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Environmental impacts in domestic birds: Towards homeostasis, efficiency and well-being

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Environmental impacts in domestic birds: Towards homeostasis, efficiency and well-being

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Editorial: Environmental impacts in domestic birds: towards homeostasis, efficiency and well-being

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Editorial on the Research Topic
Environmental impacts in domestic birds: towards homeostasis, efficiency and well-being

A *sine qua non* is that environmental stressors exert a negative effect on animals via the hypothalamo-pituitary-adrenal axis. Such stresses include cold stress, heat stress, high stocking density, feed restriction environmental and pollutants (Akinyemi and Adewole, 2021). This Research Topic provides a compendium of 11 papers and reviews covering aspects of environmental impacts in domestic birds: towards homeostasis, efficiency and wellbeing. Effects of the following environmental factors were investigated: heat stress, lighting (intensity or duration), isolation stress, pathogens, and pesticides with both physiological and/or behavioral responses determined.

Geoffrey Harris developed the schema of control of the release of anterior pituitary hormones by hypothalamic releasing hormones passing from terminals in the median eminence of the hypothalamus in the hypophyseal portal blood vessels Watts (2015). Arguably implicit to the model were the views that releasing hormones would be specific for individual anterior pituitary hormones and that there would be one, or perhaps two (one stimulatory and one inhibitory), releasing hormones per anterior pituitary hormone. An example of such a control system is the hypothalamo-pituitary- adrenocortical axis with corticotropin releasing hormone (CRH) binding to CRH receptors 1 (CRH-R1) stimulating the release of adrenocorticotropic hormone (ACTH) which, in turn, stimulates the synthesis of glucocorticoids (Kang and Kuenzel, 2014). In addition to this system, there are other neuropeptides participating in the responses to stressors including met-enkephalin and related peptides (Pierzchała-Koziec and Scanes, 2023).

There are multiple members of the CRH family of genes/peptides across the vertebrates: CRH in eutherian mammals (CRH 1 in non-eutherian mammal and other vertebrates), CRH 2 in multiple vertebrate groups but neither in eutherian mammals nor teleost fish, urocortin

(UCN) 1, 2, and 3 (Seasholtz et al., 2002; Cardoso et al., 2016); the last common ancestor between these vertebrate classes existing 290 million years ago (Davis et al., 2012). In chickens, the expressed precursors of four peptides have been identified: CRH 1 (Vandenborne et al., 2005), CRH 2 (Genbank accession KU887752), urocortin (Genbank accession XM_046939396), and urocortin 3 (Grommen et al., 2017) (for sequences of the chicken members of the CRH family of peptides see Figure 1). Across the vertebrates, there are two forms of CRH with CRH 2 having been reported in multiple vertebrates including platypus and opossum (class: Mammalia), lizard (class: Reptilia), coelacanth (class: Actinistia) spotted gar (class: Actinopterygii), elephant shark (class: Chondrichthyes) (Cardoso et al., 2016) together with chicken (class: Aves) (Genbank accession KU887752). In the present Research Topic, there are marked changes in expression of CRH 2 in several hypothalamic nuclei in chickens exposed to the stress of restraint (Kadhim and Kuenzel); "*this being first report in any vertebrate species that following a stressor there occurs significant increases of CRH2 gene expression in two neural structures that play a role in regulating the stress response, the nucleus of the hippocampal commissure and the paraventicular hypothalamic nucleus, resulting in a sustained increase in the stress hormone, corticosterone*" (W.J. Kunzel, personal communication).

There is increasing information of gene expression in neurons within the hypothalamic stress related nuclei in birds. For instance, the premammillary (PMM) nucleus contains neurons expressing enzymes required for the synthesis of dopamine and melatonin together with expression of the light sensitive opsin (Kang). This is consistent with neurons in the premammillary being dual sensory-neurosecretory units (Kang). Moreover, there was greater expression of tryptophan hydroxylase 2, tyrosine hydroxylase and glucocorticoid receptor in the ventral tegmental area in birds raised on low that higher light intensity or on variable light intensity (Kang et al.).

There is cross-talk between the hypothalamo pituitary adrenocortical and thyroid axes in birds with thyrotropin releasing hormone (TRH) receptor 2 present in avian thyrotropes. Chicken thyrotropes not only express CRH-R2 but also both CRH (CRH 1) and urocortin 3 (another ligand for the CRH-R) stimulate thyrotropin release (De Groef et al., 2003). Somatostatin inhibits thyrotropin release in response to either TRH or CRH (De Groef et al., 2005). In the present Research Topic, immobilization stress is demonstrated to induce increases in circulating concentrations of corticosterone and the expression of thyrotropin β subunit in the anterior pituitary gland together with that of the TRH receptor 1 and 3 in the anterior pituitary gland of chickens (Kadhim and Kuenzel).

Pathogens represent a stressor affecting poultry. There were marked differences in ileal and cecal microbiome between conventional broiler chickens and slow growing birds and following *salmonella* challenge (Sheets et al.). *Salmonella* challenge was associated with depressed growth rate in convention broiler chicks but not slow growing chickens (Snyder et al.).

Heat stress adversely affects poultry. In this Research Topic, differences in the responses to heat stress were reported between unselected broiler type chickens and chickens highly selected for growth (Brugaletta et al.). Both growth and feed intake were decreased in 1995 random bred (equivalent to broiler chickens of the 1990s), and fast growing modern random bred (a mixture of the broilers of the 2020s) subjected to heat stress but did not influence growth or feed intake in either Jungle fowl, Athens Canadian Random Bred (equivalent to broiler chickens of the 1950s) (Brugaletta et al.). However, hypothalamic expression of feeding related neither neuropeptides nor their receptors were not affected by elevated environmental temperatures (Brugaletta et al.).

Heat stress was accompanied by increases in the plasma concentration of both corticosterone and cortisol but decreases in egg production in domestic female ducks (Oluwagbenga et al.). Body condition scoring rubric for welfare assessment was employed also to assess the welfare of the birds (Oluwagbenga et al.). Heat stress depressed welfare as assessed by feather quality and cleaniless together with foot pad condition (Oluwagbenga et al.). Chronic heat stress was accompanied by increases in the norepinephrine concentrations in the egg albumin but not yolk of domestic ducks However, there were no effect of chronic heat stress on the concentrations in the norepinephrine precursor, L-3,4-dihydroxyphenylalanine (l-dopa) in either the albumin and yolk (Lyte et al.).

Domesticated birds respond to two characteristics of light, namely, duration and intensity. Transfer of mountain ducks from a long-daylength (17L5D) to continuous lighting (24L:0D) was accompanied by increased plasma concentrations of gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), prolactin, progesterone (P_4), testosterone and estradiol (E_2) (Liufu et al.). Hypothalamic expression of TRH was increased in ducks on 24L:0D but decreased in 8L:16D (Liufu et al.). Similarly, expression of TSH β sub-unit in the anterior pituitary gland was increased in ducks on 24L:0D but decreased in 8L:16D (Liufu et al.). It is questioned whether continuous lighting is stressful.

FIGURE 1
Corticotropin releasing hormone (CRH) family members in chickens: 1—CRH 1 (Vandenborne et al., 2005), 2—CRH 2 (Genbank accession KU887752), 3—urocortin (Genbank accession XM_046939396), 4—urocortin 3 (Grommen et al., 2017). Blue indicates structure of CRH 1 or identical amino acids in other peptides. Red indicates differences to CRH 1.

Light intensity influences birds. For instance, the incidence of dust bathing was greater in broiler chickens raised with a variable light intensity in different parts of the pen compared to either those raised on either 5 or 20 lux light intensity (Kang et al.). Moreover, the responses to a novel object test were greater in young broiler chickens raised under a low light intensity compared to a higher light intensity which in turn were greater than birds under a variable lighting regimen (Kang et al.). There was greater expression of tryptophan hydroxylase 2 in both the caudal raphe nucleus and the dorsal raphe nucleus (Kang et al.). There were also differences with birds on natural lighting (Kang et al.). It is also questioned whether low and/or high light intensities and/or an environment with little differences in light intensity are stressful.

Fear is a stressor. Line differences in fear related behaviors in laying hens such as latency following tonic immobility and vocalization following isolation stress (Brown et al.). In addition, the were line/genetic differences in plasma concentrations of corticosterone, heterophil to lymphocyte ratio and physical asymmetry (Brown et al.).

Chickens are used as models for the effects of potential toxicants in the environment on reproduction. An example of this is a definitive study by Fréville et al. Glyphosate is a widely used herbicide and is consequently present in the environment. There were multiple effects of the addition of glyphosate-based herbicide to the feed of laying hens including increases in the relative weight of the gizzard, in the plasma concentrations of the oxidative stress parameter, thiobarbituric acid reactive substances (TBARS) in the liver and muscle, plasma concentrations of aminomethylphosphonic acid (AMPA) and cecal concentrations of short chain fatty acids (Fréville et al.). Moreover, in a companion paper, the same group reports differences in growth rate, circulating concentrations of triglyceride and the adipokinin, chemerin, together with abdominal adiposity and behaviors in the progeny of hens that had previously been treated with glyphosate-based herbicide compared to control hens (Estienne et al., 2023).

Author contributions

SW: Conceptualization, Writing–review and editing, Project administration, Supervision. GF: Conceptualization, Writing–review and editing. SO: Conceptualization, Writing–review and editing. DK: Writing–review and editing. GA: Writing–review and editing. KP-K: Writing–review and editing. CS: Writing–review and editing, Conceptualization, Writing–original draft.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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PERSPECTIVE
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Central Nervous System Associated With Light Perception and Physiological Responses of Birds

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Environmental light that animal receives (i.e., photoperiod and light intensity) has recently been shown that it affects avian central nervous system for the physiological responses to the environment by up or downregulation of dopamine and serotonin activities, and this, in turn, affects the reproductive function and stress-related behavior of birds. In this study, the author speculated on the intriguing possibility that one of the proposed avian deep-brain photoreceptors (DBPs), i.e., melanopsin (Opn4), may play roles in the dual sensory-neurosecretory cells in the hypothalamus, midbrain, and brain stem for the behavior and physiological responses of birds by light. Specifically, the author has shown that the direct light perception of premammillary nucleus dopamine-melatonin (PMM DA-Mel) neurons is associated with the reproductive activation in birds. Although further research is required to establish the functional role of Opn4 in the ventral tegmental area (VTA), dorsal raphe nucleus, and caudal raphe nucleus in the light perception and physiological responses of birds, it is an exciting prospect because the previous results in birds support this hypothesis that Opn4 in the midbrain DA and serotonin neurons may play significant roles on the light-induced welfare of birds.

Keywords: light, melanopsin (Opn4), premammillary nucleus, ventral tegmental area, raphe nucleus, dopamine, serotonin, welfare

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INTRODUCTION

Light perception and integration of photic information in the diurnal animals are critical for their proper adaptation to the environment, and therefore, animals can respond to daily and annual environmental change (Chmura et al., 2019; Hussein et al., 2021). Light plays a central role in modulating animal behavior and is a critical environmental factor that can affect the physiological processes, performance, and welfare of many animals and birds (Wilson and Cunningham, 1980; Manser, 1996; Deep et al., 2010; Fernandes et al., 2013; Aulsebrook et al., 2021). The physiological roles and effects of light include facilitating sight, regulating reproductive hormone release, and affecting social behavior. The most visible physiological effects of light on birds are the effect of photoperiod and light intensity on the seasonal reproduction, health, and behavior of birds (Deep et al., 2010; Olanrewaju et al., 2018; ViviD and Bentley, 2018).

Several studies provide evidence that light can affect the central physiology of animals independent of retinal function (Chiu et al., 1975; Routtenberg et al., 1978; Underwood et al., 1984; Wade et al., 1988; Fernandes et al., 2013). In avian species, photoperiodic synchronization is achieved independently of the pineal melatonin through direct light perception by avian deep-brain photoreceptors (DBPs), which project directly to the median eminence near the pars tuberalis (PT) in the anterior pituitary (Kang et al., 2010; Nakane et al., 2010; Chmura et al., 2019). However, evidence is not available regarding the pathway used by the photoperiodic message to reach the PT independently of pineal melatonin in mammals. The melatonin-independent photoperiodic entrainment of the annual thyroid-stimulating hormone (TSH) rhythm was reported in the European hamster, suggesting the presence of the non-visual DBPs in mammals (Saenz De Miera et al., 2018). Interestingly, encephalopsin (Opn3) was found to be expressed in different areas of the rodent brain, indicating a potential role of Opn3 in the non-visual photic process due to the changes in light (Blackshaw and Snyder, 1999; Nissila et al., 2012).

The initiation of light-induced physiological change is particularly important for diurnal animals such as mammals and birds. However, those within the avian brain have not been studied extensively. In this study, the author explored and derived how non-visual photoreceptive cells in the avian brain may connect to circuits controlling the aspects of feeding and emotional behaviors, which will provide an intriguing perspective on how environmental light can be a critical cue for the welfare of birds.

EFFECT OF LIGHT ON THE BEHAVIOR AND PHYSIOLOGY OF BIRDS

Light information characterizing the particular day length (i.e., photoperiod) and intensity can be stored within the organism and subsequently used to provide time signals for the adjustments of the physiological behavior of animals (Farner and Wingfield, 1980; Gwinner, 1989; Brandstatter et al., 2000). Animals must be able to discriminate between short and long days to perform photoperiodic time measurement. The differences of circadian changes related to the reproductive activation between mammals and avian species were well-reviewed by recent reports (Ikegami and Yoshimura, 2013; Kuenzel et al., 2015; ViviD and Bentley, 2018). In comparison with mammals, the avian circadian pacemaking system seems to be more complicated, being composed of at least three major components containing autonomous circadian oscillators as follows: the pineal gland, the retina, and a central nervous hypothalamic component possibly equivalent to the mammalian suprachiasmatic nucleus (SCN). The avian pineal organ contains photoreceptors with different photopigments including melanopsin (Opn4, an opsin-based photopigment), and synthesizes and secretes melatonin which is regulated by light (Sato, 2001; Kang et al., 2007, 2010).

The effects of artificial light on wild birds are critical for their various biological responses. Especially, artificial light at night alters natural light/dark cycles to be problematic for many avian species, suggesting that disrupting circadian rhythms causes multiple direct and indirect physiological consequences of birds because the unnatural sleep deprivation is associated with cardiovascular disease and endocrine disruption and has a profound effect on the circadian expression of genes associated with the immune and stress response (Dominoni et al., 2016).

Light intensity has a significant effect on the behavior, diurnal activity, and immune function of chickens (Blatchford et al., 2009). When birds are in the higher light intensity, they show a more dramatic circadian rhythm, spending more time active, eating and drinking, walking, foraging, and preening during the photophase (light), and resting more time during the scotophase (dark) compared with birds kept at lower light intensities (Alvino et al., 2009; Blatchford et al., 2009; Rault et al., 2017). The rhythms of the multiunit neuronal activity in the premammillary nucleus (PMM) of the caudal hypothalamus of temperate zone bird were demonstrated to show the photoperiod-dependent durations of high activity (Kang et al., 2007; El Halawani et al., 2009). Moreover, in the follow-up confirmation study, low light intensity (10 lux) could not activate PMM in the turkey hypothalamus even in long-day photoperiod (Moore et al., 2018), indicating that light intensity is also a key stimulant of initiation of avian reproductive function as well as photoperiod in avian species. Melanopsin (Opn4) is one of the DBPs which was characterized in the PMM of female turkey (Kang et al., 2007, 2009, 2010; El Halawani et al., 2009; Leclerc et al., 2010).

AVIAN DBP Opn4 FOR LIGHT PERCEPTION

The primary system to detect avian photoperiodic information has been thought to be non-retinal, non-pineal DBPs (Benoit and Assenmacher, 1953; Menaker et al., 1970; Yokoyama et al., 1978). Three DBPs (i.e., Opn4, Opsin 5, and Vertebrate ancient opsin) were proposed in the avian brain that responds to photoperiodic information affecting the onset and development of the reproductive function, and all three types of DBPs appear to be involved in priming the neuroendocrine system to activate the reproductive functions of birds (Halford et al., 2009; Kang et al., 2010; Nakane et al., 2010; Kang and Kuenzel, 2015). In this study, the author focused only on Opn4.

Melanopsin (Opn4) was first discovered by Provencio et al. (1998) in the photosensitive melanophores of *Xenopus* skin. *In situ* hybridization studies demonstrated that Opn4 mRNA is also expressed in other photosensitive tissues, such as the retina, the magnocellular preoptic nucleus, and the SCN in the brain (Brown and Robinson, 2004). Later, several studies make Opn4 an attractive candidate for circadian photopigment and non-visual photic responses (Gooley et al., 2003; Hannibal et al., 2013). In non-mammalian vertebrates, Opn4 has two isoforms, namely, mammal-like Opn4m and *Xenopus*-like Opn4x (Bellingham et al., 2006).

