Pituitary prolactin drives transcriptional changes in the reproductive axis of parenting pigeons

by Rayna M Harris [1], Suzanne H. Austin [2], Andrew S. Lang, Victoria S. Farrar, April Booth, Tanner Feustel, Matthew D. MacManes, Rebecca M. Calisi [3]

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).

OR

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Reproduction is an essential process for all living things, but only a subset of vertebrates and invertebrates require parental care for offspring survival. Identifying the mechanisms that regulate parental care behavior is like a thing for scientists in many disciplines (ecology, neuroscience, psychology), but it is hard to disentangle the mechanisms that regulate reproduction from those that regulate parental care. Or maybe that contrast doesn't work...

Cumbia livia has been used by many scientists to study evolutionary processes and behavior stuff. Building on previous research, we sought to identify the transcriptional mechanisms that regulate parental care in the hypothalamic-pituitary-gonadal axis (or the reproductive axis). With a highly replicated experimental design, we aimed to distinguish the reproductive process from the behavioral process.

Discuss parental care candidate genes and reproductive candidate genes. Cite some literature. Oxytocin and arginine vasopressin are known to regulate pair-bonding. Oxytocin also promotes cervical contractions. These all occur before birth of the offspring and therefore do not count as parental care according to that book I read. Oh crap, but they are also involved in pair-bonding with offspring... how to dissociate reproduction and parental care..... Prolactin is known for promoting lactation, hence it's name. In mammals, typically (or always?) only the female lactates to provide nutrition for the offspring thus even in bi-parental species where both parents provide parental care, only the female provides breastfeeding-related care. In Cumbia livia, however (maybe other species) both males and females produce "crop milk" thus both males and females can feed and care for their offspring in a way that other species cannot. Indeed, prolactin promotes this pseudo-lactation. In some species lactation promotes.

Results

Characterization of parental care

To characterize transcriptional activity, 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 by giving each datapoint a location in a two-dimensional map (van der Maaten and Hinton 2008). t-SNE is widely using in single-cell data process for identify related cell types. Here, we use t-SNE to identify variation associated with tissue, sex, and treatment (Fig. 1j).

Our results suggest that tissue is the greatest source of variation in the dataset, with hypothalamic, pituitary, and gonadal samples all forming distinct clusters, with only a few pituitary and gonad samples falling outside the 95% confidence interval (Fig. 1b). Sex-differences are most prounounced in the gonads with male and female samples form two discrete clusters (Fig. 1c). Treatment accounts for much less of the variation than tissue and sex, and even after removing sex as a variatble, treatment effects in all three tissues are hard to visualize simultatenously (Fig. 1d,e), so we next looked at each tissue and sex separately. In the hypothalamus, control samples form tight clusters that stands out from all other timepoints that are largely overlapping in space (Fig. 1h). The pituitary shows the strongest treatment effect with one cluster of the "earlier" parental timepoints (nest-building, lay, incubation days 3 and 9), a second cluster of "later" parental timpoints (incubation day 17, hatch, and nestling care days 5 and 9; Fig. 1i). Like the hypothalamus, in the gonads, the control timepoints appear to be much less variable that the parental timepoints, but there is no clear separation of gonadal samples by parental stage (Fig. 1j). While the tSNE analysis reveals broad patterns across all levels of the experiment, additional tools are needed it hone in on the specific differences associated with parental care in each tissue. Differential gene expression analysis can shed insight into specific differences between treatment groups and thus deepen our understand of the transitions between reproduction and parental care behaviors. We used DESEq2 to identify genes whose expression changed significantly between sequential timepoints in each tissue and sex separately (Fig. 2a).

