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Sex-biased transcriptomic response of the reproductive axis to stress

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ABSTRACT

Stress is a well-known cause of reproductive dysfunction in many species, including birds, rodents, and humans, though males and females may respond differently. A powerful way to investigate how stress affects reproduction is by examining its effects on a biological system essential for regulating reproduction, the hypothalamic-pituitary-gonadal (HPG) axis. Often this is done by observing how a stressor affects the amount of glucocorticoids, such as cortisol or corticosterone, circulating in the blood and their relationship with a handful of known HPG-producing reproductive hormones, like testosterone and estradiol. Until now, we have lacked a full understanding of how stress affects all genomic activity of the HPG axis and how this might differ between the sexes. We leveraged a highly replicated and sex-balanced experimental approach to test how male and female rock doves (*Columba livia*) respond to restraint stress at the level of their transcriptome. Females exhibit increased genomic responsiveness to stress at all levels of their HPG axis as compared to males, and these responsive genes are mostly unique to females. Reasons for this may be due to fluctuations in the female endocrine environment over the reproductive cycle and/or their evolutionary history, including parental investment and the potential for maternal effects. Direct links between genome to phenome cause and effect cannot be ascertained at this stage; however, the data we report provide a vital genomic foundation on which sex-specific reproductive dysfunction and adaptation in the face of stress can be further experimentally studied, as well as novel gene targets for genetic intervention and therapy investigations.

1. Introduction

Stress can disrupt reproduction in multiple, complex ways (Geraghty and Kaufer, 2015; Johnson et al., 1992; Toufexis et al., 2014). The perception of a stressor activates the hypothalamic-pituitary-adrenal (HPA) axis, which results in a synthesis of metabolic hormones, including glucocorticoids (Sapolsky et al., 2000). Glucocorticoid hormones (cortisol in humans, corticosterone in birds and rodents) are synthesized by the adrenal cortex and exert both rapid and gradual actions on vertebrate physiology (de Kloet et al., 2008). This activation of the HPA system can cause suppression of the reproductive system, i.e., the hypothalamic-pituitary-gonadal (HPG) axis, at multiple levels (Fig. 1), including inhibiting gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus, suppressing luteinizing hormone (LH) and follicle stimulating hormone (FSH) release from the pituitary, sex steroid hormone synthesis in the gonads, and ultimately reducing or eliminating sexual behavior and reproduction (Geraghty et al., 2015; Retana-Márquez et al., 2003; Rivier and Rivest, 1991; Viau, 2002). However, questions remain as to 1) how stress affects all gene activity of the HPG axis, and 2) if these effects are sex-specific. Evidence suggests regulatory mechanisms of the HPG system under stress can be sex-specific (e.g. human: (Verma et al., 2011); rodent: (Patchev and Almeida, 1998); birds: (Dickens and Bentley, 2014; Schmidt et al., 2014)), but the full extent of sex-biased changes is still largely unknown. In general, males have dominated animal studies (Beery and Zucker, 2011; Ranganathan and Kumar, 2015; Zucker and Beery, 2010), obscuring discovery of potential sex differences that could inform and guide further research and clinical studies (Clayton and Collins, 2014).

Here, we tested the effects of stress on the genomic activity of the HPG axis of male versus female rock doves (*Columba livia*). Doves have been historically used to study reproductive behavior (Ball and Silver, 1983; Buntin et al., 1993; Darwin, 1968; Lehrman, 1955) and now are proving to be a valuable model for genomics research (Domyan and Shapiro, 2016; Gillespie et al., 2013; MacManes et al., 2017; Shapiro et al., 2013). The effects of stress on reproduction can vary by species and between the sexes in accordance with age, reproductive strategy, evolutionary history, and in response to multiple social and environmental factors (Wingfield and Sapolsky 2003). Subjects used in our study were of similar age, and rock doves in general are similar in many

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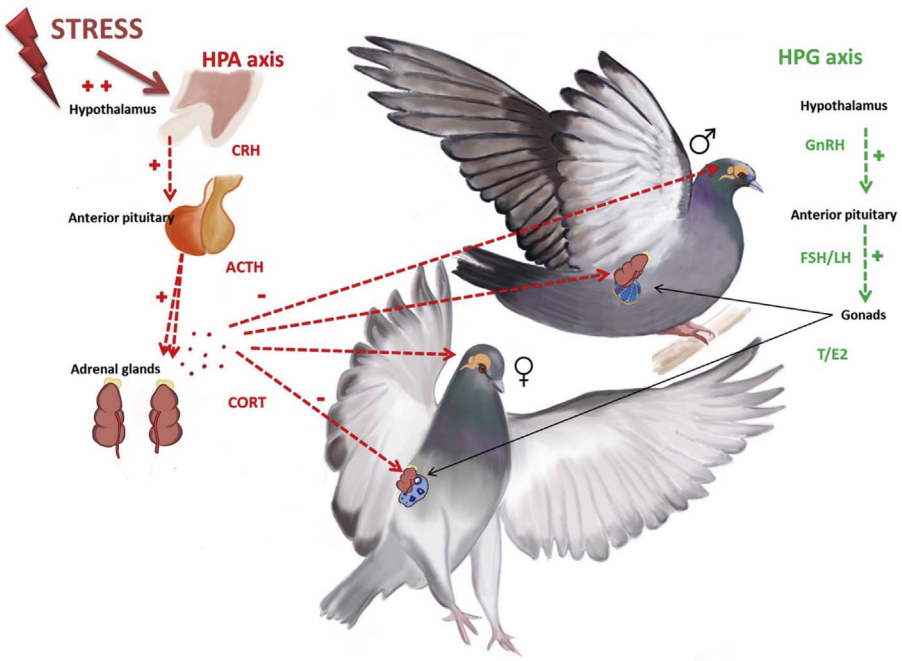