Avian Opn4 expression and functional role in the photoperiodic activation of reproductive function were reported in several avian species (Bailey and Cassone, 2005; Kang et al., 2010; Potter et al., 2018; Nakane et al., 2019). A recent study on Japanese quail showed the possible functional role of

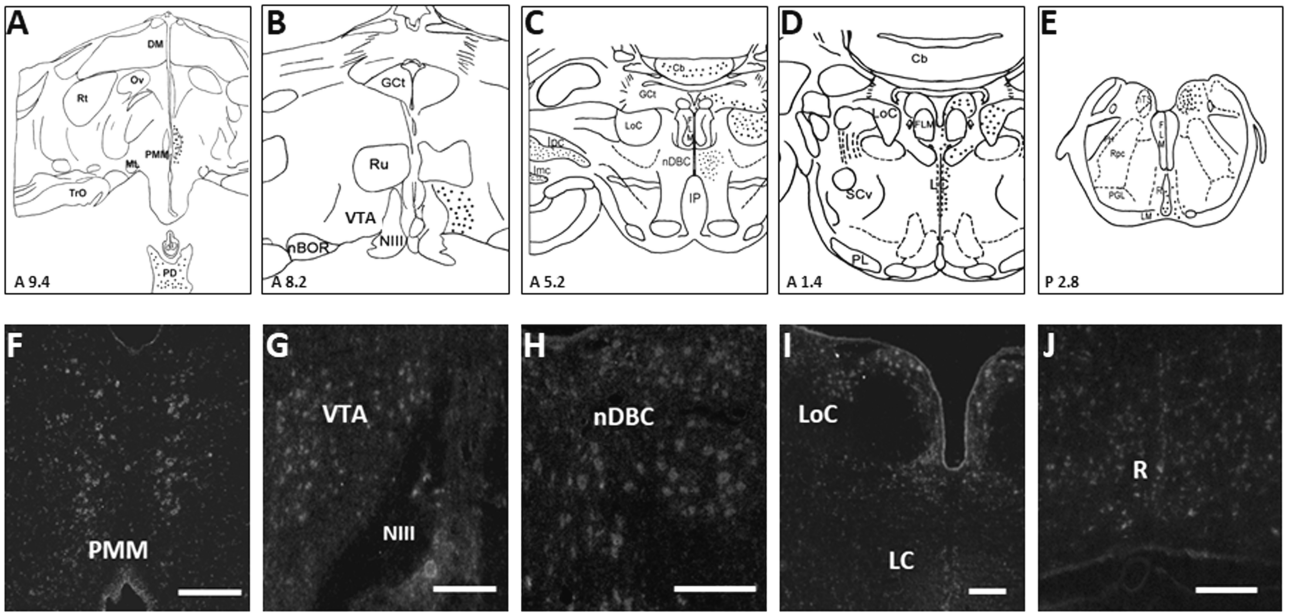

FIGURE 1 | Schematic drawings of rostral-to-caudal **(A–E)** coronal sections, showing the distribution of turkey *Xenopus*-like melanopsin (tOpn4x) expression labeled neurons (filled circles) of the turkey hen. Coronal illustrations were drawn from an unpublished turkey brain atlas with nomenclature taken from a chicken atlas (Kuenzel and Masson, 1988) and the revised nomenclature for avian brains (Reiner et al., 2004). Representative photomicrographs **(F–J)** showing the distribution of tOpn4x mRNA labeled neurons in the PMM, VTA, nDBC, LoC, LC, and R (refer to the abbreviations given below). A specific tOpn4x cRNA probe was used for *in situ* hybridization histochemistry (ISH). Darkfield photomicrographs of turkey brain sections processed for ISH with ^{33}P-labeled tOpn4 antisense cRNA probes. Scale bar: 100 μm **(G,H,J)**, 200 μm **(F,I)**. The following abbreviations are used in the figure: Cb, cerebellum; DM, dorsomedial hypothalamic nucleus; MLF, medial longitudinal fasciculus; GCt, mesencephalic central gray; Ipc, parvocellular nucleus Isthmi; Imc, magnocellular nucleus isthmi; IP, interpeduncular nucleus; LC, caudal linear nucleus; LM, medial lemniscus; LoC, locus coeruleus; ML, lateral mammillary nucleus; nBOR, nucleus of the basal optic root; nDBC, nucleus decussationis brachiorum conjunctivorum; NIII, third cranial nerve; nTS, nucleus of the solitary tract; Ov, nucleus ovoidalis; PD, pars distalis; PH, plexus of Horsley; PL, lateral pontine nuclei; PMM, premammillary nucleus; R, raphe nucleus; Rpc, parvocellular reticular nucleus; Rt, nucleus rotundus; Ru, nucleus ruber; SCv, nucleus subcoeruleus ventralis; TrO, tractus opticus; VTA, ventral tegmental area (Modified from Kang et al., 2010).

Opn4 in the mediobasal hypothalamus by evaluating an action spectrum for the expression of photoperiodically controlled beta subunit of TSH in the PT of the pituitary gland (Nakane et al., 2019). Interestingly, it has been suggested that Opn4 may have additional physiological roles beyond the reproductive system in the Pekin duck (Van Wyk and Frakey, 2021).

In mammals, specific populations within PMM were genetically defined as dopaminergic (DAergic) neurons and activated in specific social contexts and functions *via* glutamate release to regulate social interactions; moreover, mammalian PMM has a projection of the catecholaminergic input from locus coeruleus (LoC) (Sobrinho and Canteras, 2011; Soden et al., 2016).

Avian PMM neurons co-express both dopamine and melatonin (DA-MEL, Kang et al., 2007) and are activated by light provided during the photosensitive phase for reproductive stimulation (Thayananuphat et al., 2007b). The regulation of rhythmic DAergic/melatoninergic (MELergic) activity may involve clock genes, which localize and cycle rhythmically within DA/MEL neurons (Leclerc et al., 2010). Moreover, light pulses that are provided during the photosensitive phase for reproductive stimulation activate these neurons, as indicated by the induction of *c-fos* (Thayananuphat et al., 2007a) and the upregulation of *Cry1* and *Per3* genes (Leclerc et al., 2010). Dopamine and MEL expressing neurons of avian PMM have been shown to have dual functionality, which consists of sensory of light information by Opn4 and neurosecretory functions by the diurnal activities of DA and MEL (Kang et al., 2007, 2009, 2010; **Figures 1A,F**), suggesting that PMM may be a conserved dual sensory-neurosecretory unit in avian species as suggested in the lower vertebrates (Tessmar-Raible et al., 2007; Conzelmann et al., 2013).

Opn4 EXPRESSION IN THE DOPAMINERGIC AND SEROTONERGIC NUCLEI AND ITS POSSIBLE ROLES IN THE WELFARE OF BIRDS

Photoreceptor Opn4 was observed in the brain areas that are associated with DA and serotonin [5-hydroxytryptamine (5-HT)] in birds (Kang et al., 2010), which were not appreciated hitherto (**Figures 1B–E,G–J**). It may be of interest to speculate that direct light perception may be involved in the physiological function of DA and 5-HT neurons in the avian brain. Light-induced feed intake in birds may be directly stimulated by central Opn4 because tryptophan hydroxylase 2 (TPH2: rate-limiting enzyme of serotonin biosynthesis) in

the dorsal raphe nucleus (DRN) is also associated with food intake and energy balance (Flores et al., 2018; Liu et al., 2021).

Dopamine is predominantly synthesized in the ventral tegmental area (VTA) and substantia nigra (SN) of the midbrain. Dopaminergic neurons in the VTA integrate complex inputs to convert multiple signals that influence motivated behaviors *via* various neural projections underlying the different functions of these neurons in psychological processes and brain diseases (Beier et al., 2015; Bouarab et al., 2019). In mammals, the important roles of DA neurons were discovered in numerous behavioral or psychological processes other than rewards, such as aversion, depression, fear, social behavior, stress, and movement coordination (Pani et al., 2000; Bromberg-Martin et al., 2010; Zweifel et al., 2011; Lammel et al., 2012; Chaudhury et al., 2013; Matsumoto and Takada, 2013; Friedman et al., 2014; Walsh et al., 2014; Grace, 2016; Holly and Miczek, 2016). The major brain structures associated with positive emotion are the amygdala complex and nucleus accumbens (Janak and Tye, 2015). Importantly, the nucleus accumbens is the terminal site of the DAergic mesolimbic axis originating in the VTA (Ikemoto, 2007; Holly and Miczek, 2016). Ventral tegmental area neurons have long been implicated in feeding behaviors, and major neurons are DAergic neurons (about 60% of VTA neurons) (Ungless and Grace, 2012; Meye and Adan, 2014). In addition to DAergic neurons, VTA also contains gamma-aminobutyric acid (GABA) and glutamate neurons that account for about 35 and 2–3% of VTA neurons, respectively (Nair-Roberts et al., 2008; Taylor et al., 2014; Miranda-Barrientos et al., 2021). Besides DA, GABA, and glutamate neurons, several studies reported serotonergic (5-HTergic) neurons in the VTA of mammalian and avian brains (Kang et al., 2009; Carkaci-Salli et al., 2011; Morales and Margolis, 2017; Smith et al., 2019). Interestingly, the optogenetic activation of VTA GABAergic neurons stimulates food intake and anxiety-like behavior in mice (Chen et al., 2020).

The avian VTA contains cell bodies that label positively for tyrosine hydroxylase (TH; the rate-limiting enzyme in catecholamine biosynthesis) but not DA-β-hydroxylase (which is involved in converting DA to norepinephrine), indicating that the major population of avian VTA is DAergic neurons (Kang et al., 2009, **Figure 2**). The electrophysiological and pharmacological properties of VTA neurons have been studied using whole-cell recordings in the brain slices of birds (zebra finch) (Gale and Perkel, 2006), showing that zebra finch VTA DAergic neurons possess physiological properties very similar to those of mammalian DAergic neurons and also contain non-DAergic neurons similar to GABAergic neurons in the mammalian VTA. In addition, avian VTA DAergic neurons densely innervate the striatal areas of the basal ganglia and project more moderately to several other regions of the telencephalon, and the pharmacological agents and lesions targeting the DAergic system have many similar behavioral effects in birds and mammals (Durstewitz et al., 1999). Therefore, these results provide strong evidence for anatomical, physiological, and functional similarities between the VTA DAergic systems of mammals and birds (Gale and Perkel, 2006).

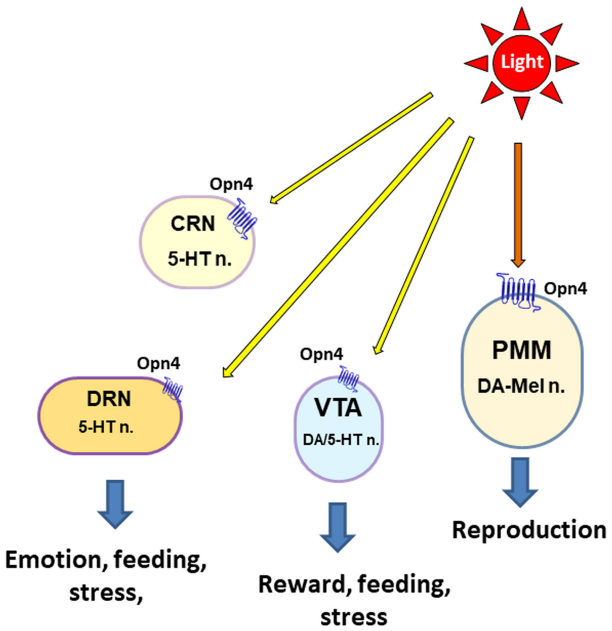

FIGURE 2 | Schematic overview of extraocular light perception in the midbrain and brain stem of avian species for the physiological response. The following abbreviations are used in the figure: 5-HT, serotonin; DA, dopamine; CRN, caudal raphe nucleus; DRN, dorsal raphe nucleus; n, neuron; PMM, premammillary nucleus; VTA, ventral tegmental area.

The distribution of 5-HT immunoreactivity and TPH2 mRNA expression was reported in the avian brain such as VTA, DRN, and caudal raphe nucleus (CRN) (Cozzi et al., 1991; Challet et al., 1996; Kang et al., 2009). The presence of TPH2-positive neurons in the VTA may provide an area of further investigation involving interactions between 5-HTergic and DAergic systems within the VTA (Carkaci-Salli et al., 2011). Serotonin is one of the main neurotransmitters to regulate the parasympathetic nervous system (PNS) and is involved in emotional states caused by stress, pain, or the availability of food (Chamas et al., 1999; Mosienko et al., 2012), while DA acts on the sympathetic nervous system (SNS). Serotonergic neurons can be identified based on the presence of TPH2 mRNA expression, and thereby the TPH2 expression levels can be used as a specific marker for 5-HT generation (Chamas et al., 1999; Kang et al., 2009, 2020; Carkaci-Salli et al., 2011; Liu et al., 2021). The DRN is a heterogeneous brain stem nucleus located in the midbrain and pons, which is involved in the control of various physiological functions, such as learning and memory (Michelsen et al., 2008). The most abundant neurotransmitter in the DRN is serotonin, and the TPH2 mRNA expression was observed in the avian DRN such as nucleus decussationis brachiorum conjunctivorum (nDBC), LoC, and caudal linear nucleus (LC) (Kang et al., 2009, **Figure 2**).

The presence of both DA and 5-HT systems in the VTA indicates that avian VTA is the critical area of the midbrain involved in the welfare of avian species (Kang et al., 2009, 2020; Carkaci-Salli et al., 2011). Several studies have proposed that DA and 5-HT could serve as positive indicators of animal welfare (Algers et al., 2007; Boissy et al., 2007; Polter and Kauer, 2014). Stress and negative experience alter the 5-HT metabolism in the brain by stimulating 5-HT turnover in the areas innervated by 5-HTergic neurons (Clement et al., 1993; Inoue et al., 1994; Amat et al., 1998). In mammals, repeated immobilization stress increased the *TPH2* gene expression levels in the raphe nuclei of the brain stem (Chamas et al., 1999; Walther et al., 2003), indicating the elevation of 5-HT metabolism. In the recent study of DA and 5-HT activity, 5-HTergic and DAergic activities respond differently to light intensity and light intensity preference, and these results suggest the beneficial effects of dual intensity lighting program on the protection of the central nervous system of birds (Kang et al., 2020).

PERSPECTIVE

Animals explore their surroundings to secure resources such as food, water, and shelter, and the regulation of their reproductive system for producing offspring depends on the environment day-and-night light condition.

The data discussed in this study and the previous light intensity study (Kang et al., 2020) suggest the possible roles of Opn4 in the VTA and DRN/CRN on the direct light perception for the physiological responses of birds such as feeding behavior and welfare. Although this observation makes the hypothesis that Opn4 is a positive candidate photoreceptor associated with direct light perception in the ancient brain (i.e., hypothalamus and brain stem) of birds, the functional role of Opn4 should be tested in the future study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SK contributed to the conception, drafted the manuscript, edited and revised the manuscript, and approved the final version of the manuscript.

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Effect of Cyclic Heat Stress on Feeding-Related Hypothalamic Neuropeptides of Three Broiler Populations and Their Ancestor Jungle Fowl

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Heat stress (HS) has been increasingly jeopardizing the sustainability of the poultry production. Moreover, modern high-performing chickens are far less able to withstand HS than their predecessors due to higher growth rate and metabolic rates. Performance losses caused by HS are mainly ascribed to decreases in feed consumption. Since feed intake is tightly controlled by the hypothalamic centers of hunger and satiety, we sought to determine the effect of chronic cyclic HS on the expression of feeding-related hypothalamic neuropeptides (FRHN) in unselected chickens (i.e., the ancestor junglefowl—JF) and three broiler lines from diverse stages of genetic selection (i.e., the slow growing ACRB, the moderate growing 95RN, and the fast growing MRB). From 29 to 56 days, birds (n = 150 birds for each population) were subjected to either thermoneutral (TN, 25°C) or cyclic heat stress (HS, 36°C, 0900–1,800 h) conditions. Molecular data were analyzed by two-way ANOVA with interaction between the main factors, namely environmental temperature and line. The expression of major FHRN, like neuropeptide Y, agouti-related peptide, proopiomelanocortin, and cocaine and amphetamine regulated transcript remained unchanged. However, melanocortin receptor 1 exhibited a line-dependent decreasing trend from JF to MRB under both TN and HS (p = 0.09), adiponectin expression showed a distinct trend toward significance with 95RB exhibiting the highest mRNA level irrespective of the environmental temperature (p = 0.08), and JF had a greater mRNA abundance of visfatin than ACRB under TN ($p < 0.05$). The hypothalamic integration of circadian information, acclimation to long-lasting HS exposure, stable hypothalamic pathways unaffected by evolution and genetic selection, focus on mRNA abundances, and use of the entire hypothalamus masking gene expression in specific hypothalamic nuclei are all possible explanations for the lack of variations observed in this study. In conclusion, this is the first assessment of the impacts of heat stress on feeding-related hypothalamic neuropeptides of chicken, with a valuable and informative comparison between the ancestor junglefowl and three differently performing broiler lines.