In the hypothalamus only two sequential stage comparisons results in a sizable change in expression; in females, 1927 genes changes their expression betwen hatch to nesting care day 5 (with 1076 decreasing and 851 increased their expression), and in the male hypothalamus,87 genes were diferentially expressed bewteen incubation days 9 and 17 (with 38 decreasing and 49 increasing their expression (Fig. 2b)). In the pituitary, differential gene expression differenes are greatest when compariing incubation days 9 and 17, and this difference is more pronounced in females (Fig. 2c). The next largest difference in the pituitary is between hatch and n5. The female putitary shows moderate chagnes from nest-bilding to lay to incuabation day 3 (Fig. 2c). The male pituitary changes more that the female pitutiary changes between nestling care days 5 and 9 (Fig. 2c). About 2000 genes also change their expression in the female pituitary and about 425 in the male pituitary when comparing incubation days 9 and 17 (Fig. 2). In the female gonads, 270 genes are decreased while only 56 are

increased when comparing incubation days 9 and 17(Fig. 2). Many more significant changes in gene expression in the female gonad occur between nest-building and lay and between lay and clutch completion (incubation day 3) than in any other comparison (Fig. 2).

Internal physiology versus external stimuli hypothesis

We show that samples from the pituitary on incubation day 17 cluster more closely with hatch and nestling care day 5 samples than with incubation days 3 and 9. This suggest that, in the pituitary, internal mechanisms that prepare for chick arrival have the greatest effect on gene expression rather than suggesting that gene expression responds to changes in the external enviornament.

Blah 3, blah, 4 blah.

Potential implications for cancer research

These results confirmed prolactin (PRL) gene expression fluctuates throughout parental care in a manner that is consistent with its role in promoting lactation and maintaining parental behaviors. Interestingly, we identified about 100 genes whose expression was correlated with PRL, including (BRCA1, which is associated with breast cancer). Additionally, we identified thousands of genes whose expression changed over the course of parental care. Exploring the relationship between PRL and other differentially expressed genes could provide important insights into the `symphony` of gene expression that regulates behavior at the organismal and cellular level.

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.

OR

In the hormone experiment, we replicated the peak in prolactin. Then, unexpectedly or can I find this elsewhere, we confirmed that prolactin gene expression in the pituitary is strongly correlated with circulating prolactin. Oh, maybe do stats comparing the curve of prolactin hormone and prolactin gene expression. could use those same stats that I used for hormone papers or maybe just show correlations...

hypothesis-driven. this research is hypothesis-driven... we had these hypotheses and came to these conclusions... Add GO analysis comparing observed versus expected hits for a given go term. 5

A data-driven analysis using PCA identifies prolactin gene expression in the pituitary is the major explainer of variation in our entire dataset. This leads us to ask what genes are correlated with prolactin and what genes are differentially expressed when comparing samples that had higher or lower levels of prolactin. We found 100 genes that mirrored the prolactin curve. GO analysis suggested that these genes regulate the cell cycle. Many are associated with breast- and testicular cancers. This suggests/confirms? that there may be a strong link between prolactin and cancer in the tissues that receive strong inputs from the pituitary (like the mammary glands, the gonads, and the adrenals). this research uses a readily available data-driven approach for identifying the most variable genes. our data-driven approach supported existing knowledge and shed light on new gene-gene interactions

that need future investigation and could potentially yield new insight into unsolved problems in basic biology and biomedicine.

Our software tool provides a unique oportunity for professionals with differnt levels of proficiency in bioinformatics to itneract with the data to advance science and medicien. Futuremore, we turn our data into sounds, providing a unique insight into transcripional variation that cannot be captured with the traditional data viauzliations that are the foundation of omics science communication.

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].

Findable, accessible, interoperable, and reusable

In addition to making our data and code open by sharing it in open-access repositories (e.g GitHub and that repo were are using that hopefully, Andrew has the link to), we also wanted to make it findable, accessible, and interoperable, and reusable (the tenants of FAIR data)[9]. Our first step in doing this was to create a "binderized"[3] GitHub repository where the R scripts can be executed in an RStudio server on the cloud. This means that no local installations or downloads are required, ensuring (or at least increasing the likelihood of) interoperability and reproducibility. To make the data more reusable and accessible to those familiar and unfamiliar with R, we created Shiny application (define) that allows users to interactively pick a gene of interest and visualize their expression level in this data set. (If Rayna adds a download button, or a new shiny tab then the can get the raw data too.)

