The reproductive and parental care transcriptome of the rock dove

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Abstract In animals that exhibit a parental care reproductive strategy, successful rearing of offspring typically involves a shift from sexual behaviors to more caring and nurturing ones. Decades of work have highlighted the role of particular hormones in the maintenance of parental care behavior, the majority of which are produced by a biological system essential for reproduction, the hypothalamic-pituitary-gonadal (HPG) axis. However, we know very little about when and how the HPG axis transitions into parental care behaviors in any vertebrate, and this knowledge is fundamentally important to our understanding of the mechanisms mediating parental care. First, we characterized the transcriptome of the HPG axis in both mothers and fathers over 9 points of the parental care stage using a socially monogamous and bi-parental model, the rock dove (Columba livia). Then, we experimentally manipulated offspring presence at 7 different time points using classic offspring replacement and removal manipulations to uncover cause and effect of these transcriptional changes. We offer the most complete characterization and understanding of changes in neural, pituitary and gonadal transcription and translation ever reported in any vertebrate over the course of parental care.

Introduction

Parental care is essential to many organisms' reproductive strategy and predominates avian and mammalian groups (Clutton-Brock, 1991; Royle et al., 2012). Depending on the species, reproductive and parental behaviors may include territory establishment, courtship and mating, nest-building, nest defense, incubation, and the brooding and provisioning of young. The goal of these behaviors, either directly or indirectly, is the successful production of offspring resulting in a direct fitness benefit (Reviewed in Santos, E.S.A, 2012). Underlying these behaviors are the anatomical and physiological changes that facilitate the transition into and across reproductive and parenting stages. These changes have fascinated biologists and inspired a prodigious body of research. Much of this research has focused on understanding reproduction and parental care from an endocrine and neuroendocrine perspective (reviewed in Ondrasek 2016, Kelly 2020, Wingfield, Silver, Buntin). Reproduction and parental care is largely regulated by a set of three tissues: The Hypothalamus in the brain, the Pituitary, and the Gonads (testes and ovaries). Together, these tissues are often referred to as the 'reproductive' axis or the HPG. To activate reproduction, the HPG initiates an endocrine cascade starting with the production and release of gonadotropin-releasing hormone (GnRH) in the hypothalamus which, in turn, stimulates the anterior pituitary to produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH travel to the gonads via the bloodstream where they induce gonadal growth and steroidogenesis of estradiol, testosterone and progesterone). These sex steroids have well established roles in mating behavior, nest-building, territory defense, and egg-laying in birds (reviewed in Buntin). Another key hormone, prolactin, is produced in the pituitary and is associated with the induction and maintenance of behaviors such as incubation in birds and lactation (in doves and mammals) (birds: reviewed in Angelier, Austin et al. in prep, mammals: Austin and Word). Other hormones like vasopressin and oxytocin are related to affiliative behaviors (Yoshihara C, 2018) while dopamine, serotonin aggressive behaviors or avoidance (Rilling, JK, 2013; Adams DB, 2006). While these and many other hormones have been reported to play integral roles in various reproductive and parental care behaviors, we have yet to come close to conceptualizing the larger physiological and genomic coordination of events that yield such behaviors.

To begin to address this larger goal, we investigated genomic changes in the reproductive axis using the model of the rock dove (Columba livia). This species is socially and genetically monogamous and has bi-parental incubation and offspring care thereby making investigations of sexual variation across reproduction possible. Doves and pigeons have been used extensively to study the physiological mechanisms of reproduction and parental care, including the proximate and ultimate mechanisms that regulate associated behaviors (cite a bunch of new and old papers or books). Our team has produced a series of papers with the goal of illuminating how transcriptomic activity in the reproductive axis varied by sex (MacManes et al. 2016), in response to a stressor (Calisi et al. 2017, Lang et al. 2020), and following a hormonal manipulation (Austin et al. in prep). By using a genomic approach that spans the reproductive timeline, we have the ability to elucidate a transcriptional symphony that coordinates