Keywords: broiler chicken, ancestor, heat stress, feed intake, hypothalamic neuropeptides

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INTRODUCTION

Numerous factors have contributed to the formidable expansion of poultry meat production. However, genetic selection has been widely deemed to be one of the most important drivers for this incomparable success in the history of livestock industry. When it comes to broiler chicken genetic advancements, enlightening papers published by Havenstein et al., 1994a,b, 2003a,b) certainly come to mind of poultry scientists and professionals. These authors clearly showed the impressive improvement in broiler's growth rate, feed efficiency, and yield ascribable to breeding programs carried out since the 1950s. Their findings have also been recently supported by Zuidhof et al. (2014). Tallentire et al. (2018) have even posited that the biological limits for further improvements are about to be reached. Moreover, these outstanding enhancements have come at a price in terms of animals' resilience and welfare. For instance, modern high-performing chickens are far less able to cope with high environmental temperatures than their predecessors (Cahaner and Leenstra, 1992; Yunis and Cahaner, 1999). The reason for this greater sensitivity to hot conditions has primarily been attributed to faster growth and higher metabolic rate characterizing the currently available lines (Yahav, 2009). Even though their body has undergone selection-mediated changes, chickens still are homeothermic animals lacking sweat glands and relying on sensible heat loss—through conduction, radiation, and convection (Renaudeau et al., 2012; Rostagno, 2020)—and latent heat loss—via evaporation of water from the respiratory tract (Rajaei-Sharifabadi et al., 2017)—to thermoregulate.

Heat stress (HS) is a thermoregulatory failure resulting from a negative balance between heat dissipated and metabolic heat generated by animals. Besides being potentially lethal, HS unfavorably affects the behavior, physiology, productive potential, and well-being of chickens (Renaudeau et al., 2012; Rostagno, 2020). The voluntary reduction in feed intake is an evolutionary conserved strategy adopted by animals to reduce the thermogenesis linked to digestion, absorption, and nutrient utilization (Baumgard and Rhoads, 2013). Performance losses of heat-stressed poultry have commonly been ascribed to decreases in feed consumption (Dale and Fuller, 1980), although pair-feeding experiments have revealed that almost half of them are anorexia-unrelated (Dale and Fuller, 1980; Geraert et al., 1996; Renaudeau et al., 2012).

Given the extraordinary strides made by genetic selection, higher proneness of modern broilers to HS, and remarkable HS-caused modifications in feed intake of poultry, we hypothesized that the hypothalamus, known as a vital control center of feeding behavior and energy homeostasis (Simpson et al., 2008; Waterson and Horvath, 2015; Timper and Brüning, 2017; Luquet et al., 2019), can be differently affected in unselected chickens and genotypes from diverse breeding era stages. Hence, we sought to determine here the effect of chronic cyclic HS on the expression of feeding-related hypothalamic neuropeptides (FRHN) in three broiler populations and their common ancestor, namely the jungle fowl.

TABLE 1 | Experimental design and assignment of lines to chambers and pens.

Chamber	Treatment	Pen	Line
1	HS	1	ACRB
		2	MRB
2	TN	3	JF
		4	MRB
3	HS	5	ACRB
		6	95RAN
4	TN	7	JF
		8	95RAN
5	HS	9	95RAN
		10	MRB
6	HS	11	ACRB
		12	JF
7	TN	13	ACRB
		14	95RAN
8	HS	15	JF
		16	MRB
9	TN	17	ACRB
		18	MRB
10	TN	19	JF
		20	ACRB
11	HS	21	JF
		22	95RAN
12	TN	23	MRB
		24	95RAN

MATERIALS AND METHODS

Chicken Populations

The four chicken lines employed in this study are maintained, bred, and hatched at the poultry facilities of the University of Arkansas. Each generation has been randomly mated except for full and half sibs in order to curtail the inbreeding. The slow-growth Athens Canadian Random Bred (ACRB) represented a commercial broiler from the 1950s (Collins et al., 2016). The moderate growing 1995 random bred (95RN), obtained from 7 parent stock male and 6 parent stock female lines, was used as representative of meat-type chickens available in the 1990s (Harford et al., 2014). The third line was the high yielding and fast growing modern random bred (MRB) conceived and developed at the University of Arkansas as a mixture of broiler hybrids currently used. The fourth populations was the ancestor of the domestic chicken, that is the South East Asian junglefowl (JF) (Gyles et al., 1967; Wall and Anthony, 1995). The present study was approved by the University of Arkansas Animal Care and Use Committee (protocols 18,083 and 16,084), and conducted in compliance with the guide for the care and use of laboratory animals of the National Institutes of Health.

Animal Husbandry and Experimental Design

At hatch, 600 vent-sexed male chicks were individually wing-tagged with a number code, grouped by line (n = 150), and randomly placed in 12 environmentally controlled chambers

divided in 2 equally sized pens (25 birds/pen). Each pen represented a replicate for a 2 × 4 experimental design with environmental temperature and line as main factors. Pens were equipped with a feeder and a drinker, while the floor was covered with wood shavings as bedding material. All birds were manually fed and watered *ad libitum* on a daily basis. A two-phase feeding program, based on commercially available starter (0–28 days) and finisher diets (29–56 days), was used. The artificial photoperiod was 23L:1D during the first 7 days, while 20L:4D for the remainder of the trial. The environmental temperature was progressively reduced in all chambers from 32°C (0–3 days), to 31°C (4–6 days), 29°C (7–10 days), 27°C (11–14 days), and 25°C (15–28 days). Later, from 29 to 56 days, birds were exposed to either thermoneutral (TN, 25°C) or cyclic heat stress (HS, 36°C, 0900–1,800 h) conditions. Therefore, 6 rooms were kept at TN, whereas the others cyclically subjected to HS (**Table 1**).

Productive Performance Measurement and Hypothalamic Sample Collection

Body weight (BW) and feed intake (FI) were recorded weekly. The number and BW of dead or culled birds were considered while computing the feed conversion ratio (FCR) of each replicate as previously reported (Abdelli et al., 2021). Sample collection was carried out at the end of the grow-out period (56 days) after feeding all animals and applying, in the appropriate rooms, HS for at least 2 h. Two birds per pen—i.e., 6 birds from each combination of environmental temperature and line—were randomly selected and euthanized by cervical dislocation. Hypothalamic samples were harvested and processed following the techniques illustrated by Piekarski et al. (2016). Briefly, the brain was pulled from the skull and submerged in 2-methylbutane (Sigma, St. Louis, MO) in dry ice for 60 s. This treatment preserved the brain structure and provided firmness necessary to make precise cuts for hypothalamus extraction. The hypothalamic dissection was performed according to the stereotaxic atlas of the chick brain authored by Kuenzel and Masson (1988). Brain samples were placed on a cold metal plate with the ventral side exposed for dissection. The hypothalamus was dissected with an anterior cut at the corticoseptomesencephalic tract (also known as septopalliomesencephalic tract) and a posterior cut at the third oculomotor nerve. Laterally, 2 mm from the brain midline, two cuts were performed on either side. Dorsally, a 5 mm cut from the brain base was performed to get the entire hypothalamus.

RNA Isolation, Reverse Transcription, and Real-Time Quantitative PCR

The analytical process described in this section was conducted along the lines of previous studies published by our laboratory (Rajaei-Sharifabadi et al., 2017; Greene et al., 2019, 2020). Total RNA was isolated from the hypothalamic samples ($n = 48$) by way of Trizol reagent (Life Technologies, Carlsbad, CA) according to the recommendations provided by the manufacturer. RNA purity and concentrations were assessed via Take3 micro-volume plate and Synergy HT multimode microplate reader (BioTek, Winooski, VT). One sample belonging to JF-HS group showed poor RNA quality and, therefore, was excluded. RNA samples were RQ1 RNase-free DNase treated (Promega, Madison, WI), and 1 μg RNA was reverse transcribed by means of qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). The reverse transcription reaction was performed at 42°C for 30 min followed by a 5 min incubation at 85°C. Real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR System) was carried out in a total 12.5 μL reaction using 2.5 μL of cDNA, 0.5 μL of each forward and reverse specific primer, and SYBR Green Master Mix (Thermo-Fisher Scientific, Rockford, IL). The chicken-specific oligonucleotide primers used in this study are listed in **Table 2**. The qPCR cycling settings were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 60 s). Relative expression of target genes was calculated via the comparative C_T method reviewed by Schmittgen and Livak (2008), using 18S rRNA as a housekeeping gene and JF-TN group as calibrator.

Statistical Analysis

Performance and mRNA expression data were analyzed through two-way ANOVA with interaction between the main factors, namely environmental temperature, and line. The pen (i.e., the replicate) and sampled animal were the experimental units for performance and mRNA expression analysis, respectively. The significance level was set at 0.05. Tukey's honestly significant difference test was used as *post hoc*. These analyses were carried out in R (R Core Team, 2020).

RESULTS

Heat Stress Differentially Depressed Feed Intake and Altered the Expression of Feeding-Related Hypothalamic Neuropeptides

As previously shown by our group (Abdelli et al., 2021) and summarized in **Table 3**, chronic cyclic HS significantly decreased cumulative FI in both MRB and 95RB, but not in JF and ACRB. The highest cumulative FI was observed in MRB, followed by 95RB, and JF and ACRB regardless of the environmental conditions. These changes in FI resulted in similar modulation of BW, with highest weight observed in MRB, 95RB, and lowest weight in ACRB and JF (**Table 3**). At molecular level, neither environmental temperature nor line significantly influenced the hypothalamic expression of neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC), and cocaine and amphetamine regulated transcript (CART) (**Figure 1** and **Supplementary Table 1**). The mRNA expression of melanocortin receptors (MC1-5R) genes was not affected in a significant fashion, even though MC1R exhibited a line-dependent decreasing trend from JF to MRB under both TN and HS ($p = 0.09$) (**Figure 2** and **Supplementary Table 1**). The expression of orexin (ORX), orexin receptors 1 and 2 (ORXR1-2), and corticotropin releasing hormone (CRH) genes appeared to be unaffected by environmental temperature and line (**Figure 3** and **Supplementary Table 1**). Similarly, no

TABLE 2 | List of qPCR chicken-specific oligonucleotide primers.

Gene	Accession number[†]	Primer sequence (5'→3')	Orientation[§]	Product size (bp)
NPY	NM_205473	CATGCAGGGCACCATGAG	F	55
		CAGCGACAAGGCGAAAGTC	R	
AgRP	AB029443	GCGGGAGCTTTCACAGAACA	F	58
		CGACAGGATTGACCCCAAAA	R	
POMC	AB019555	GCCAGACCCCGCTGATG	F	56
		CTTGTAGGCGCTTTTGACGAT	R	
CART	KC249966	GCTGGAGAAGCTGAAGAGCAA	F	60
		GGCACCTGCCCGAACTT	R	
ORX	AB056748	CCAGGAGCACGCTGAGAAG	F	67
		CCCATCTCAGTAAAAGCTCTTTGC	R	
ORXR1	AB110634	TGCGCTACCTCTGGAAGGA	F	58
		GCGATCAGCGCCCATTC	R	
ORXR2	XM_004945362	AAGTGCTGAAGCAACCATTGC	F	61
		AAGGCCACACTCTCCCTTCTG	R	
CRH	NM_001123031	TCAGCACCAGAGCCATCACA	F	74
		GCTCTATAAAAATAAAGAGGTGACATCAGA	R	
Ghrelin	AY303688	CACTCCTGCTCACATACAAGTTCA	F	75
		TCATATGTACACCTGTGGCAGAAA	R	
GHSR	NM_204394	GCACAAATCGGCAAGGAAA	F	61
		GTGACATCTCCCAGCAAATCC	R	
MC1R	NM_001031462	GCTCTGCCTCATTGGCTTCT	F	76
		TGCCAGCGCGAACATGT	R	
MC2R	NM_001031515	GCTGTTGGGCCCCCTTT	F	60
		AAGGGTTGTGTGGGCAAAAC	R	
MC3R	AB017137	GCCTCCCTTTACGTTCACATGT	F	59
		GCTGCGATGCGCTTCAC	R	
MC4R	NM_001031514	CCTCGGGAGGCTGCTATGA	F	62
		GATGCCCAGAGTCACAAACACTT	R	
MC5R	NM_001031015	GCCCTGCGTTACCACAACAT	F	63
		CCAAATGCATGCAATGATAAGC	R	
Ob-R	NM_204323	GCAAGACCCTCTCCCTTATCTCT	F	70
		TCTGTGAAAGCATCATCCTGATCT	R	
Adip	AY786316	ATGGACAAAAGGGAGACAAAGG	F	64
		TCCAGCACCCATATACCCAAA	R	
AdipR1	NM_001031027	CCGGGCAAATTCGACATC	F	58
		CCACCACGAGCACATGGA	R	
AdipR2	NM_001007854	TTGCCACTCGGAAGGTGTTT	F	60
		AGTGCAATGCCAGAATAATCCA	R	
Visfatin	NM_001030728	CCGGTAGCTGATCCAAACAAA	F	65
		CCAGCAGGTGTCCTATGCAA	R	
NPGL	AB909129	CCCTCAGTGCTGGGAATCC	F	61
		AGAAATGCGAGGCTTCCTCAT	R	
NPGM	XM_040665724.1	CACGGGCTGGTGGAAATG	F	65
		ATGAAGTCCCAGAGAGCAATGAC	R	
18S	AF173612	TCCCCTCCCGTTACTTGGAT	F	60
		GCGCTCGTCGGCATGTA	R	

[†]*Accession number refers to GenBank (National Center for Biotechnology Information—NCBI).*

[§]*F, forward; R, reverse.*

NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, proopiomelanocortin; CART, cocaine and amphetamine regulated transcript; ORX, orexin; ORXR1, orexin receptor 1; ORXR2, orexin receptor 2; CRH, corticotropin releasing hormone; GHR, growth hormone receptor; GHSR, growth hormone secretagogue receptor; MC1R, melanocortin receptor 1; MC2R, melanocortin receptor 2; MC3R, melanocortin receptor 3; MC4R, melanocortin receptor 4; MC5R, melanocortin receptor 5; Ob-R, leptin receptor; Adip, adiponectin; AdipR1, adiponectin receptors 1; AdipR2, adiponectin receptors 2; NPGL, neurosecretory protein GL; NPGM, neurosecretory protein GM.

TABLE 3 | Effect of cyclic heat stress on cumulative feed intake (FI), final body weight (BW), and cumulative feed conversion ratio (FCR) (n = 3 replicate/group).

Trait	TN				HS				SEM	*p*-value		
	JF	ACRB	95RB	MRB	JF	ACRB	95RB	MRB		Env. temp.	Line	Env. temp. × Line
Cumulative FI (kg/bird)	1.98^{e}	1.95^{e}	5.96^{c}	7.77^{a}	1.99^{e}	1.86^{e}	5.40^{d}	7.06^{b}	0.09	<0.001	<0.001	0.002
Final BW (kg/bird)	0.88^{e}	0.97^{e}	3.19^{c}	5.01^{a}	0.86^{e}	0.94^{e}	2.95^{d}	4.46^{b}	0.04	<0.001	<0.001	<0.001
Cumulative FCR	2.35^{a}	2.08^{b}	1.89^{bc}	1.56^{d}	2.40^{a}	2.06^{bc}	1.85^{c}	1.60^{d}	0.04	0.819	<0.001	0.719

Within a row, means with different superscripts are significantly different ($p < 0.05$).
HS, heat stress; TN, thermoneutral.