Fig. 1. Depiction of the hypothalamic-pituitary-adrenal (HPA) axis and hypothalamic-pituitary-gonadal axis (HPG). Plus signs denote a stimulatory influence on axis activity in the direction indicated by dashed arrows; negative signs denote the potential negative effects of corticosterone (CORT) on HPG activity. Illustration by Natalia Duque.

ways; they are monomorphic, monogamous, and exhibit a biparental care strategy (Johnston, 1992). Thus, we might presume that male and female rock doves respond to stress in a similar manner. However, variation females experience in their reproductive cycle coupled with their evolutionary history, which includes increased parental investment and potential trade-offs of reproductive success for survival, led us to anticipate otherwise. We predicted that the HPG axis, a system intimately connected with reproductive behaviors and processes under selection, would exhibit sex-specific responses to stress at the level of genomic activity.

We exposed sexually mature males and females to 30 min of restraint stress, which successfully activates the stress response as measured through significantly increased circulating plasma glucocorticoids. We compared the genomic expression of the HPG axis of stressed females and males to each other and in comparison with unstressed controls. We report patterns of tissue-specific and sexually dimorphic gene expression, with females showing a greater stress response at the level of the transcriptome in all three tissues - the hypothalamus, pituitary, and gonads - as compared to males. These data offer a valuable resource to advance stress and reproductive research with the potential for devising future therapeutic strategies to ameliorate stress-induced HPG axis dysfunction.

2. Materials and methods

2.1. Animal Collection Methods

Subjects were housed at the University of California, Davis, in large aviaries (1.5 × 1.2 × 2.1 m), with 8 sexually reproductive adult pairs per aviary. Food and water were provided ad libitum. To control for reproductive stage and potential circadian rhythm confounds, males and females sampled were paired but lacked eggs or chicks, and sampling occurred between 0900 and 1000 (PST) following animal care and handling protocols (UC Davis IACUC permit #18895). A total of 48 subjects were sampled for this study (stress treatment group: 12 male, 12 female; control group: 12 male, 12 female). Subjects in the baseline, or "unstressed," group were sampled within 5 min of entering their cage. Subjects in the stress treatment group were restrained in cloth bags for 30 min prior to sampling. To sample tissue, subjects were first anesthetized using isoflurane until unresponsive (< 2 min), at which point they were decapitated. Trunk blood was collected to assay for plasma corticosterone concentrations. Brains, pituitaries, and gonads were then immediately extracted and placed on dry ice, then transferred to −80 °C until further processing. Brains were sectioned coronally on a cryostat (Leica CM 1860) at 100 μm to best visualize and biopsy the hypothalamus in its entirety, its identity and location confirmed using a stereotaxic atlas of the pigeon brain (Karten and Hodos, 1967). Techniques used to harvest and sequence rock dove hypothalamic, pituitary, and gonadal tissue have been previously validated and reported by our research group (MacManes et al., 2017). In brief, hypothalamic tissue and adjoining lateral septum were collected beginning at the point of bifurcation of the septopallio-mesencephalic tract and ending after the cerebellum became apparent. Hypothalamic sections, pituitaries, and gonads were preserved in RNALater and shipped from the UC Davis to the University of New Hampshire for further processing. Tissue was sequenced from these biopsied brain sections as well as from whole homogenized testes and ovaries, the latter comprised of tissue from the oviduct and ovarian follicles.

2.2. Hormone assay

Fresh blood was centrifuged at 4 °C for 10 min. Plasma was removed and stored at −80C. Plasma was assayed for corticosterone using radioimmunoassay (RIA), informed by a serial dilution conducted prior to the assay. A dilution of 1:20 was used in a commercially available Corticosterone RIA kit (MP Biomedicals, Orangeburg, NY) to determine corticosterone levels (ng/ml). The assay was validated for cross-reactivity with *C. livia* corticosterone and the limit of detection was estimated at 0.0385 ng/ml. A two-way ANOVA was used to determine if our stress treatment significantly ($P > 0.05$) increased circulating corticosterone as compared to controls.

2.3. Illumina library preparation and sequencing

Tissues frozen in RNALater were thawed on ice in an RNAse-free work environment. Total RNA was extracted using a standard Trizol