterize reproduction and parental care, birds were sampled at 8 timepoints across the parental care cycle (Fig. 1a) as perviously described in Austin et al 2020. Briefly, nest-building pairs where at least was individual was seen carrying nesting material or shaping a nest (bldg); the day the 1st egg was laid (lay), clutch completion (also known as incubation day 3 (inc.d3)), mid-incubation (inc.d9), late incubation (inc.d17), the day the first chick hatched (where hatch), nestling care days 5 and 9 (n5 and n9). Additionally, a non-breeding (control) timepoint was adding using control birds from previously published studies (2,3).

To test our hypothesis that gene expression is govern more by internal mechanisms than external cues, we conducted multiple offpsring replacement or removal manipuations.... Add details here.

To control for circadian rhythm confounds, each member of the pair was collected between 0900-1200 (PST). We sampled a total of 331 birds (16 treatments with ~10 pairs per treatment). Pairs were captured and collected simultaneously within 5 min of entering their cage, were anesthetized using isoflurane until unresponsive (<2 min), and then decapitated. Trunk blood, brains, pituitaries, and gonads were collected. Processing and analysis of truck blood is described in Austin et al. 2020.

RNA sequencing analysis

Processing of brains, pituitaries, and gonads for RNA sequenceing is described in detail in Lang et al. 2020. Briefly, RNA from the hypothalamus, pituitary, and gonads was processed for Illumina sequencing using the NEB Next Ultra Directional RNA Library Prep Kit. Reads were pseudomapped (insert Kalisto citation) to the Rock Dove transcriptome v1.1.0 whose transcripts were annotated with genes from Gallus gallus genome v5.

Statistical analyses and modeling were perforeming using R[6;8]. Limma was use to process all samples using the model ~ tissue * sex * treatment (citation). t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis was conducted using a perplexity of 10[7]. Principal Component Analysis (PCA) was conducted using the top 500 most varying genes (citation). DESeq2 was used to model gene express for sex and tissue combination separately using the model ~treatment \citep{Love2014). Weighted correlation network analysis (WGCNA) was used to identify set of genes with simliar patterns of gene expression over the course of reprodution and parental care (citation). ANOVAs were used to test whether specific genes were differnetially expressed between between sequential timepoints.

Data availablility

RNA sequencing data for available through the European Nucleotide Archive (ENA) project ID PRJEB16136 and XXXXX. Code and data for reproducing the results described in the manuscript are available at `https://github.com/macmanes-lab/DoveParentsRNAseq` (upload to zendo and cite). Data can be explored in the cloud using Binder[3].

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Figures

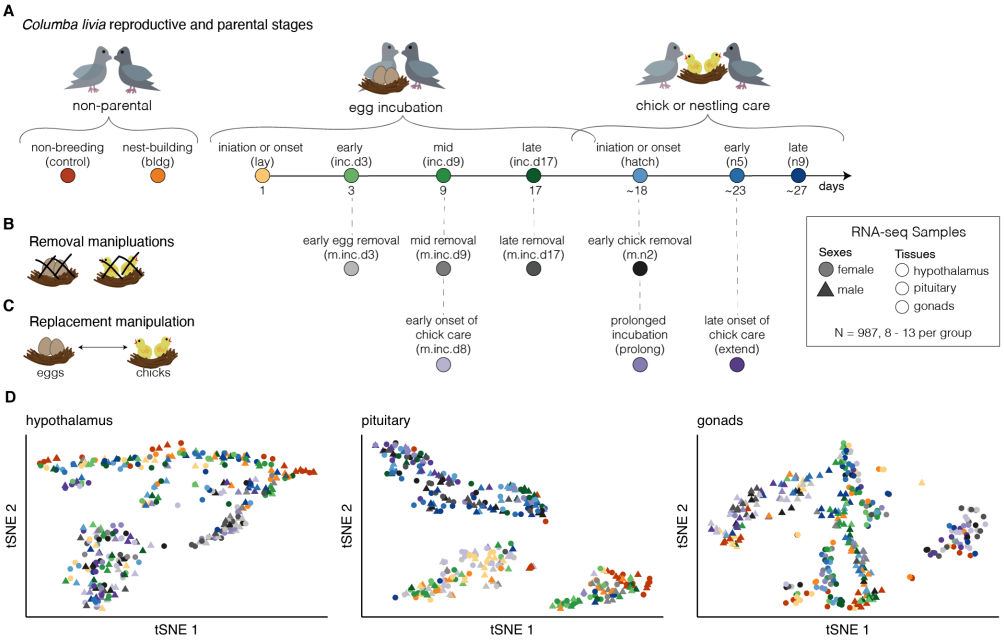

Figure 1. Experimental design. A) We investigated nine timepoints that spanned the majority of reproductive efforts in this species. These time-points consisted of a "control" or non-parenting state (from MacManes et al 2016 and Calisi et al. 2017), nest-building, clutch initiation or the onset of incubation, clutch completion and early incubation, mid-incubation, late incubation, initiation of nestling care, early nestling care, and mid-nestling care. To teste whether external or internal factors were regulating gene activity, we also conducted a series of offspring removal (B) and replacement (C) manipulations to test whether transcriptional changes were dependent upon offspring presence or were regulated by an internal clock. (D) We used t-Distributed Stochastic Neighbor Embedding (t-SNE) to reduce the dimensionality of the transcriptomes from the hypothalamus, pituitary, and gonads of male and female rock. Circles and triangles represent female and male samples, respectively, and points are colored by treatment.