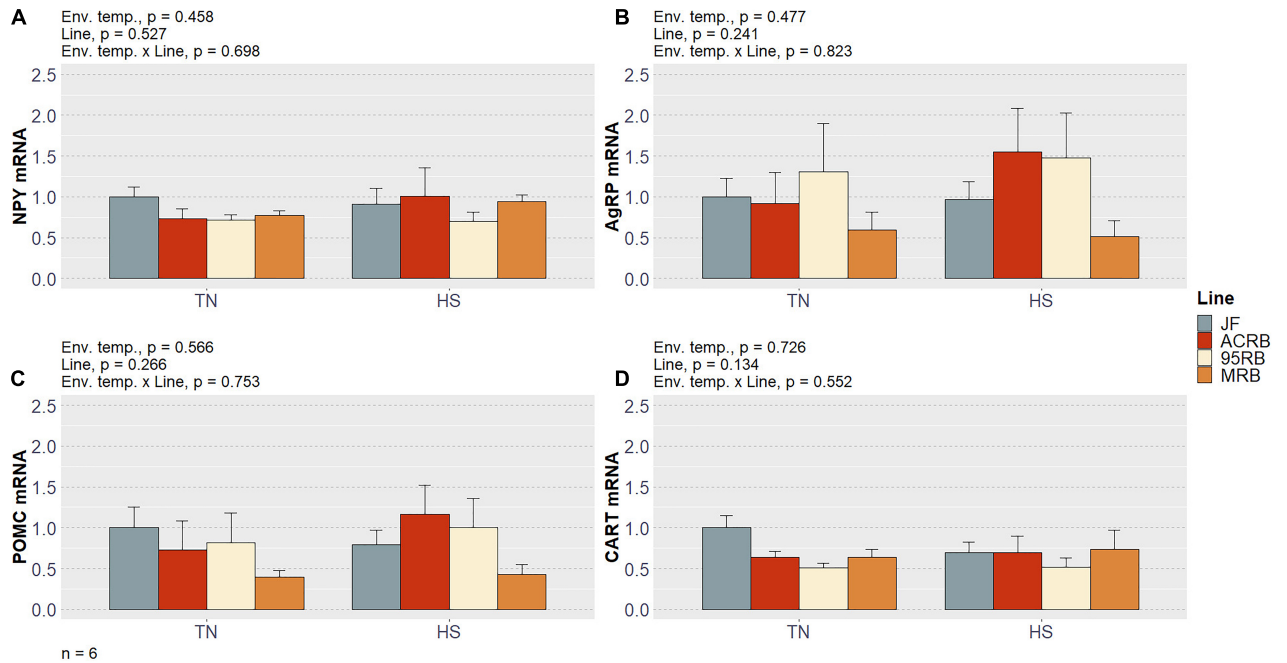

FIGURE 1 | Effect of cyclic heat stress on the hypothalamic expression of NPY **(A)**, AgRP **(B)**, POMC **(C)**, and CART **(D)** in JF and modern broilers. The mRNA abundances were determined by qPCR using $2^{-\Delta\Delta Ct}$ method. JF-TN group was used as a calibrator. Data are mean ± SEM (n = 6 birds/group). HS, heat stress; TN, thermoneutral.

significant differences were found for the expression of ghrelin and its receptor GHSR (**Figure 4** and **Supplementary Table 1**). While the expression of leptin receptor (Ob-R) and adiponectin receptors 1 and 2 (AdipR1-2) remained unchanged, adiponectin (Adip) expression showed a distinct trend toward significance with 95RB exhibiting the highest mRNA level irrespective of the environmental temperature (p = 0.08) (**Figure 5** and **Supplementary Table 1**). The environmental temperature did not affect the expression of visfatin and neurosecretory proteins GL and GM (NPGL and NPGM, respectively) (**Figures 6B,C** and **Supplementary Table 1**). However, under TN, JF had a greater mRNA abundance of visfatin than ACRB ($p < 0.05$) (**Figure 6A** and **Supplementary Table 1**).

DISCUSSION

A 70-year genetic progress has been instrumental in making the poultry sector a cost-effective source of high-quality and affordable proteins worldwide. Intensive genetic selection programs have produced broiler chickens capable of efficiently converting feed into muscle mass (Clavijo and Flórez, 2018) with tremendously high growth rates and processing yields (Havenstein et al., 1994a,b, 2003a,b; Zuidhof et al., 2014; Tallentire et al., 2016). Results reported in our previously published papers (Orlowski et al., 2020; Abdelli et al., 2021) are consistent with the improvements in productive performance and slaughter traits ascribable to selection. By outperforming the other lines regardless of the environmental temperature, MRB corroborated the valuable outcomes of selection. Although they have become very efficient, modern broilers are facing unintended drawbacks caused by genetic selection.

In addition to welfare issues detailed by Hartcher and Lum (2020), current broilers are more sensitive to elevated ambient temperatures due to high metabolic rates (Cahaner and Leenstra, 1992; Yunis and Cahaner, 1999; Yahav, 2009). Even though selection has remodeled the broiler chicken body (Zuidhof et al., 2014), this homeothermic bird still employs the same

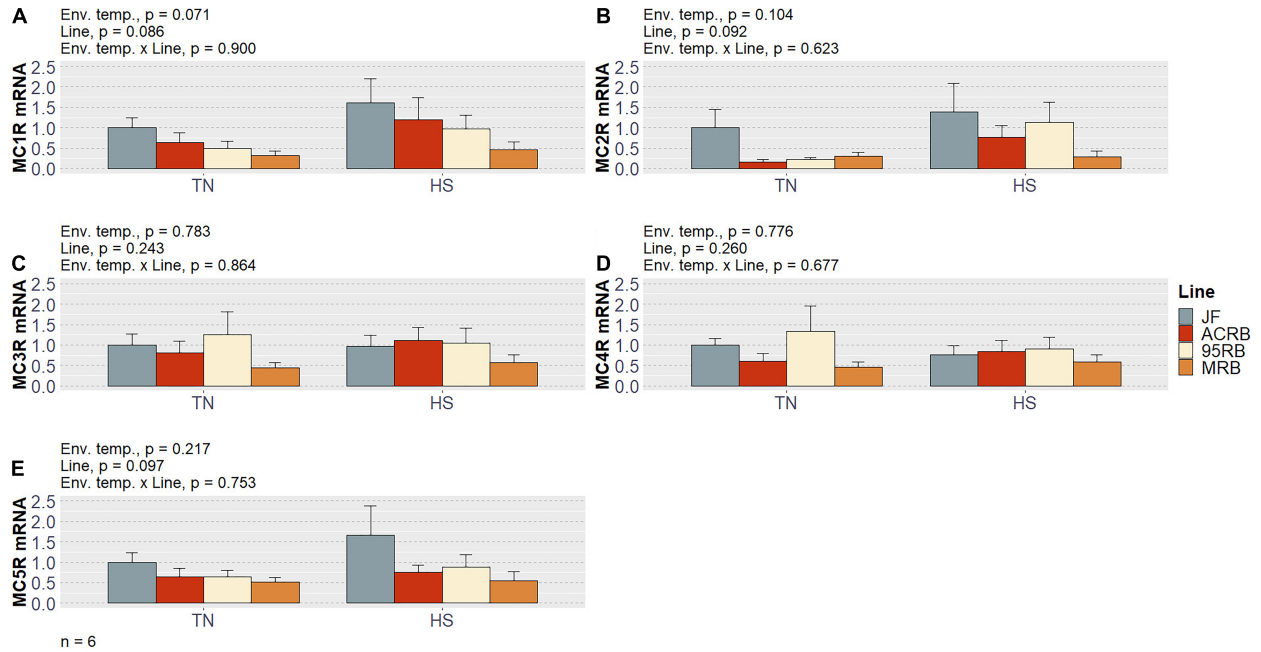

FIGURE 2 | Effect of cyclic heat stress on the hypothalamic expression of the melanocortin receptor system in JF and modern broilers. The expression of MC1R **(A)**, MC2R **(B)**, MC3R **(C)**, MC4R **(D)**, and MC5R gene **(E)** was determined by qPCR using $2^{-\Delta\Delta Ct}$ method. JF-TN group was used as a calibrator. Data are mean ± SEM (n = 6 birds/group). HS, heat stress; TN, thermoneutral.

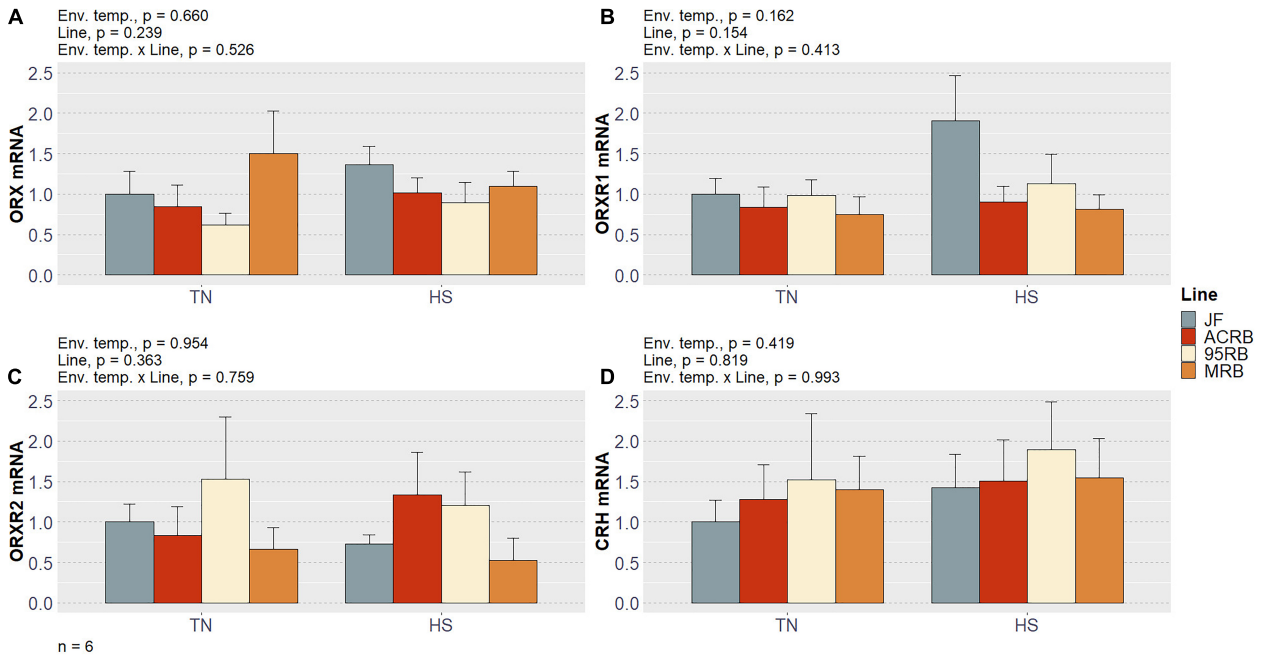

FIGURE 3 | Effect of cyclic heat stress on the hypothalamic expression of the orexin system and CRH in JF and modern broilers. The expression of ORX **(A)**, ORXR1 **(B)**, ORXR2 **(C)**, and CRH gene **(D)** was determined by qPCR using $2^{-\Delta\Delta Ct}$ method. JF-TN group was used as a calibrator. Data are mean ± SEM (n = 6 birds/group). HS, heat stress; TN, thermoneutral.

thermoregulatory apparatus and strategies as its undomesticated forebear (Renaudeau et al., 2012; Rajaei-Sharifabadi et al., 2017; Rostagno, 2020). When exposed to HS, endothermic animals generally reduce feed consumption to limit the metabolic heat released by the gastrointestinal activities and use of nutrients (Baumgard and Rhoads, 2013).

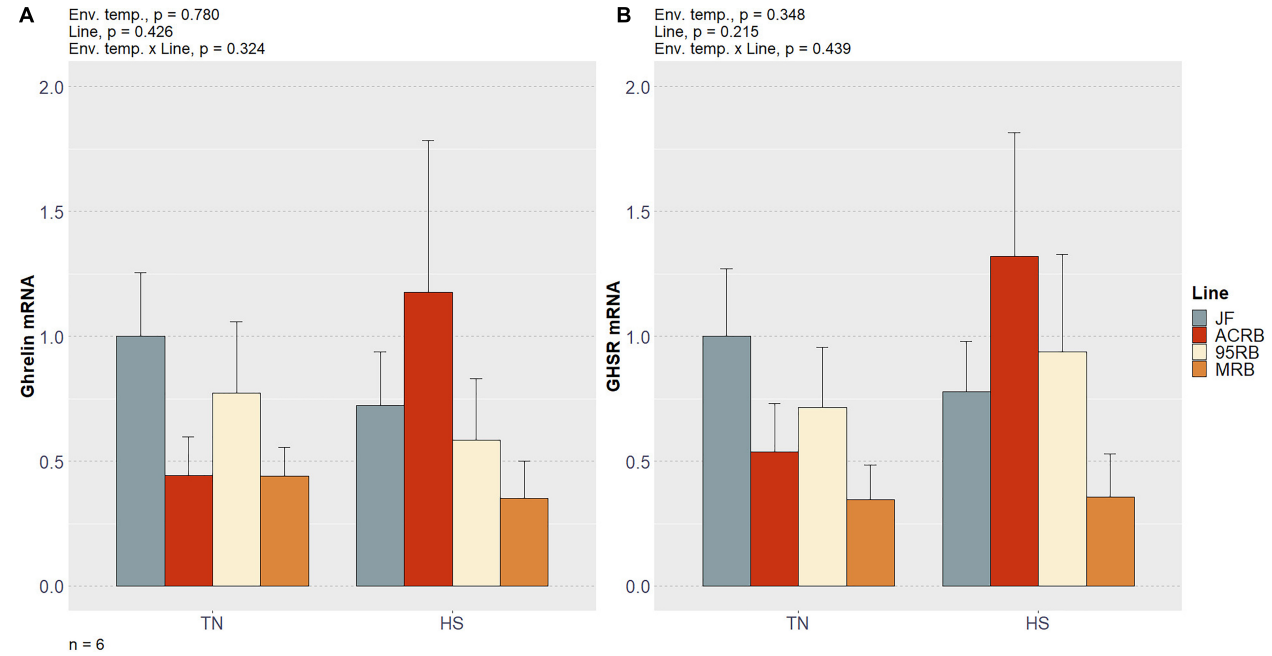

FIGURE 4 | Effect of cyclic heat stress on the hypothalamic expression of ghrelin **(A)** and its receptor **(B)** in JF and modern broilers. The mRNA abundances were determined by qPCR using $2^{-\Delta\Delta Ct}$ method. JF-TN group was used as a calibrator. Data are mean ± SEM (n = 6 birds/group). HS, heat stress; TN, thermoneutral.

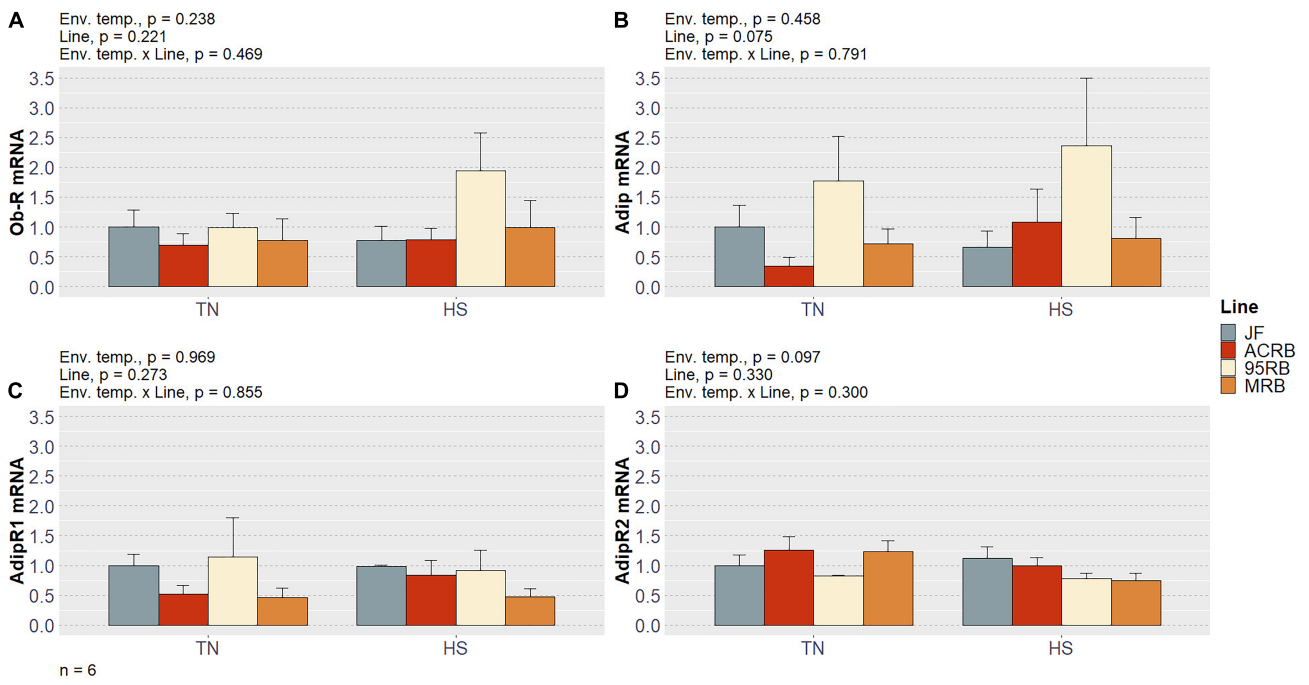

FIGURE 5 | Effect of cyclic heat stress on the hypothalamic expression of adiponectin system and leptin receptor in JF and modern broilers. The expression of Ob-R **(A)**, Adip **(B)**, Adip-R1 **(C)**, and Adip-R2 gene **(D)** was determined by qPCR using $2^{-\Delta\Delta Ct}$ method. JF-TN group was used as a calibrator. Data are mean ± SEM (n = 6 birds/group). HS, heat stress; TN, thermoneutral.