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the anatomical, physiological and behavioral changes associated with transitioning from non-breeding to parenting in a vertebrate species. The aims of this research were three-fold: Our first aim was to produce a high quality and reproducible transcriptional characterization of the HPG over reproduction and parental care in male and female rock doves. We were specifically interested in understanding how gene activity in the HPG changed as individuals transitioned from a sexually mature, non-reproductive state into reproduction and parenting. To accomplish this aim, we investigated nine time points that spanned the majority of reproductive efforts in this species. These time points consisted of 1) a control or non-parenting state (from MacManes et al 2016 and Calisi et al. 2017), 2) nest-building, 3) clutch initiation and onset of incubation, 4) clutch completion and early incubation, 5) mid-incubation, 6) late incubation, 7) initiation of nestling care, 8) early nestling care, and 9) mid-nestling care. Because the majority of time points included in this study involved some form of direct offspring care, for simplicity, we will discuss these as parental care rather than differentiating between reproductive and parenting behaviors. However, it should be understood that parental care remains only one component of a larger set of reproductive behaviors. Our second aim focused on characterizing the major patterns of transcriptional changes between the tissues, sexes, and parental stages. We specifically investigate how gene expression in the HPG changed across various transition points. For example, from non-breeding to mating and nest-building behaviors, from nest-building to egg-laying to incubation, from incubation to nestling care, and from highly dependent nestlings (brooding and crop milk production) to older, thermoregulatory independent nestlings that are fed a regurgitant of seeds. Our third aim was to identify the drivers underlying changes in transcription. Specifically, we tested whether external or internal factors were regulating gene activity. We conducted a series of offspring removal and replacement manipulations to test whether transcriptional changes were dependent upon offspring presence or were regulated by an internal clock.

Additionally, because these data are extensive, we took the opportunity to provide them in an easily accessible form that can be used for independent data exploration by the reader. Given the homology of genes and gene networks shared between rock doves and other vertebrate species, the findings from these RNA-seq experiments could be of interest to scientists and clinicians studying diverse systems, particularly in non-model organisms like our study species. Overall, this study provides a deeper understanding of the interplay of genes across parenting and has important implications for understanding molecular processes that underlie behavioral transitions.

Results

The rock dove reproduction and parental care transcriptome

We created the rock dove transcriptome by sequenced 987 RNA samples from the hypothalamus, pituitary and gonads from the female and male of 166 rock dove pairs across (Fig. 1). These samples come from nine timepoints that encapsulate important transitions in reproduction (Fig. 1A), four offspring removal manipulations (Fig. 1B), and three offspring replacement manipulations (Fig. 1C) to determine the influence of internal mechanisms and external stimuli on gene expression.

Using T-distributed Stochastic Neighbor Embedding (tSNE) we show broad patterns of variation due to treatment or sex in each tissue (Fig. 1D). In the hypothalamus, tSNE dimension visually separates most of the samples that were subject to an offspring manipulation from those from naturally occurring parental stages. In the pituitary however, samples cluster more by wether they were before incubation day 9 or after incubation day 17. Neither the hypothalamus or pituitary show strong sex differences at this level, but the gonads samples from very distinct, sex-specific clusters.

Discussion

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[2;4]. 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[3]. 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[1].

Table 1: Changes in candidate gene expression over the course of parental care in rock doves. Significant changes in gene expression are shown for X candidate genes. Positive and negative changes are denoted with (+) and (-) respectively for female (F) and male (M) hypothalamus (H), pituitary (P) and gonads (G). Comparisons are denoted in the column names. The first group is the reference group.

gene	bldg-lay	lay-inc3	inc3-inc9	inc9-inc17	inc17-hatch	hatch-n5	n5-n9
AVPR2						FP(+)	
DIO2		FG(-)					
DIO3	FP(-)						
GNRHR	FP(-)						
PRL				FP(+); MP(+)			
PRLH	FP(-)	FP(+)		FP(+)			

Table 2: Changes in candidate gene expression in response to removal. Significant changess in gene expression are shown for X candidate genes. Positive and negative changes are denoted with (+) and (-) respectively for female (F) and male (M) hypothalamus (H), pituitary (P) and gonads (G). Comparisons are denoted in the column names. The first group is the reference group.

gene	inc.d3_m.inc.d3	inc.d9_m.inc.d9	inc.d17_m.inc.d17	hatch_m.n2
AVPR1B				FP(+)
AVPR2				FP(+)
DIO3			FP(+); MH(-)	FH(-); FP(+)
ESR2			FH(-); MH(-)	
FSHB			FP(-)	FH(-); FP(-)
GABRQ			FH(+)	FH(+)
JAK2			FP(+)	FP(+)
LHCGR			FH(-)	FH(-); MH(-)
NPVF	FH(-)		FH(-)	FH(-); MH(-)
NPY	FP(-)			MP(+)
PRL			FP(-)	MP(-)
PRLH				MH(+)
SERPINA4			FP(+)	FP(+)

Tables

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Figures

Table 3: Changes in candidate gene expression in response to replacement. Significant changess in gene expression are shown for X candidate genes. Positive and negative changes are denoted with (+) and (-) respectively for female (F) and male (M) hypothalamus (H), pituitary (P) and gonads (G). Comparisons are denoted in the column names. The first group is the reference group.