This saves researcher hours of computing time downloading files and running scripts if they had to start from the links we provided above to the raw data and scripts. Finally, we musical representations of the data to our software so that uses who are not familiar with interpreting the complex graphs typically associated with the RNA-seq project can immerse themselves in data exploration. We call this software application "Musical Genes". We believe our approach is a novel way of communicating scientific information to broad audiences 6.

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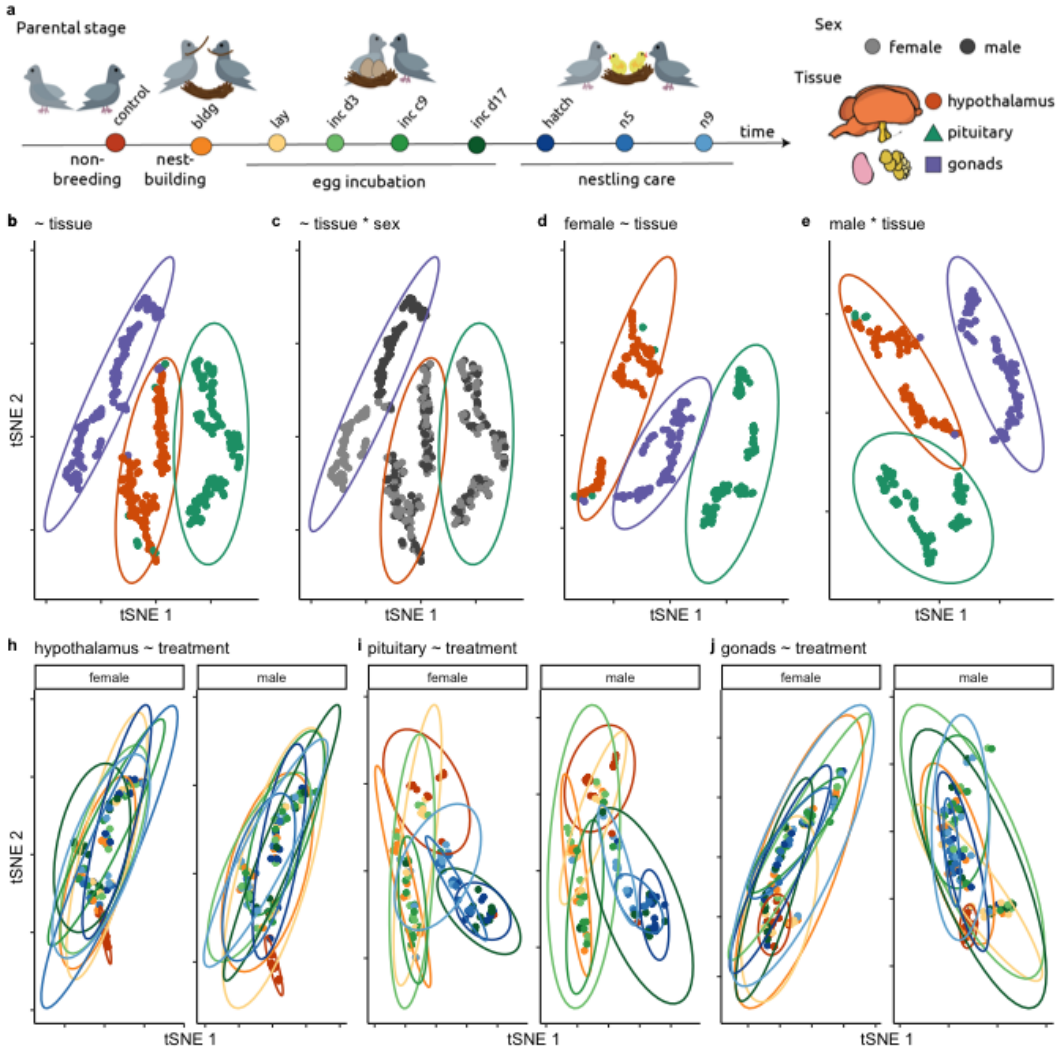