As recently shown by Abdelli et al. (2021) and summarized here (**Table 3**), the significant drops in FI of 95RB and MRB are a clear reflection of a more pronounced HS susceptibility of hyper-selected broilers than their predecessors. Since FI is tightly controlled by the hypothalamic centers of hunger and satiety (Simpson et al., 2008; Waterson and Horvath, 2015;

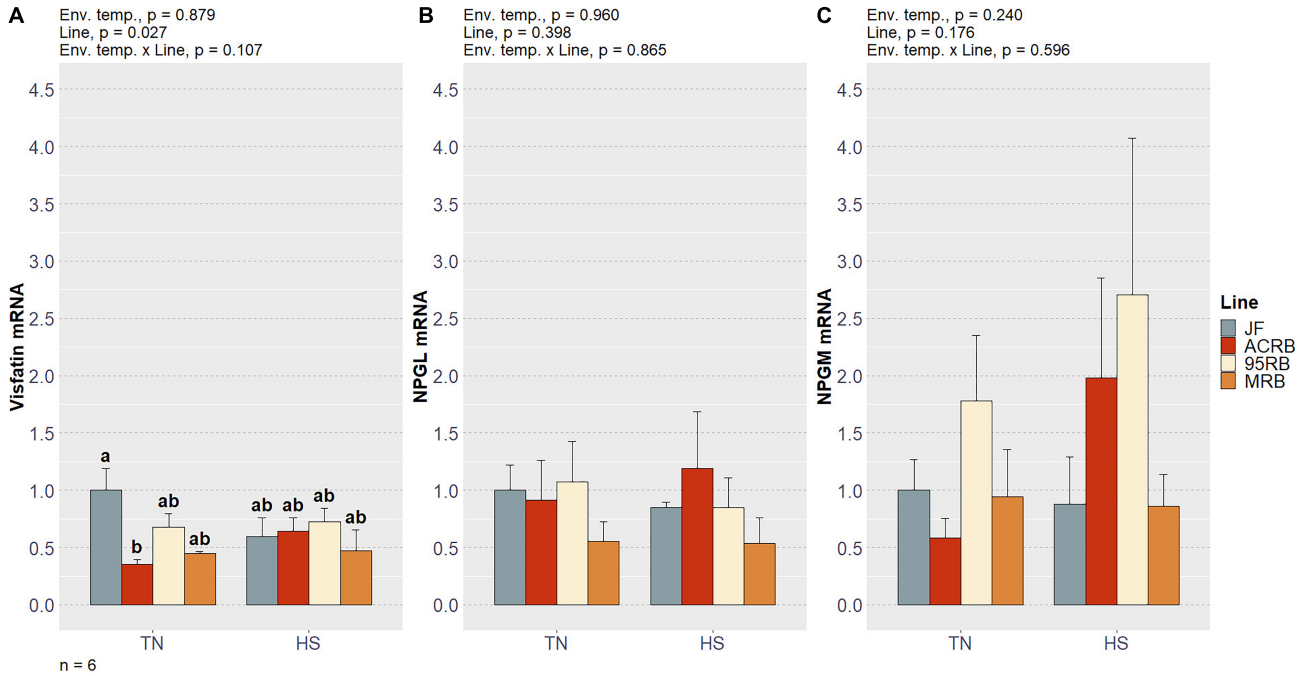

FIGURE 6 | Effect of cyclic heat stress on the hypothalamic expression of visfatin **(A)**, NPGL **(B)**, and NPGM **(C)** in JF and modern broilers. The mRNA abundances were determined by qPCR using $2^{-\Delta\Delta Ct}$ method. JF-TN group was used as a calibrator. Data are mean ± SEM (n = 6 birds/group). HS, heat stress; TN, thermoneutral.

Timper and Brüning, 2017; Luquet et al., 2019) through a myriad of (an)orexigenic neuropeptides (Arora and Anubhuti, 2006; Furuse et al., 2007; Tachibana and Tsutsui, 2016; Delgado et al., 2017), we sought to determine the expression profile of FRHN in four chicken populations undergoing chronic cyclic HS.

NPY, AgRP, POMC, and CART represent the most renowned FRHN. NPY and AgRP are orexigenic peptides co-expressed by a subpopulation of neurons located in the infundibular nucleus (IN) of the avian hypothalamus (equivalent to the mammalian arcuate nucleus—ARC). Notably, NPY has been considered to be the most potent appetite-stimulating factor since the pioneering work of Stanley and Leibowitz (1985) dating back to mid-1980s. On the other hand, POMC and CART, produced by another subpopulation of IN neurons, act as anorexigenic or anorectic peptides (Delgado et al., 2017; Gaspar and Velloso, 2018). Since in this study no variation was detected for the expression of these major FHRN, it could conceivably be assumed that they are not linked to FI differences of the four lines exposed to either TN or HS.

POMC-derived ligands, collectively named melanocortins, bind to five melanocortin receptors (i.e., MC1-5R) to play their anorexigenic role (Tachibana and Tsutsui, 2016). In this study, MC1R showed a line-dependent trend under both environmental conditions, with a gradually decreasing mRNA abundance across the four lines. We have also previously reported an overexpression of melanocortin receptors in male quails selected for low feed efficiency (Blankenship et al., 2015). Future research on hypothalamic melanocortin system and its different expression in poor- and high-performing poultry is therefore warranted.

Orexin system, encompassing ORX and its receptors, was unaffected in our experimental conditions. While mammalian orexins—also known as hypocretins—have been shown to considerably stimulate appetite, the effect of orexins on chicken feeding behavior is still unclear and matter of debate (Furuse et al., 1999). Nonetheless, we have recently found that ORX system is central in both muscular and hepatic energy metabolism of avian species (Lassiter et al., 2015; Greene et al., 2020).

CRH, widely known for its involvement in stress response (Weninger et al., 1999), has been shown to possess a substantial anorectic effect in chicken (Furuse et al., 1997). Interestingly, Tachibana and Tsutsui (2016) posited that CRH can be a downstream mediator for the anorexigenic neural pathway in chicken brain. The environmental stressor applied in this study was expected to increase the hypothalamic expression of CRH, with a consequent CRH-mediated role in FI depression exhibited by 95RB and MRB subjected to HS. However, no differences were found in mRNA level of CRH.

Some neuropeptides can play divergent roles according to the animal class (Volkoff et al., 2005; Luquet et al., 2019). Ghrelin, a peripheral peptidergic hormone mainly released by the gastric mucosa (Arora and Anubhuti, 2006), is a clear example of these discrepancies (Tachibana and Tsutsui, 2016). Contrary to mammals and many fishes, ghrelin suppresses FI in chicken (Furuse et al., 2001; Tachibana and Tsutsui, 2016) and quails (Shousha et al., 2005) via a cascade of events discovered by Saito et al. (2005). They found out that the intracerebroventricular

injection of ghrelin induces a CRH-dependent activation of the hypothalamic-pituitary-adrenal axis resulting in corticosterone secretion and, eventually, hyperactivity and anorexia in chicks. These researchers also confirmed earlier findings demonstrating that ghrelin is expressed in chicken brain (Kaiya et al., 2002). Moreover, at hypothalamic and pituitary level, ghrelin has been shown to bind to GHSR, a G-protein coupled receptor that modulates the release of growth hormone (Wren et al., 2000). In the present study, neither ghrelin nor GHSR hypothalamic expression exhibited significant line-dependent differences under both TN and HS.

Leptin receptor (Ob-R) has been shown to have a pivotal role in feeding control of chicken. Indeed, Lei et al. (2018) have lately demonstrated that the administration of anti-Ob-R antibodies promotes FI in growing chickens. We have previously examined the targets of leptin in chicken hypothalamus (Dridi et al., 2005) revealing, among other things, that it downregulates the expression of its own receptor. In this study, however, the between-line differences in Ob-R mRNA level were not significant under both environmental conditions.

When it comes to adipose tissue-derived peptides involved in regulation of feeding behavior and energy homeostasis, Adip and visfatin are also worth mentioning. In addition to adipocytes, Adip is expressed in several chicken tissues (Maddineni et al., 2005) while its receptors, viz. AdipR1-2, are abundantly present in the hypothalamus (Kadowaki et al., 2008). Kadowaki et al. (2008) thoroughly reviewed the appetite stimulation, longer-term fat modulation, and starvation signaling properties of Adip system, along with its antagonistic effect to leptin. Interestingly, we detected a tendency of 95RB to overexpress Adip regardless of the environmental temperature. It is difficult to interpret this result and further investigations are required to clarify the role of Adip system in hypothalamic FI modulation of chicken.

Visfatin, the second adipokine mentioned before, has been shown to have a wide array of physiological and pathophysiological functions (Dahl et al., 2012). In contrast to mammals, visfatin stimulates appetite in chicken (Cline et al., 2008). Furthermore, we have formerly established that visfatin is ubiquitously expressed in chicken and interconnected with numerous regulatory factors of energy balance (Ons et al., 2010). Here, the observed difference in visfatin expression between JF and ACRB provides further evidence to reasonably believe that visfatin modulates the feeding behavior of chicken. While the involvement of several FRHN in feeding regulation has been verified and their mechanism of action successfully elucidated, many others are still under study or have not even been identified (Parker and Bloom, 2012).

In this regard, two novel hypothalamic neuropeptides, called NPGL and NPGM, have been lately discovered in chicken (Ukena et al., 2014; Shikano et al., 2018). Shikano et al. (2017, 2018) found that NPGL is orexigenic, whereas NPGM has anorectic properties. Although we did not detect a connection between these new FRHN and the observed FI differences, additional studies on chicken NPGL and NPGM are strongly recommended.

There are several possible explanations for the lack of variations observed in this study. The first speculation is based on the hypothalamic integration of circadian information (Bechtold and Loudon, 2013; Delgado et al., 2017). The fact that birds have been fed prior to sampling and sacrificed at different times due to unavoidable operational needs may have remarkably impinged on the hypothalamus, resulting in a flattening of FRHN expression. A second reason might be related to the persistence of the environmental stressor: birds might have gradually acclimatized to the long-lasting exposure to high ambient temperature, thereby accommodating their physiological and hypothalamic response to chronic HS (Sykes and Fataftah, 1986; Yahav, 2009; Collier et al., 2019). Thirdly, the homogeneity showed by some major FRHN across the four chicken populations may partly be explained by stable hypothalamic pathways unaffected by evolution and genetic selection. Fourthly, we measured only mRNA abundances, while it is plausible that protein expression might have differently been affected. Lastly, the fact that we used the whole hypothalamus might have masked the mRNA expression in specific hypothalamic nuclei.

CONCLUSION

In conclusion, this is the first assessment of the impacts of heat stress on feeding-related hypothalamic neuropeptides of chicken, with a valuable and informative comparison between the ancestor jungle fowl and three differently performing broiler lines.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The present study was approved by the University of Arkansas Animal Care and Use Committee (protocols 18,083 and 16,084), and conducted in compliance with the guide for the care and use of laboratory animals of the National Institutes of Health.

AUTHOR CONTRIBUTIONS

SD conceived and designed the study. TT, EG, SO, and SD conducted the *in vivo* experiments. GB performed the molecular analyses, analyzed the data, and wrote the first draft of the manuscript. SD edited and corrected the manuscript with a critical review by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.809341/full#supplementary-material

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Behavior and Immune Response of Conventional and Slow-Growing Broilers to *Salmonella* Typhimurium

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Fast growth rate in broiler chickens comes with welfare concerns and the contribution of growth rate to pathogen resistance and sickness behavior is relatively unknown. The objective of this study was to evaluate physiological and behavioral responses of conventional (CONV) and slow-growing (SG) male broilers challenged with *Salmonella* Typhimurium. CONV (n = 156) and SG (n = 156) chicks were raised in a pen with wood litter shavings until day 7 of age, when birds were transferred to 24 isolators (n = 11 chicks/isolator). On day 14 of age, half of the birds (n = 12 isolators) were challenged with *S.* Typhimurium (ST) and the other half (n = 12 isolators) received a control (C). On days 7, 13, 17, 21, and 24, body weight was recorded, and blood, jejunum and ileum sections were collected from 2 birds/isolator (n = 48 birds/sampling) to measure plasma IgA and IgG and intestinal histomorphology, respectively. On days 12, 16, 21, and 23, video was recorded to evaluate bird postures (sitting, standing, or locomoting) and behaviors (eating, drinking, preening, stretching, sham foraging, allopreening, and aggression). CONV birds were 70 g heavier (p = 0.03) on day 21 and 140 g heavier (p = 0.007) on day 24 than SG. On day 7, CONV jejunum villus height and crypt depth were 22 and 7 μm greater ($p \leq 0.001$), respectively, than SG. On day 24, ST ileum villus height was 95 μm shorter (p = 0.009) than C. IgA increased after day 17 for all birds and at day 21, CONV IgA was greater (p = 0.01) than SG. Although SG IgG was 344 μg/ml greater (p = 0.05) than CONV on day 7, CONV IgG increased with age ($p < 0.0001$) to greater ($p \leq 0.03$) concentrations than SG on day 21 and day 24 by 689 μg/ml and 1,474 μg/ml, respectively, while SG IgG remained at similar concentrations after day 13. Generally, a greater proportion of birds sham foraged as they aged ($p < 0.0001$). A greater proportion of CONV tended to sit (p = 0.09) and fewer locomoted ($p < 0.0001$) than SG as they aged. The results illustrate conventional and slow-growing broilers differ in their behavior, immunity, and response to *Salmonella*.

Keywords: broiler, growth rate, *Salmonella*, immune response, behavior

INTRODUCTION

The United States broiler industry is the largest globally, producing nearly 20 billion kg of chicken meat annually (NCC. National Chicken Council, 2021). As such, broilers are bred for increased feed efficiency and meat yield to satisfy consumer demand (Zuidhof et al., 2014), resulting in conventional broilers that reach market weight at 42 days of age (Aviagen, 2018). However, selection for fast growth comes with trade-offs, as breeds more intensively selected for growth rate have been observed to have greater mortality and culls (Dixon, 2020), greater incidences of hock burn (Weimer et al., 2020), greater disease susceptibility (Swinkels et al., 2007), and poorer immune function (Cheema et al., 2003). Thus, the welfare of conventional, fast-growing broilers has been called into question and compared against slow-growing broilers in recent years.

Torrey et al. (2021) studied the growth, efficiency, and mortality of sixteen broiler breeds to distinguish the outcomes of selection for growth. Body weight and feed efficiency varied, and the conventional breeds were up to 1,264 g heavier and were more feed efficient (by 43 points) than the other breeds, yet mortality did not differ amongst all breeds (Torrey et al., 2021). Other researchers have found greater plasma concentrations of enzymes (aspartate transaminase, creatine kinase, lactate dehydrogenase, and creatinine) and lower tibia ash in a conventional breed compared to four slow-growing breeds, indicating reduced liver function and poorer skeletal mineralization and support relative to body mass (Mohammadigheisar et al., 2020). Dixon (2020) compared three commercial broiler breeds to a slower-growing breed and reported poorer welfare measures (observational scores for gait, feather cover, feather cleanliness, and hock burn) and greater inactivity in the conventional breeds. Despite the current parameters studies have collected regarding conventional versus slow-growing broilers, little is known about the outcomes of the selection for growth rate on resistance to human foodborne pathogens, such as *Salmonella*.