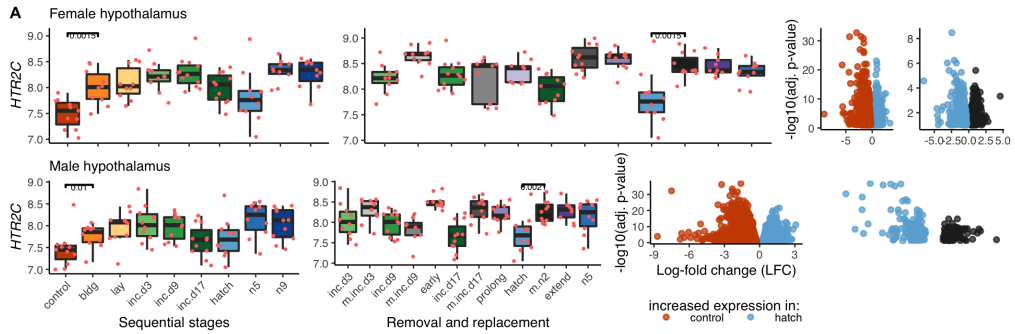

Figure 2. Hypothesis-driven and data-driven approaches to identifying changes in gene expression. Box-and-whisker plots show changes in gene expression of serotonin receptor (HTR2C) in the hypothalamus (A), prolactin (PRL) in the pituitary (B), and estrogen receptor 1 (ESR1) in the gonads.Bar and statistics are shown for one contrast of interest between treatment groups. Boxes are colored by treatment. Volcano plots show the log-fold change (LFC) and -log10(FDR) for all genes that are significantly different differentially between the aforementioned treatment groups. Boxes are colored by the treatment were gene expression was higher.

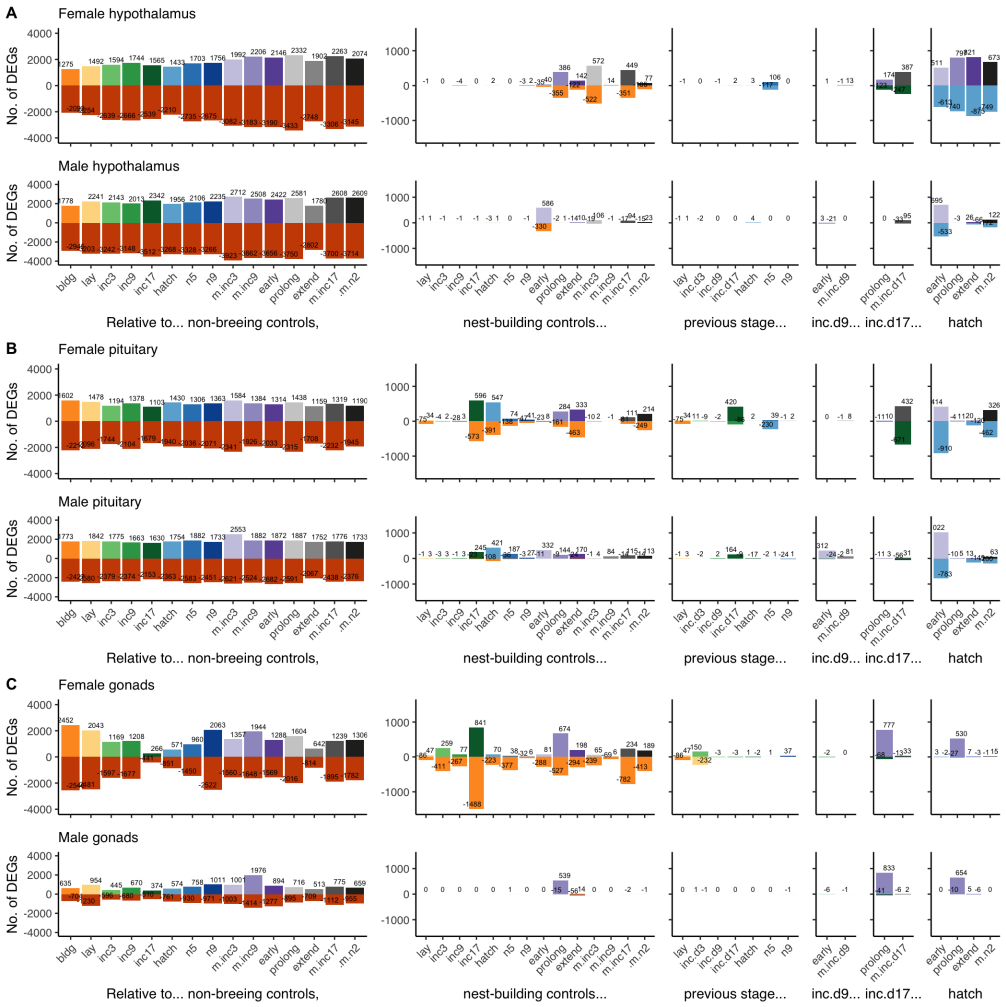

Figure 3. Summary of differentially expressed genes. Bar plots show the total number of significantly differentially expressed genes for all two-way comparisons in the hypothalamus (A), pituitary (B), gonads (C) . Contrasts are made relative to the non-breeding controls, the nest-building group, the previous stage, or the internal or external control group. Bars are colored by the treatment where gene expression was higher. A negative number of differentially expressed genes means that many genes had increase expression in the reference group.

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