Figure 1: Broad patterns of variation in hypothalamic, pituitary, and gonadal gene expression in male and female rock doves across reproductive and parental transitions. a) Experimental design. b) t-SNE plot highlighting tissue differences in gene expression. Each dot represents 1 sample and is colored by the source tissue (hypothalamus: orange, pituitary: teal, gonads: purple). c) t-SNE plot highlighting sex differences in tissue and sex, as shown in light grey (females) and dark grey (males). t-SNE plots highlight treatment effects in females (d), males (e), the hypothalamus (h), pituitary (i), and gonads (j), with samples colored by parental stage (control: red or , nest-building: orange, lay: yellow; inc.d3: light green, inc.d9: green, inc.d17: dark green, hatch: dark blue, n5: blue, and n9: light blue.

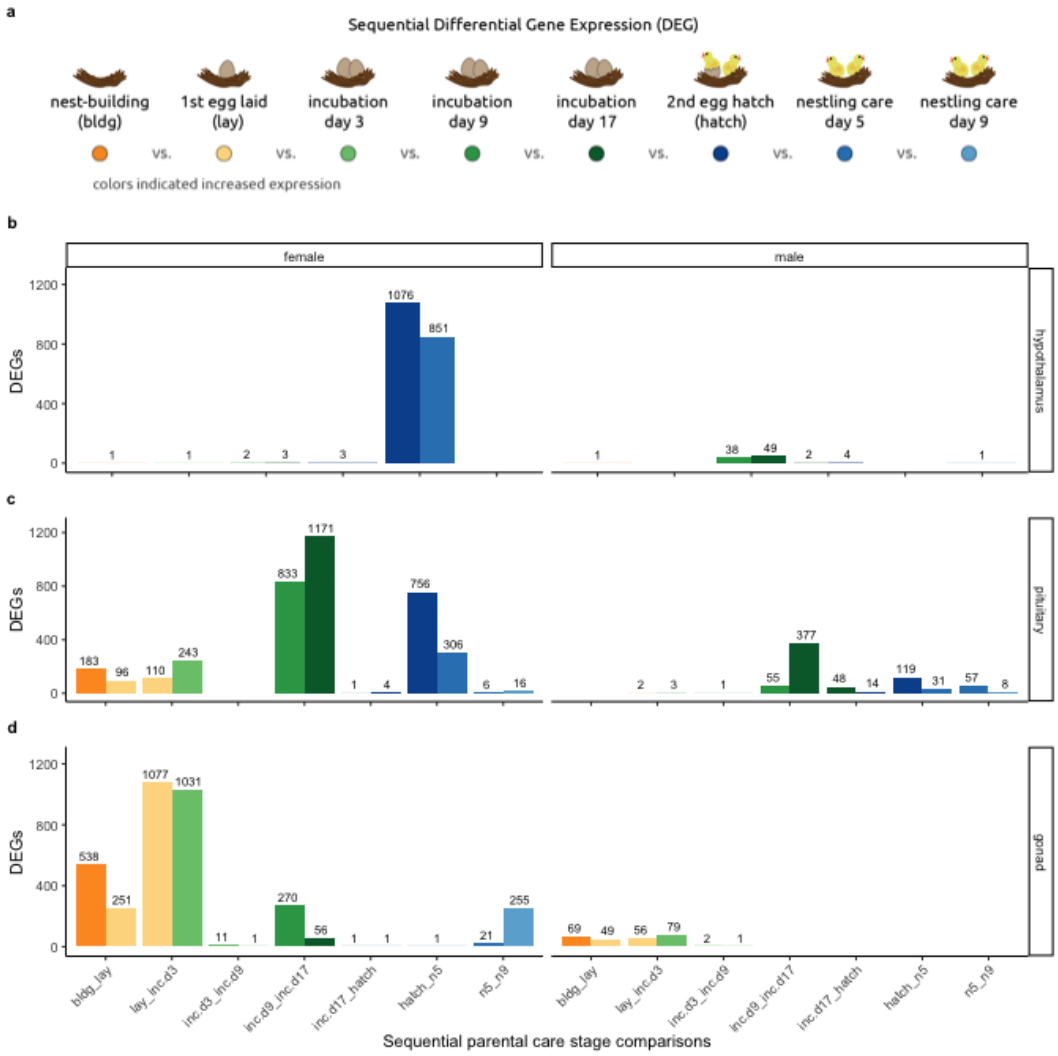


Figure 2: Differential gene expression differences between sequential parental stages. a) We calculated the differentail gene expression between each consequtive parental stage samples, for a total of 8 pair-wise comparisons for each sex in each tissue. The y-axis of these bar plots displays the total number of differentially expressed genes for each comparsison in the hypothalamus (b), pituitary (c), gonads (d). Bars are collored by the direction of the change in gene expression. Control: control: red or , nest-building (bldg): orange, lay: yellow; incubation day 3 (inc.d3): light green, inc.d9: green, inc.d17: dark green, hatch: dark blue, nestling care day 5 (n5): blue, and n9: light blue.