Salmonella enterica serovars Enteritidis and Typhimurium are a common cause of human foodborne illness globally (Knodler and Elfenbein, 2020), resulting in 1.35 million infections and 420 deaths annually in the United States (CDC. Centers for Disease Control and Prevention, 2020). Contaminated poultry products, including chicken meat and eggs, are a frequent cause (Bearson et al., 2017). *Salmonella enterica* infection rarely causes clinical symptoms in poultry (Barrow et al., 2012), and chickens are reported to be asymptomatic carriers of *Salmonella* (Shanmugasundaram et al., 2019). However, subclinical physiological and behavioral indicators may exist as *Salmonella* infection can induce immune stress in chickens, impacting welfare and performance. This can include intestinal inflammation (Gomes et al., 2014), reduced appetite (Liu et al., 2014), and impaired gut morphology (Shao et al., 2013). Thus, the prevalence of *Salmonella* in broiler flocks and products is an important production, food safety, and human health concern, but little research has focused on the pathogen resistance of broiler breeds that differ in growth rate.

Both innate and adaptive immune responses are stimulated by *Salmonella enterica* infection within the chicken gastrointestinal system, involving both cytokine action (Brisbin, 2011; Crhanova et al., 2011) and antibody responses (Hassan et al., 1991; Holt et al., 1999; Acevado-Villanueva et al., 2020). Dar et al. (2019) orally challenged 4-day old Cobb broiler chicks with *S.* Typhimurium and reported elevated serum levels of immunoglobulins (Ig) M and IgG as well as increased mRNA expression of the cytokines interferon-gamma (IFN-γ), interleukin (IL)-12, and IL-18. Immune function differences exist between broiler breeds (Cheema et al., 2003; Swinkels et al., 2007). In a study evaluating the susceptibility to intra-abdominal *Salmonella* Enteritidis infection of two commercial broiler breeds and their crosses, Swaggerty et al. (2005) found that one parent breed and one crossbred breed had a greater number of heterophils at the infection site and increased mRNA cytokine expression. Immune function can also be selectively bred (Yunis et al., 2000; Cheng et al., 2004) and an inverse relationship has been observed between growth rate and immune function (Parmentier et al., 2010; van der Most et al., 2010). When chickens from a broiler breed and a layer breed were injected with lipopolysaccharide (LPS), the layers had greater mRNA expression of pro-inflammatory cytokines IFN- γ and interleukin 1 beta (IL-1β) compared with broilers (Leshchinsky and Klasing, 2001). Bodily resource reallocation in response to infection may be a primary contributor to the differences reported in the aforementioned research (Leshchinsky and Klasing, 2001; Parmentier et al., 2010; van der Most et al., 2010), because the intensive selection of birds for enhanced growth may prioritize the allocation of bodily resources towards growth as opposed to immune function (Humphrey and Klasing, 2004).

Selection for enhanced growth rate may also result in differences in gastrointestinal tract anatomy and nutrient absorption (Yamauchi et al., 2010). Yamauchi and Isshiki (1991) compared the intestinal structure of chickens from commercial broiler and layer breeds for 30 days post-hatch and reported that by day 10, the broilers had wider villi and microvilli as well as more epithelial cell turnover in the duodenum and jejunum. On the other hand, a recent study involving five broiler breeds varying in growth rate reported no differences in jejunum morphology (Mohammadigheisar et al., 2020). Infection of the gut by pathogens such as *Salmonella* Typhimurium (Kaiser et al., 2000; Dar et al., 2019) and *Salmonella* Enteritidis (Awad et al., 2016) can damage the intestinal epithelium, potentially impairing intestinal function. However, there is little research studying breed-related differences in gut integrity and resistance to pathogenic infection.

Differences in the behavior of fast- and slow-growing broilers have been previously studied (Almeida et al., 2012; Dixon, 2020; Torrey et al., 2020; Abeyesinghe et al., 2021). Generally, activity (locomoting and standing) decreases with age and conventional broilers are less active than slow-growing broilers (Dixon, 2020; Torrey et al., 2020). Additionally, conventional broilers tend to forage less than medium- (Almeida et al., 2012) and slow-growing (Yan et al., 2021). Exploratory behaviors (foraging), alongside comfort and social behaviors, such as stretching, preening and

allopreening, can be studied to determine welfare status (Prayitno et al., 1997; Costa et al., 2012) and changes in these behaviors may precede clinical signs of disease (Abeyesinghe et al., 2021). This is particularly important because chickens (evolutionarily prey animals) rarely show sickness behavior when diseased.

Sickness behavior serves as an indicator of an immune response in action and is a sign of proinflammatory cytokine signaling (Kelley et al., 2003; Dantzer, 2004). There is a gap in knowledge regarding breed-related differences in the occurrence of sickness behaviors in broilers, especially their response to *Salmonella enterica* infection, but the selection for fast growth is reported to reduce proinflammatory cytokine expression (Leshchinsky and Klasing, 2001; Berghman, 2016). Thus, it is possible that broilers more intensively selected for fast growth rate may have reduced sickness behavior expression and otherwise display less significant changes in activity, exploratory, comfort, and social behaviors as a result of infection.

The use of conventional or slow-growing broilers is presently a hotly debated topic in animal welfare. At the same time, *Salmonella* infection in humans remains a continuous global concern, despite the rigorous industry efforts to control its spread. It is unclear if the selection for growth rate in broilers has impacted foodborne pathogen resistance. The objective of this study was to evaluate differences in body weight, immune response, gut morphology, and sickness behavior in conventional and slow-growing broiler chickens when challenged with *Salmonella* Typhimurium.

MATERIALS AND METHODS

Experimental Design, Animals, and Housing

All procedures were approved by the University of Maryland Animal Care and Use Committee (IACUC#: R-NOV-19-55). The study was a 2 × 2 split plot design. Three-hundred and twelve male day-of-hatch chicks from two breeds, conventional (**CONV**; N = 156 Ross; Aviagen) and slow-growing (**SG**; *n* = 156 Redbro; Hubbard), were placed into a single floor pen with wood shavings litter. On day 7 of age, chicks were transferred to an ABSL2 research facility where 11 birds from each breed were exclusively placed into 6 isolators in each of 4 rooms (*n* = 24 isolators). Fresh water and commercially available feed (Purina Start and Grow Non-Medicated Chicken Feed) were provided *ad libitum* to all experimental treatment groups. Ambient temperature, humidity, and photoperiod were maintained according to the Ross Broiler Management Handbook (Aviagen, 2018) throughout the study.

A nalidixic acid (NAL)-resistant culture of *Salmonella enterica* serovar Typhimurium Strain #289-1 (Cox and Blankenship, 1975) was used as the challenge treatment. On day 14 of age, CONV (N = 6 isolators) and SG birds (*n* = 6 isolators) were challenged with 1 ml of 1.3×10^8 CFU/ml in tryptic soy broth (TSB; **ST**) via oral gavage, while the controls received 1 ml sterile TSB (**C**).

Qualitative Bacteriology for *Salmonella* spp.

Qualitative bacteriology was performed day of hatch (day 0) and 1 day prior to challenge (day 13) to ensure the birds were negative for *Salmonella* spp. prior to challenge. On day 0, 30 birds (*n* = 15 birds/breed) were randomly selected and their vents aseptically swabbed. Swabs were individually incubated in 10 ml TSB diluted with phosphate-buffered saline (PBS) for 24 h at 37°C. Swabs were then plated on bismuth sulfite agar (BSA) plates and incubated at 37°C for 24 h. Qualitative bacteriology for *Salmonella* spp. was repeated on day 13 with 48 birds (*n* = 24 birds/breed) using the same protocol as on day 0. Each swab from day 13 was plated on BSA as well as BSA + NAL plates to confirm birds were negative for *Salmonella* spp. and NAL-resistant *Salmonella* Typhimurium, respectively.

Sampling and Video Recording

Mortality was recorded daily. Body weight, blood, and intestinal tissue samples were collected on days 7, 13, 17, 21, and 24 from 24 birds from each breed (*n* = 48 birds/sampling day). Birds were euthanized after recording live body weight, and blood was collected through cardiac puncture and was decanted into plasma separation tubes. Blood was centrifuged for 10 min at 2,000 × g and 15°C to separate plasma. A 2 cm longitudinal segment was removed from the jejunum (2 cm anterior to Meckel's diverticulum) and the ileum (2 cm anterior to the ileocecal junction) and transferred to tubes containing 10% buffered formalin. On days 12, 16, 20, and 23, video was recorded on 2 isolators per room (*n* = 8 isolators total, 4 CONV and 4 SG) for 1 h using GoPro (GoPro Hero 7, San Mateo, CA) cameras. Days selected for recording video preceded each sampling by 24 h.

Plasma IgA and IgG

Plasma was stored at −80°C until assayed with commercially available ELISA kits (Bethyl Laboratories Inc., Montgomery, TX) to determine immunoglobulin A (IgA) and immunoglobulin G (IgG) concentrations. The manufacturer protocol was followed using the recommended sample dilutions (1:1000 for IgA, and 1: 100,000 for IgG). Absorbance was measured on a plate reader at 450 nm. A standard curve was generated for each plate to calculate sample IgA and IgG concentrations (μg/ml).

Gut Morphology

Histological preparation was performed by Histoserv, Inc. (Germantown, MD). Slide images were taken at ×40 magnification and later measured using histological software (Qupath Quantitative Pathology & Bioimage Analysis, University of Edinburgh, United Kingdom). Five to ten villus and crypt measurements per intestinal segment (jejunum and ileum) per bird were recorded electronically for villus height and crypt depth. Villi were measured from the tip of the villus to the base at the villi-crypt junction, and crypts were measured from the villi-crypt junction to the crypt base at the basolateral membrane (Golder et al., 2011). Only well-oriented, untorn villi and their paired crypt were measured. Villus-crypt ratio (VCR) was calculated by dividing villus height by its corresponding crypt depth for each villus-crypt pair.

Behavior

Each bird per video recording was coded for mobility, production, comfort, exploratory, and social behaviors by 3

TABLE 1 | Ethogram of postures and behaviors.

Posture/Behavior[a]	Description
Sitting	Resting with hocks on the ground or lying on the side
Standing	One or both feet on the floor and immobile
Locomotion	Mobile and taking steps in any direction, at any speed of movement (walk or run). This may include jumping or lunging
Eating	Sitting or standing in front of the feeder with head over or in trough
Drinking	Sitting or standing next to the waterer and actively putting beak in the water or raising head to swallow
Preening	Sitting or standing and actively grooming oneself (body, feathers, feet, head) via pecking, running beak through feathers
Stretching	Standing while extending a leg and/or wing away from the body
Sham Foraging	Sitting, standing, or moving while physically investigating the environment by pecking or scratching
Allopreening	Sitting, standing, or moving while preening other birds
Aggression	Sitting, standing, or moving while interacting with another bird with vigorous pecking (usually directed at the recipient's head) or kicking (physical contact). Also includes threats (no contact) with erect necks, raised neck feathers, and intentional movement with a raised head by the initiator to the recipient bird

[a]*Ethogram adapted from Prayitno et al., 1997; Bizeray et al., 2002; Bokkers and Koene, 2003, Bailie et al., 2013, Baxter et al., 2019.*

individuals using instantaneous scan sampling every 15 s for the 60 min of video recorded (n = 241 scans/video), beginning 5 min after the researcher left the room. During each scan, the total number of birds performing each posture (sitting, standing, and locomoting), then each behavior (eating, drinking, preening, stretching, sham foraging, allopreening, and aggression) were recorded using the ethogram in **Table 1**. Each bird was coded for one posture and one behavior, one posture and no behavior, or not visible. Bird counts were calculated into proportions of birds performing a posture or behavior out of the total number of birds in the isolator for statistical analysis. Interobserver agreement was 95%.

Statistical Analysis

The bird was the experimental unit on day 7 and isolator was the experimental unit for subsequent ages. Due to collection errors at sampling, day 13, 17, and 21 histology data is missing, and histology results are only reported for days 7 and 24. Day 7 body weight, histology, and immune marker data were run in JMP (v14) using 1-way ANOVA for the fixed effect of breed. Day 13, 17, 21, and 24, body weight, histology (day 24 only), and immunoglobulin data were run using 2-way ANOVA for the fixed effects of breed, challenge, and their interaction with the random effect of isolator nested within room. Additionally, a model was run independent of challenge to evaluate the effects of age, breed, and their interaction with the random effect of isolated nested within room across all ages on broiler plasma immunoglobulin A and G (IgA and IgG) concentrations. Means were separated using Tukey's post-hoc adjusted LSMeans. Pearson's pairwise correlations were compared for body weight, immune markers, and histology data.

Behavior proportion data from days 12, 16, 20, and 23 were analyzed using the GLIMMIX procedure in SAS (v9.4). Behavior data was analyzed independent of challenge for the effects of age, breed, and their interaction. Means were separated using Tukey's post-hoc adjusted LSMeans and differences between measures were detected using PDIFF. Aggression and allopreening behaviors had very low frequencies and there was insufficient data for statistical analysis, so aggression and allopreening results are reported as mean numerical observations. Data were considered significant at a $p \leq 0.05$ and a tendency at $p \leq 0.10$.

TABLE 2 | Body weight (BW, g) on days 7, 13, 17, 21, and 24.

	CONV		SG			*P*-value[a]		
Age (day)	C	ST	C	ST	SEM	B	C	B*C
7	129	-	127	-	4.0	0.69	-	-
13	279	316	271	276	9.8	0.08	0.12	0.23
17	441	461	399	413	22.7	0.06	0.45	0.91
21	682[a]	709[a]	625[b]	626[b]	31.3	0.04	0.66	0.69
24	906[a]	876[a]	754[b]	747[b]	47.0	0.007	0.70	0.80

Data shown as mean BW (±SEM) of male broilers from conventional (CONV) and slow-growing (SG) breeds challenged with Salmonella Typhimurium (ST) or TSB (C) on day 14 of age.

[a]*B, breed; C, challenge; B*C, breed*challenge interaction.*

[ab]*Means sharing the same letters across each row are significantly different for the main effect of breed.*

RESULTS

Qualitative Bacteriology for *Salmonella* spp. Presence/Absence

To ensure that the chicken colony was free of exogenous *Salmonella enterica* contamination, birds were screened for the presence of *Salmonella* prior to inoculation. On day 0, all birds were negative for *Salmonella* spp. growth, while 5 (33%) CONV and 3 (20%) SG birds were positive for non-*Salmonella* spp. bacterial growth on BSA plates. One day prior to *Salmonella* challenge (day 13), 22 (92%) CONV and 22 (92%) SG birds were positive for non-*Salmonella* spp. bacteria, and all birds were negative for *Salmonella* spp. growth on BSA and BSA + NAL plates.

Mortality and Body Weight

Mortality before moving birds into ABSL2 isolators on day 7 (n = 312 birds) was 6 CONV and 1 SG. Mortality after day 7 was 2 CONV-C birds between day 19 and 24.

As anticipated, the most obvious divergence was between CONV and SG birds as they aged. Breed had an effect on BW as the birds aged (**Table 2**). CONV tended to have a greater (p = 0.09) BW than SG by 24 g on day 13. On day 17, CONV tended to have a greater BW (p = 0.06) than SG by 45 g. CONV birds,

TABLE 3 | Plasma IgA and IgG concentrations (μg/ml) on days 7, 13, 17, 21, and 24.

	CONV		SG			*P*-value[a]		
Age (day)	**C**	**ST**	**C**	**ST**	**SEM**	**B**	**C**	**B*C**
IgA (μg/ml)								
7	47	-	47	-	5	0.93	-	-
13	74[a]	59[b]	69[a]	44[b]	7	0.13	0.009	0.47
17	61	64	51	61	7	0.41	0.37	0.64
21	138[b]	168[a]	107[b]	164[a]	19	0.36	0.03	0.49
24	121[a]	115[a]	72[b]	88[b]	14	0.01	0.73	0.40
IgG (μg/ml)								
7	2693[b]	-	3037[a]	-	120	0.05	-	-
13[3]	1991	1709	1589	1578	189	0.19	0.44	0.47
17	1341	1218	1281	962	157	0.33	0.18	0.54
21	2120[a]	1597[a]	985[b]	1356[b]	284	0.03	0.79	0.13
24	3051[a]	2675[a]	1199[b]	1580[b]	334	0.0003	0.99	0.27

Data shown as mean concentrations (±SEM) of immunoglobulin in the plasma of male broilers from conventional (CONV) and slow-growing (SG) breeds challenged with S. Typhimurium (ST) or TSB (C) on day 14 of age.

*[a]B, breed; C, challenge; B*C, breed*challenge interaction.*

[ab]Means sharing the same letters across each row are significantly different.