gene	inc.d9_early	inc.d17_prolong	hatch_early	hatch_prolong	hatch_extend
AR			FP(+)		
AVPR2			FP(+)		
CRHR1		MG(+)			
ESR2		MG(+)			
GABRQ	MP(+)	FH(+)	MH(+); MP(+)	FH(+); MG(+)	FH(+)
GNRH1			FH(+)		
LHCGR		FH(-)	MH(-)	FH(-)	FH(-); MH(-)
NPVF	MH(-)	FH(-)	FH(-); MH(-)	FH(-)	FH(-)
NPY		MP(-)	MP(+)		
POMC			FH(-)	FH(-)	
PRL			FP(-); MP(-)		
PRLHR			MP(+)		
SERPINA4		MG(+)	MH(+)		

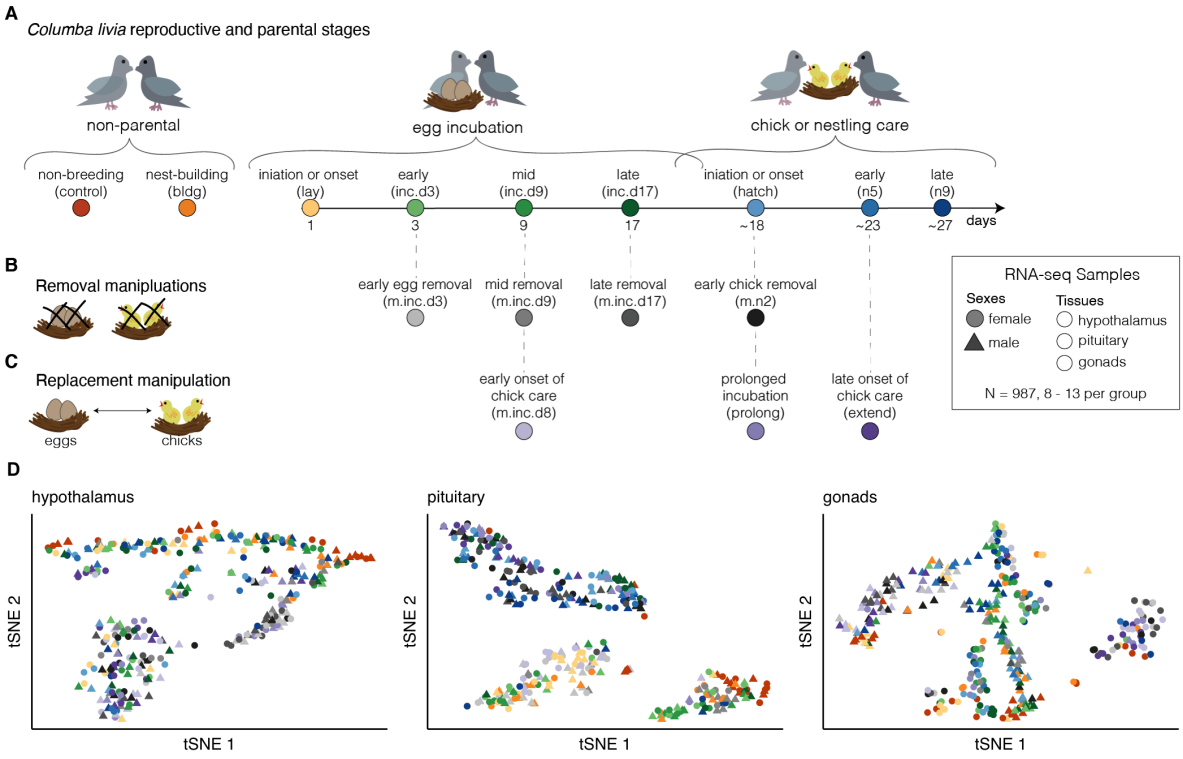

Figure 1: Experimental design. A) We investigated nine timepoints that spanned the majority of reproductive efforts in this species. These timepoints consisted of a "control" or non-parenting state (from MacManes et al 2016 and Calisi et al. 2017), nest-building, clutch initiation or the onset of incubation, 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.