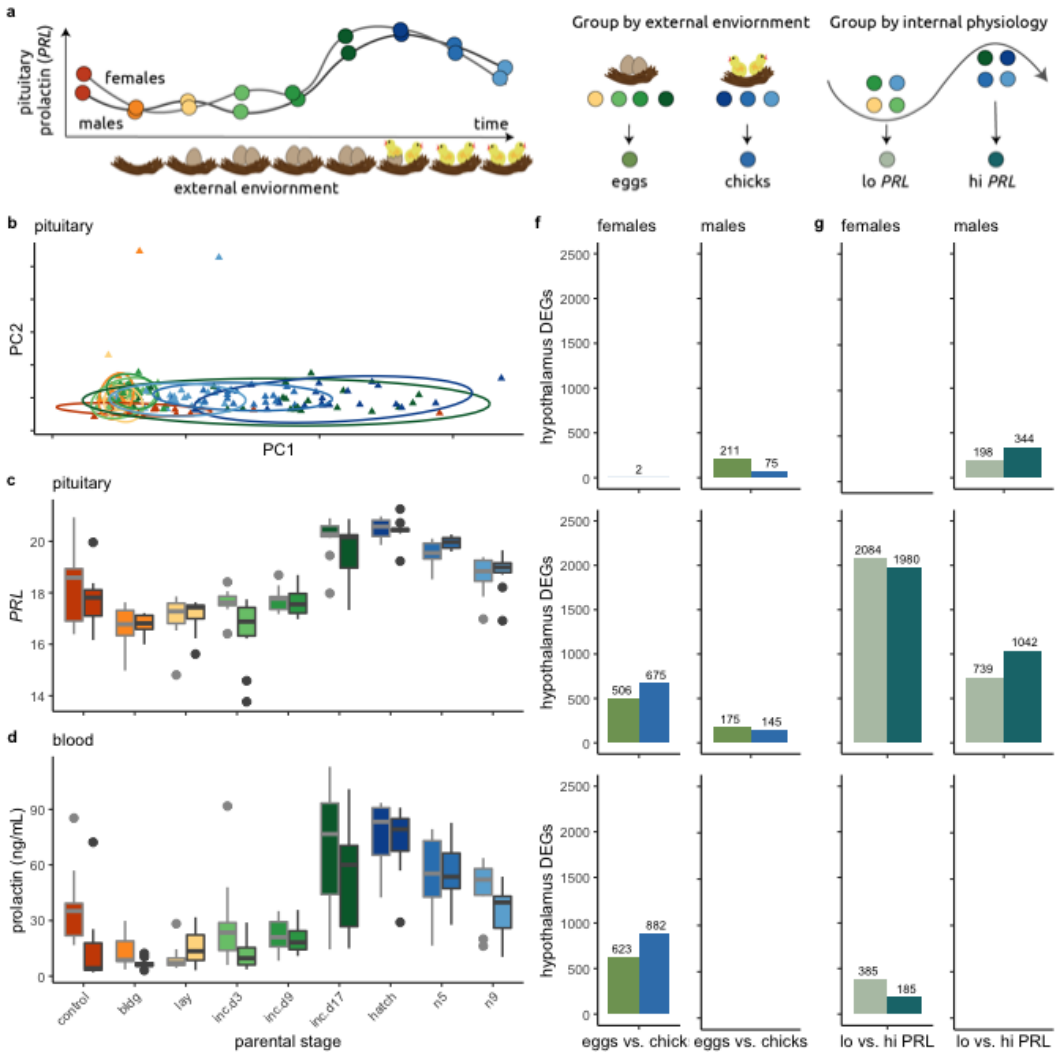