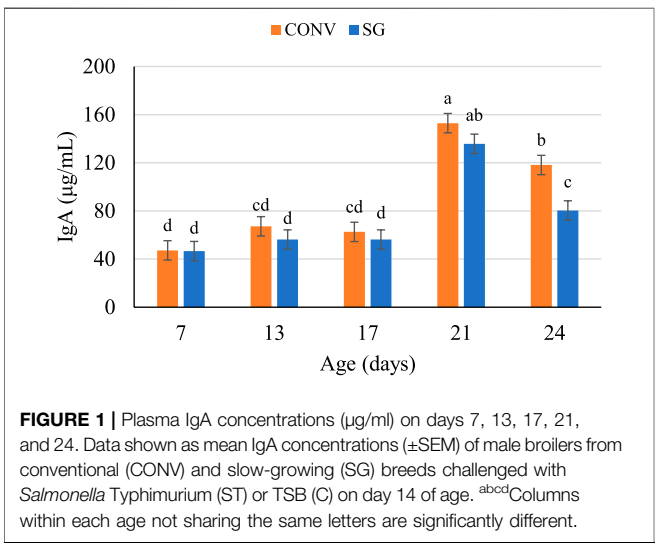

FIGURE 1 | Plasma IgA concentrations (μg/ml) on days 7, 13, 17, 21, and 24. Data shown as mean IgA concentrations (±SEM) of male broilers from conventional (CONV) and slow-growing (SG) breeds challenged with *Salmonella* Typhimurium (ST) or TSB (C) on day 14 of age. [abcd]Columns within each age not sharing the same letters are significantly different.

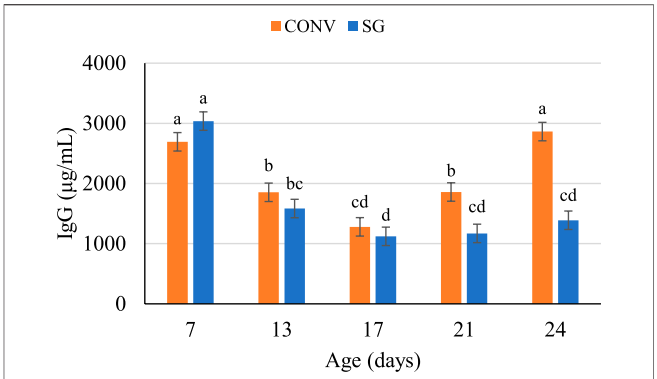

FIGURE 2 | Plasma IgG concentrations (μg/ml) on days 7, 13, 17, 21, and 24. Data shown as mean (±SEM) IgG concentrations of male broilers from conventional (CONV) and slow-growing (SG) breeds challenged with *Salmonella* Typhimurium (ST) or TSB (C) on day 14 of age. [abcd]Columns not sharing the same letters are significantly different.

regardless of bacterial challenge, had a greater (p = 0.03) BW by 70 g on day 21 and by 140 g (p = 0.007) on day 24 than SG. Conversely, *Salmonella* challenge did not affect BW in either CONV or SG chickens.

Immune Response

It was hypothesized that differences in growth rate may lead to differential immune responses or susceptibility to bacterial infection. To query the immune response to *Salmonella* spp. infection, the IgA response to infection was measured (**Table 3**). On the day prior to challenge (day 13), plasma IgA concentrations in C birds was 20 μg/ml greater (p = 0.009) than ST. As anticipated, an IgA response was mounted 1 week after bacterial challenge on day 21 where the infected chickens (ST) had greater plasma IgA levels than uninfected (C) counterparts. Plasma IgA was greater (p = 0.03) for ST birds at day 21 than C by 44 μg/ml (**Table 3**). At 10 days after infection (day 24) CONV bird IgA was greater (p = 0.01) than SG by 42 μg/ml (**Table 3**).

Independent of breed and challenge, IgA concentrations were similar between day 7 (47 μg/ml), day 13 (62 μg/ml), and day 17 (59 μg/ml), then increased (p < 0.0001) to 144 μg/ml on day 21 and decreased on day 24 (**Figure 1**). On day 24, CONV bird plasma IgA was 38 μg/ml greater (p = 0.01) than SG (**Figure 1**).

To assess the potential IgG response to *Salmonella* challenge, IgG was measured throughout the infection (**Table 3**). The initial observation was detection of elevated (maternal) IgG at day 7 that waned the day before challenge (day 13). On day 7, CONV birds had lower (p = 0.05) plasma IgG than SG by 344 μg/ml. Overall, the effect of breed on plasma IgG concentrations was significant on days 21 and 24 with the SG birds consistently demonstrating reduced plasma IgG. Day 21 and 24, CONV birds had greater ($p \leq$ 0.03) plasma IgG than SG by 688 μg/ml and 1,473 μg/ml, respectively (**Table 3**). However, *Salmonella* challenge did not induce an increase in plasma IgG between C and ST groups at any age.

There was no effect of challenge on IgG concentration across age. Independent of challenge, the effects of breed, age, and their interaction were significant (**Figure 2**). The plasma IgG of both CONV and SG birds were similar and decreased (p < 0.0001) from day 7 through 17, after which CONV and SG plasma IgG concentrations diverged (p < 0.0001) (**Figure 2**). CONV plasma IgG increased to 1,859 μg/ml on day 21 and 2,863 μg/ml on day 24, while SG plasma IgG remained lower (p < 0.0001) than CONV at concentrations of 1,170 μg/ml on day 21 and 1,389 μg/ml at day 24 (**Figure 2**).

Gut Morphology

Gut anatomy prior to infection was assessed for differences between breeds (**Figure 3**). On day 7, all histological measures were significant ($p \leq$ 0.04) for the main effect of breed. CONV jejunum villus (JV) height and jejunum crypt (JC) depth were

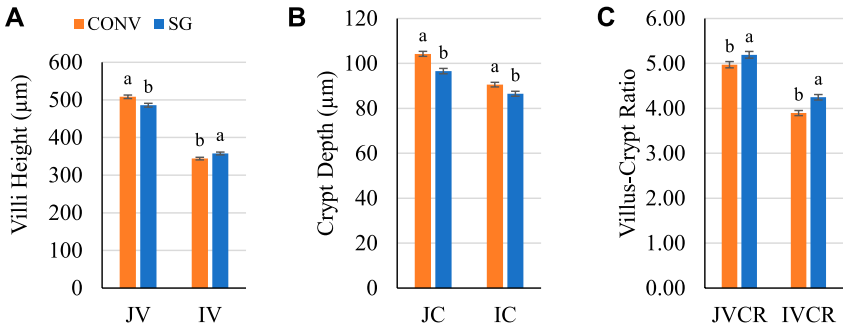

FIGURE 3 | Day 7 **(A)** villus height, **(B)** crypt depth, and **(C)** VCR. Data shown as mean (±SEM) jejunum villi height (JV; µm), jejunum crypt depth (JC; µm), jejunum villus-crypt ratio (JVCR), ileum villi height (IV; µm), ileum crypt depth (IC; µm), and ileum villus-crypt ratio (IVCR) of male broilers from conventional (CONV) and slow-growing (SG) breeds when challenged with *Salmonella* typhimurium (ST) or TSB (C) on day 14 of age. [ab]Columns not sharing the same letters are significantly different.

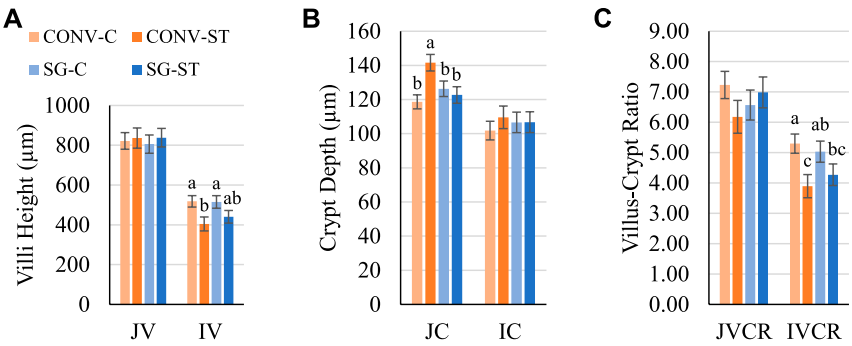

FIGURE 4 | Day 24 **(A)** villus height, **(B)** crypt depth, and **(C)** VCR. Data shown as mean (±SEM) jejunum villi height (JV; µm), jejunum crypt depth (JC; µm), jejunum villus-crypt ratio (JVCR), ileum villi height (IV; µm), ileum crypt depth (IC; µm), and ileum villus-crypt ratio (IVCR) of male broilers from conventional (CONV) and slow-growing (SG) breeds challenged with *Salmonella* Typhimurium (ST) or TSB (C) on day 14 of age. [abc]Columns within each age not sharing the same letters are significantly different.

both greater ($p \leq 0.001$) than SG by 22 and 7 µm, respectively (**Figures 3A,B**). CONV ileum villus (IV) height was shorter ($p = 0.008$) than SG by 14 µm (**Figure 3A**); however, CONV ileum crypt (IC) depth was greater ($p = 0.007$) than SG by 4 µm (**Figure 3B**). The jejunum villus-crypt ratio (JVCR) of CONV was lower ($p = 0.003$) than SG by 0.2, and CONV ileum villus-crypt ratio (IVCR) was lower ($p < 0.0001$) than SG by 0.4 (**Figure 3C**).

To determine if these differences in gut anatomy persisted as the birds grew and to identify potential differential responses to *Salmonella* infection, jejunal and ileal villi anatomy were assessed again. On day 24, the main effect of challenge was significant for IV height but not JV height (**Figure 4**). C IV height was greater ($p = 0.009$) than ST IV by 94 µm (**Figure 4A**). CONV-C IV height was 114 µm greater ($p = 0.009$) than CONV-ST (**Figure 4A**). There was no effect of breed within challenge group. The effect of challenge was also significant for JC depth among CONV birds but not SG on day 24 (**Figure 4B**). CONV-C JC depth was greater ($p = 0.05$) than CONV-ST JC by 23 µm (**Figure 4B**). The effect of challenge on IVCR was significant on day 24 in which C IVCR was greater ($p = 0.007$) than ST IVCR by 1.1 (**Figure 4C**). CONV-C IVCR was 1.4 greater ($p = 0.007$) than CONV-ST on day 24.

Correlations

To collate the data from **Figures 1**–**4** and **Tables 2**, **3**, correlation analyses to identify potential correlations between metrics were performed (**Table 4**). Although weak in strength, plasma IgA concentrations were correlated with several measures. Independent of breed, there were positive correlations between IgA and BW ($r = 0.43$; $p \leq 0.01$), JV ($r = 0.41$; $p \leq 0.01$), JVCR ($r = 0.35$; $p \leq 0.01$), and IV ($r = 0.32$; $p \leq 0.01$). Independent of breed, IgG did not correlate with any measure. CONV plasma IgA was positively correlated with BW ($r = 0.46$; $p \leq 0.01$), JV ($r = 0.35$; $p \leq 0.05$), JVCR ($r = 0.33$; $p \leq 0.05$), IV ($r = 0.31$; $p \leq 0.05$), and IgG ($r = 0.21$; $p \leq 0.05$). Within SG, plasma IgA positively correlated with BW ($r = 0.34$; $p \leq 0.01$), JV ($r = 0.45$: $p \leq 0.01$), and IC ($r = 0.43$; $p \leq 0.05$). SG plasma IgG, negatively correlated with BW ($r = -0.38$; $p \leq 0.01$), IV ($r = -0.54$; $p \leq 0.01$), and IVCR ($r = -0.42$; $p \leq 0.05$).

Behavior

Finally, to query for strain- or infection-associated differences in behavior, observation with quantification of common chicken behaviors was performed. There were minimal effects of challenge on the proportions of postures or behaviors

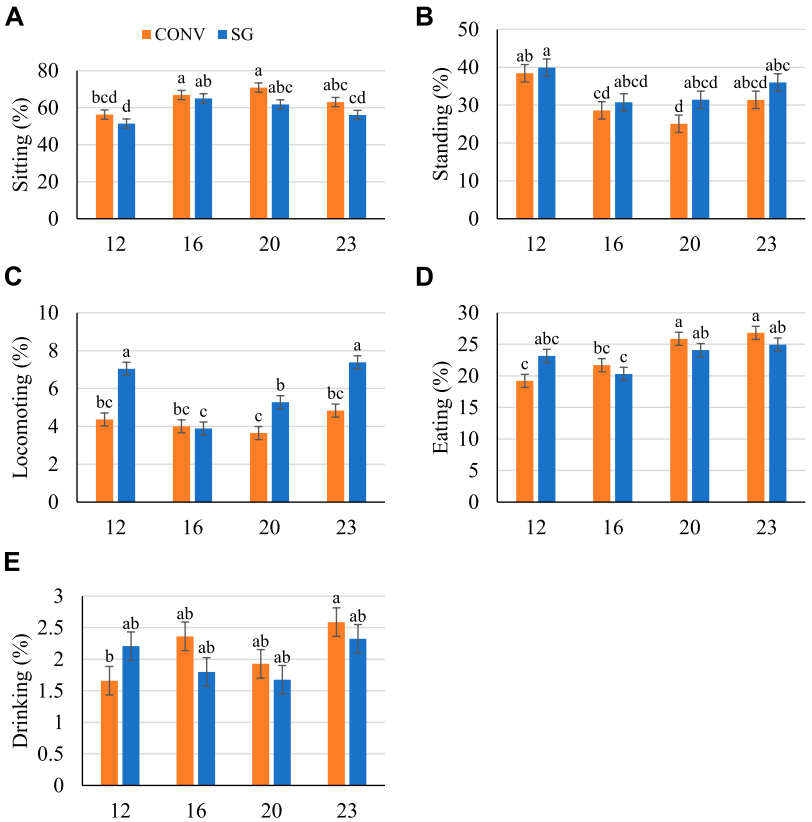

FIGURE 5 | Proportion (%) of birds **(A)** sitting, **(B)** standing, **(C)** locomoting, **(D)** eating, and **(E)** drinking on days 12, 16, 20, and 23. Data shown as mean (±SEM) of male broilers from conventional (CONV) and slow-growing (SG) breeds challenged with *Salmonella* Typhimurium (ST) or TSB (C) on day 14 of age. [abcd]Columns not sharing the same letters are significantly different.

exhibited in this study. Age and breed had much stronger effects and the main effects of breed, age, and their interaction are reported (**Figures 5**, **6**).

Across all days, more CONV birds tended to sit more ($p < 0.10$) than SG (**Figure 5A**). There was no effect of breed on standing behavior, and both breeds generally sat more ($p < 0.0001$) and stood less ($p < 0.0001$) on days 16 and 20 compared with other days (**Figures 5A,B**). Fewer ($p < 0.0001$) CONV birds locomoted than SG on days 12, 20, and 23 by 2.7, 1.6, and 2.6%, respectively (**Figure 5C**). There was no difference between breeds in proportion of birds eating, but more ($p < 0.0001$) birds ate as they aged from 21.8% on day 12 to 25.9% on day 23 (**Figure 5D**). The proportion of birds drinking generally increased ($p = 0.01$) from 1.93% on day 12 to 2.46% on day 23 (**Figure 5E**).

There was no effect of breed on the proportion of birds preening (**Figure 6A**), however, a numerically greater proportion of SG birds were allopreening on days 12, 20 and 23 than CONV (**Figure 6B**). Overall, more ($p = 0.007$) SG stretched than CONV by 0.3% (**Figure 6C**). A similar proportion of birds were sham foraging on days 12 through 20 (between 1 and 2%) and a greater ($p = 0.04$) proportion of SG birds sham foraged on day 23 than CONV (**Figure 6D**). Despite the low proportion of birds sham foraging, at day 23 fewer ($p < 0.05$) SG-ST birds sham foraged than SG-C by 1.6% (data not shown). Additionally, very low proportions of birds were observed exhibiting aggression (less than 0.5%), but a numerically greater numerical proportion of SG birds exhibited aggression than CONV at every age (**Figure 6E**).

DISCUSSION

The selection for rapid growth in modern broilers has resulted in global animal welfare concerns and consideration towards the use of slower-growing breeds. Additionally, *Salmonella enterica* remains an ever-present threat to both the broiler industry and human health. Health and welfare are very interrelated, but these relationships may not always be direct. As such, the differences in physiological and behavioral indicators of health and welfare in two breeds of broiler chickens varying in growth rate when challenged with *Salmonella* Typhimurium were evaluated.