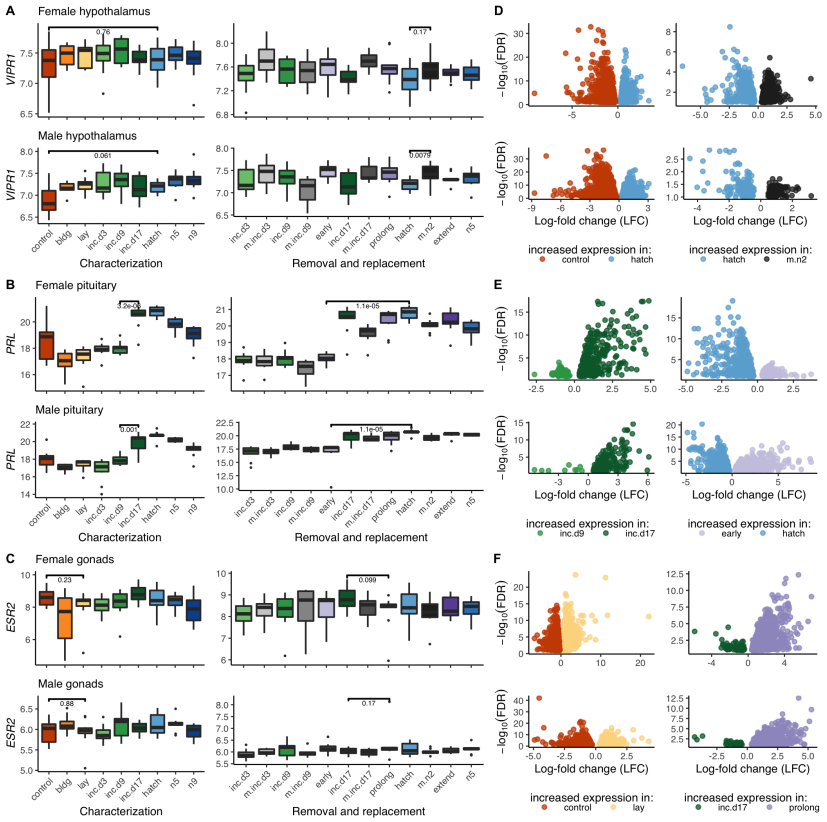

Figure 2: Hypothesis-driven and data-driven approaches to identifying changes in gene expression.Box-and-whisker plots show changes in gene expression of serotonin receptor (HTR2C) in the hypothalamus (A), prolactin (PRL) in the pituitary (B), and estrogen receptor 1 (ESR1) in the gonads.Bar and statistics 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.

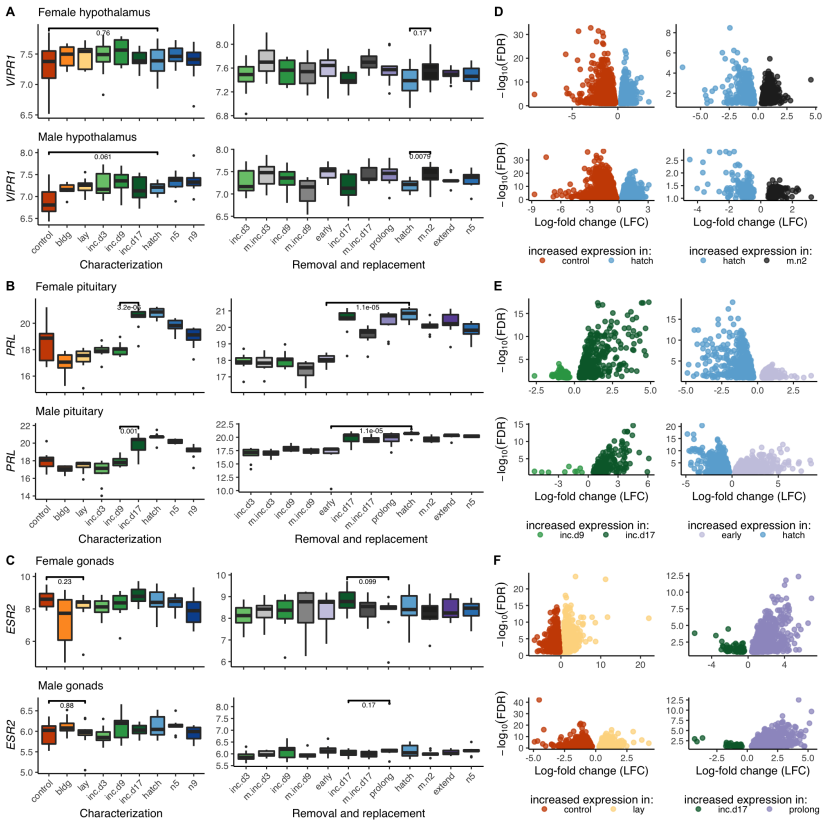

Figure 3: Summary of differentially expressed genes. Bar plots show the total number of significantly differentially expressed genes for all two-way comparisons in the hypothalamus (A), pituitary (B), gonads (C) . Contrasts are made relative to the non-breeding controls, the nest-building group, the 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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