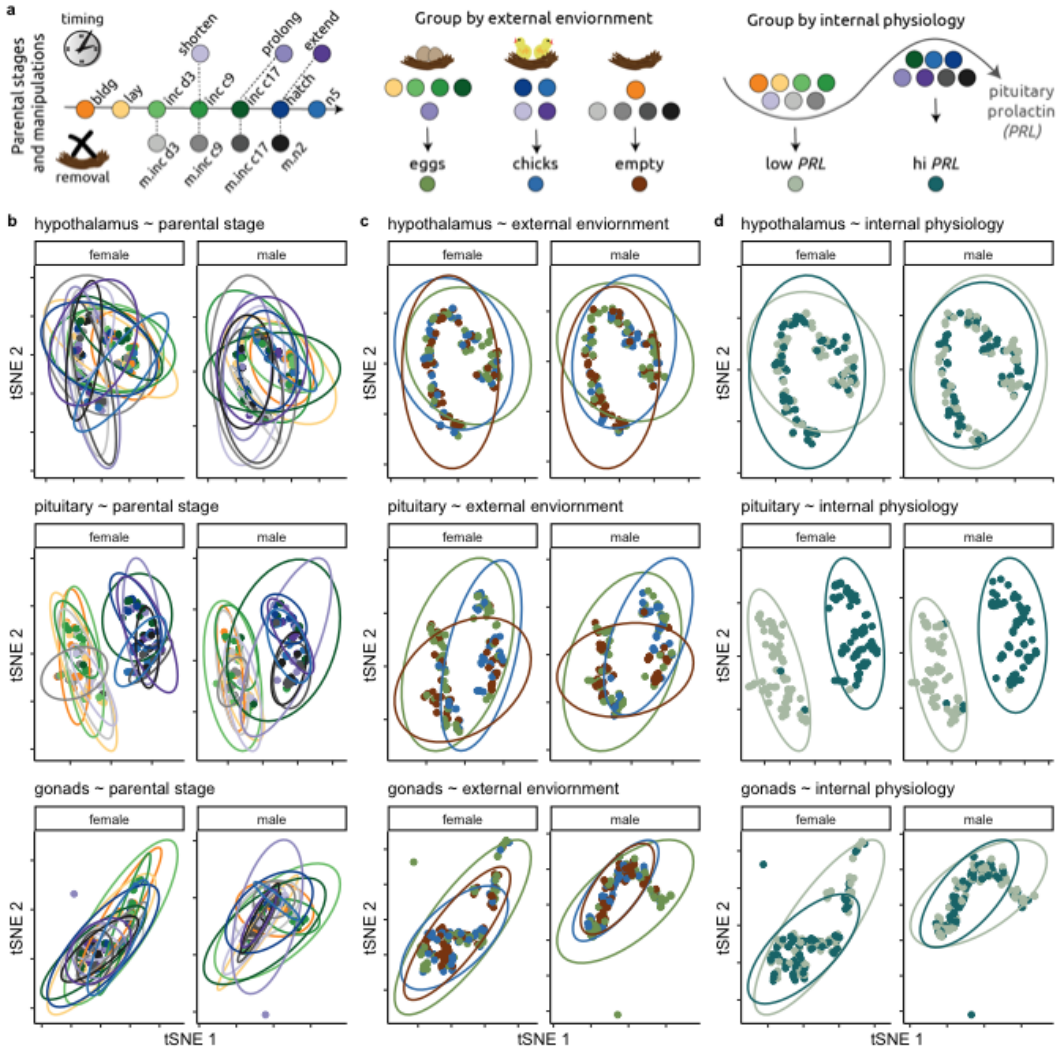


Figure 3: Another figure



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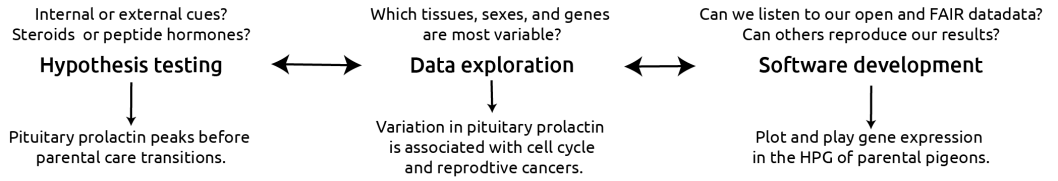

Figure 5: Hypothesis-driven research, data exploration and software development. We integrate hypothesis driven experimental testing with data-driven exploration and sofware develop to make new discoveries and advance our understanding reproduction and parental care.

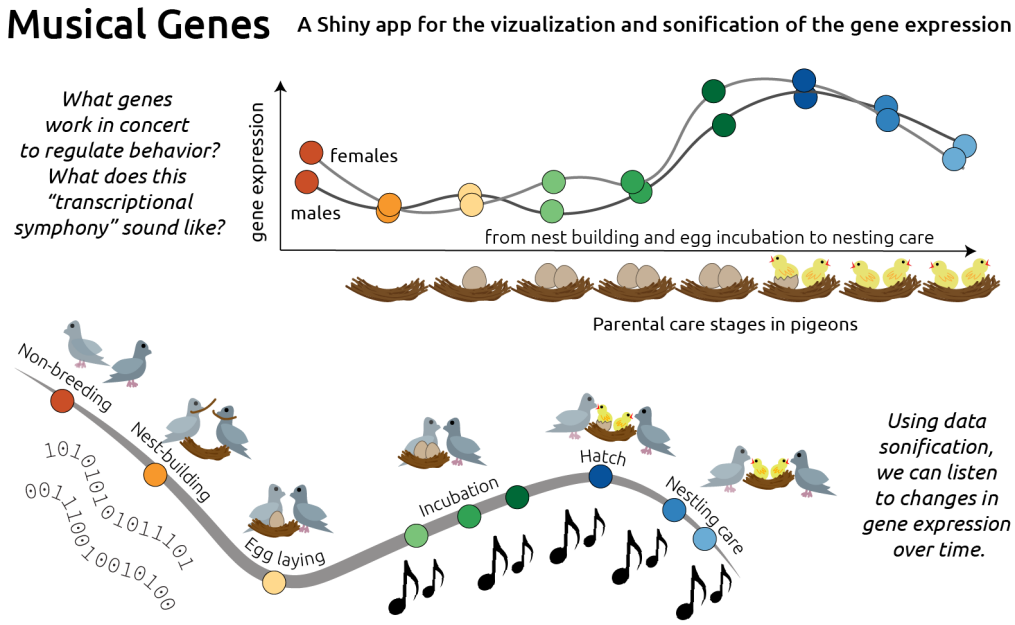

Figure 6: Shiny Musical Genes. Musical Genes is a R-based Shiny application for vizualizing and sonifying gene expression associated with transitions through reproduction and parental care.

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