As expected, *Salmonella* Typhimurium challenge did not impact morbidity, mortality, or body weight, and conventional broilers weighed more than slow-growing by day 13. However, both breeds had lower body weight and body weight gain than expected (Aviagen, 2019), which may be attributed to the feed

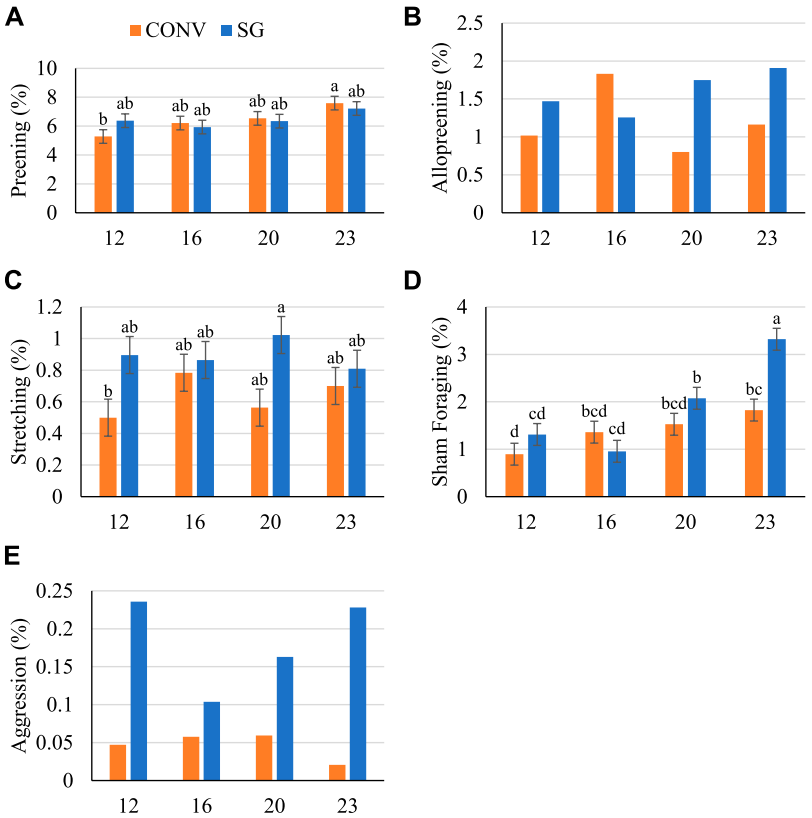

FIGURE 6 | Proportion (%) of birds **(A)** preening, **(B)** allopreening, **(C)** stretching, **(D)** sham foraging, and **(E)** exhibiting aggression on days 12, 16, 20, and 23. Data shown as mean (±SEM) of male broilers from conventional (CONV) and slow-growing (SG) breeds challenged with *Salmonella* Typhimurium (ST) or TSB (C) on day 14 of age. [abcd]Columns not sharing the same letters are significantly different.

TABLE 4 | Correlations (r) between plasma IgA and IgG concentrations (μg/ml) and body weight (BW; g), jejunum villus height (μm), jejunum crypt depth (μm), jejunum villus-crypt ratio, ileum villus height (μm), ileum crypt depth (μm), ileum villus-crypt ratio, and plasma IgA and IgG concentrations of male broilers from conventional (CONV) and slow-growing (SG) breeds on day 7 and 24 when challenged with *Salmonella* Typhimurium (ST) or TSB (C) on day 14 of age.

	BW[a]	JV	JC	JVCR	IV	IC	IVCR	IgA	IgG
Both breeds									
IgA	0.43**	0.41**	0.21	0.35**	0.32**	0.28	0.12	1.00**	0.05
IgG	-0.08	-0.14	-0.02	-0.12	-0.14	0.01	-0.15	0.05	1.00**
CONV									
IgA	0.46**	0.35*	0.17	0.33*	0.31*	0.14	0.23	1.00**	0.21*
IgG	0.10	0.06	0.12	0.01	0.10	0.18	-0.01	0.21*	1.00**
SG									
IgA	0.34**	0.45**	0.22	0.33	0.32	0.43*	-0.06	1.00**	-0.17
IgG	-0.38**	-0.34	-0.15	-0.31	-0.54**	-0.17	-0.42*	-0.17	1.00**

[a]*BW, body weight; JV, jejunum villus height; JC, jejunum crypt depth; JVCR, jejunum villus-crypt ratio; IV, ileum villus height; IC, ileum crypt depth; IVCR, ileum villus crypt ratio.* *$p \leq 0.05$; **$p \leq 0.01$.

provided in this study. The feed used in this study was a commercial backyard chicken feed, which was not specifically formulated for each dietary phase commonly provided to commercial broilers. In a study by Torrey et al. (2021) evaluating differences in 16 broiler breeds varying in growth rate, all birds were provided a diet formulated for a moderate slow-growing breed and, similar to our study, reduced growth occurred among the conventional breeds. Given the relationship between gut morphology and absorptive function of the intestines (Yamauchi et al., 2010), this may have also caused the birds in this study to have shorter villi and crypt measures than broilers of the same age or younger in other studies (Fasina et al., 2010; Lee et al., 2010; Golder et al., 2011).

The conventional and slow-growing breeds differed in their immunoglobulin response. Antibody concentrations increase with age in the chicken (Parmentier et al., 2004), and adult

chickens are expected to have plasma IgA concentrations of 600 μg/ml and plasma IgG (IgY) concentrations of 4,500–5,000 μg/ml (Tizard, 2002). The plasma immunoglobulin concentrations in this study at 24 days of age appear consistent with these findings, given that broilers are not fully mature at this age.

Avian IgA is the most predominant immunoglobulin in intestinal secretions, protecting intestinal surfaces from pathogen invasion (Tizard, 2002). Both breeds had elevated IgA responses to challenge on day 21 (1-week post-challenge) but the conventional breed had greater IgA concentrations than the slow-growing breed at day 24. Peaks in antibody levels such as IgA are generally associated with pathogen clearance (van der Most et al., 2010). If this is the case, the responses indicate the slow-growing breed may have a more effective IgA response to *S.* Typhimurium challenge than the conventional breed. However, a strong humoral immune response may not necessarily be effective or indicative of strong immune function (Barrow et al., 2012).

Within the control group of both breeds, baseline IgA concentrations were slightly elevated at day 13, which was 24 h prior to challenge. Individual variation of randomly sampled control birds could have contributed to this difference, but time of day at sampling could have also caused elevated IgA concentrations. Melatonin is known to have a role in circadian rhythm but also modulates the immune system in mammals, capable of influencing antibody production (Cernysiov et al., 2009). In laying hens administered melatonin intraperitoneally at 70 weeks of age, plasma IgA, IgG, and IgM levels were elevated (Hao et al., 2020). Circulating melatonin levels in the chicken increase during dark hours and melatonin production decreases with light stimulation (Pelham, 1975). In the present study, sampling occurred in the morning with all control-assigned birds sampled before the challenge-assigned birds. Thus, control birds were sampled earlier in the day and these birds could have had greater concentrations of melatonin, leading to greater plasma IgA levels than the later-sampled challenge birds. However, this pattern was not observed on any other day. Plasma IgA concentrations peaked in both challenge and control treatments on day 21 but were greater in challenge birds than in control birds. Located beneath intestinal mucosal surfaces, B-cells produce IgA in the intestines, of which some ends up in the bloodstream (Tizard, 2002). Thus, elevated plasma IgA levels in the bloodstream can reflect a response to *S.* Typhimurium infection in the intestines of challenged birds.

Another vital component of the chicken immune response to infection is IgG (Gharaibeh and Mahmoud, 2013). At day 7, IgG concentrations were greater in slow-growing birds than conventional, which may reflect a greater natural level of maternal antibodies in the blood. IgG, also termed IgY (immunoglobulin of the yolk), is a maternal antibody transferred to chicks through the egg yolk and lasts up to 10 days post-hatch in the chick, providing critical early life immune protection to chicks until immunocompetence is attained (Carlander et al., 1999; Gharaibeh and Mahmoud, 2013). Greater levels of maternal antibodies can reflect stronger early life immune protection (Marcq et al., 2011) and maternal IgG is especially important in broiler health due to the short lifespan of broilers in commercial production systems (Gharaibeh and Mahmoud, 2013). Broiler secondary immune organs, such as the spleen and cecal tonsils, and the resulting humoral response are not mature enough to mount an immune response until approximately 12 days of age (Mast and Goddeeris, 1999). Furthermore, the broiler immune response is not fully developed until approximately 30 days of age (Song et al., 2021). In the present study, maternal antibodies may have persisted at elevated concentrations in the slow-growing breed up to day 7 than in the conventional breed, indicating the slow-growing breed may have stronger and longer-lasting early life immune protection.

There was no apparent plasma IgG response to *S.* Typhimurium challenge in either breed. The present study was limited to the use of commercial ELISA kits to measure plasma immunoglobulin response. Thus, all plasma IgA and IgG results in the current study reflected total plasma concentrations of either immunoglobulin but not *S.* Typhimurium antigen-specific immunoglobulins, and actual immunoglobulin responses to the challenge may have been masked by baseline circulating levels of immunoglobulin. This is especially the case for IgG due the naturally high baseline levels of circulating IgG in the chicken (Tizard, 2002).

IgG concentrations increased after day 17 in the conventional breed but not the slow-growing breed. The selection for increased growth rate in broilers may have resulted in increased lymphoid organ development or earlier immune system maturation in the conventional breed. However, the rate of development or heightened antibody levels do not necessarily correspond with a stronger or more efficient immune response (van der Most et al., 2010; Barrow et al., 2012). Further research is needed to investigate the relationship between growth rate and the development of immunocompetence in broilers.

The conventional and slow-growing breeds differed in both ileum and jejunum histomorphological measures at day 7. The conventional breed had greater jejunal villus height and crypt depth than the slow-growing breed, which may suggest greater absorptive efficiency relative to the birds from this breed's enhanced growth rate. On the other hand, the slow-growing breed had greater ileum villus height but shallower ileum crypts than the conventional breed. Growth-related differences in gut morphological measures, particularly villus height, have been documented, noting that selection for growth in broilers has resulted in longer villi when compared to White Leghorns (Yamauchi and Isshiki, 1991). Between both intestinal segments, however, the slow-growing breed had greater day 7 villus height to crypt depth ratios than the conventional breed, which could indicate reduced cellular turnover in the jejunum and ileum (Seyyedin and Nazem, 2017). Gut morphology measures from the intermediate days would have provided further insight on the development of intestinal structure within each breed as the birds aged.

The breed intestinal histomorphological differences at day 7 disappeared by day 24 (10 days post-challenge). However, challenge impacted gut morphology, especially in the

conventional breed. Conventional birds challenged with *S.* Typhimurium had deeper jejunum crypts than controls and slow-growing birds, which may indicate increased enterocyte production in the crypts of conventional birds to compensate for cellular loss at the villi tips due to infection Fernando and McCraw (1973). Fernando and McCraw (1973) found that 14-week-old male White Leghorn chicks infected with *Eimeria acervulina* had reduced jejunal villus heights and increased crypt depths, which then returned to normal or greater lengths and depths 5–6 days post-infection. However, challenge did not appear to impact jejunum villus height in either breed in the present study. Others have noted no significant differences in any gut morphology measures in broilers challenged with *Salmonella* Enteritidis (Gomes et al., 2014).

Correlations were only reported for the relationships between antibody concentrations and all other measures, as the relationship between gut morphology and body weight is well-established (Yamauchi and Isshiki, 1991; Yamauchi et al., 2010). There was a positive correlation between IgA concentrations and body weight among both breeds, which was slightly stronger in the conventional breed. Additionally, the correlation between IgG and body weight was negative in the slow-growing breed, but a very weak and insignificant positive correlation was detected in the conventional breed. IgA and IgG concentrations positively correlated with one another in the conventional breed, but not the slow-growing breed. Combined, these support the possibility that selection for increased growth rate in the conventional breed may unintentionally select for earlier development of immunocompetence. Plasma IgG concentrations had little to no significant relation to gut morphological measures, except in the slow-growing breed where IgG and ileum villus height (and ileum villus height to crypt depth ratio) negatively correlated. On the other hand, plasma IgA concentration positively correlated with multiple gut morphology measures, such as jejunum villus height. This may reflect a relationship between intestinal morphology and intestinal humoral secretions, as plasma IgA concentration can be representative of intestinal IgA concentration (Tizard, 2002).

Few behavioral differences between breeds were found in this study, and there was a minimal effect of challenge on behavior. When comparing the first (day 12) to the last (day 23) of video recording days, more birds sat and fewer stood as they aged. However, the proportion of birds locomoting was similar between the first and last day. Generally, locomotion-type behaviors, such as walking or running, also become less frequent as broilers age (Bokkers and Koene, 2003; Dixon, 2020). The birds in this study were housed in biosafety isolators with space allowance and stocking density according to the Ag Guide (FASS. Federation of Animal Science Societies, 2010), but isolator space still restricted opportunities for movement. In a study involving male Peterson Arbor Acre broilers, Pelham (1975) found that broilers housed in smaller pens utilized less total space than broilers housed in larger pens, likely due to reduced movement opportunities. It is possible that space limitations affected the frequency at which locomotion occurred and was observed. Generally, slow-growing birds sat less and locomoted more than birds from the conventional breed in the current study. These differences in locomotive activity between breeds that vary in growth rate have been reported in previous research (Bokkers and Koene, 2003; Wallenbeck et al., 2016; Dixon, 2020; Yan et al., 2021).

A unique finding in this study was more birds sat and fewer stood and locomoted following the oral gavage (days 16 and 20), independent of both breed and challenge. We hypothesize that this was a behavioral indicator of stress following gavage at day 14. The use of a gavage to deliver a substance orally is an invasive and stressful event and has been evidenced by reports of increased plasma corticosterone of rats orally gavaged with corn oil (Brown et al., 2000). Stress may cause long-term behavioral consequences, resulting in reduced activity manifested through increased sitting. For example, Ross broiler chickens that underwent heat stress at 32°C for 10 h daily from 15 to 43 days of age displayed increased sitting and reduced walking and standing (Wang et al., 2018). The inclusion of a negative (no gavage) control group would have been beneficial to this study to determine if the gavage process caused stress because it is unknown to what extent the gavage causes stress in chickens and research is needed to further investigate these effects.

The proportion of birds sham foraging generally increased with age, and it was observed that this was more pronounced among slow-growing birds. The slow-growing breed sham foraged more on each successive day, except day 16. Breed differences in foraging behavior are more obvious between broilers and layers. When a hybrid broiler breed and layer breed were provided free access to feed and to feed mixed in wood shavings, the broilers showed less foraging and greater inactivity than the layers (Lindqvist et al., 2006). Foraging behavior has been reported to vary between broiler genotypes, with a tendency for slower-growing broiler breeds to forage or engage in exploratory behaviors more than medium- (Almeida et al., 2012) and conventional breeds (Yan et al., 2021). However, other studies have reported no differences between conventional and slow-growing broiler foraging behavior (Wallenbeck et al., 2016).

The slow-growing broilers exhibited more social and agonistic behaviors than conventional in the current study. For example, more slow-growing birds engaged in allopreening (except on day 16) and aggression than conventional. Increases in aggression and displacement preening (preening that results from frustration) can indicate environment-related frustration in confined chickens (Calder and Albright, 2021), which may indicate the slow-growing breed was more affected by the housing environment or other factors in the present study. However, the observed proportions of these behaviors across all ages were very small (less than 2%) and a larger study involving more birds may have resulted in more representative time budgets. Domesticated farm animals, such as broilers, are less prone to respond behaviorally to sickness (Berghman, 2016). In this study, *S.* Typhimurium challenge did not induce sickness behaviors, such as a ruffled feathers or hunched posture, as neither behavior was observed. All results of this study were impacted by the limited study length of 24 days. Measures taken beyond the end of the present study through market age would have provided

greater clarity as to breed related differences and the response to *S.* Typhimurium infection regarding body weight, immune response, gut morphology, and behavior. Further research is needed to investigate differences between conventional and slow-growing broiler breeds to determine the effect of *S.* Typhimurium infection on the frequency of social and agonistic behaviors.

The results of this study indicate that meaningful genotypic and phenotypic differences exist in conventional broilers compared to slow-growing broilers with regards to body weight, immune response, gut morphology, and behavior when challenged with *Salmonella* Typhimurium. In the present study, broilers from the conventional breed were heavier, had greater jejunum villus height with lower crypt depth, and had earlier increased IgG concentrations on days 21 and 24 of age, which may indicate earlier or faster immune development than the slow-growing breed. The slow-growing birds appeared to be more resilient to *Salmonella* challenge in that their jejunum crypt depth was unaffected by challenge, and they had greater plasma concentrations of maternal IgG, indicating greater early life immune protection. The slow-growing breed sat less and engaged in more sham foraging, allopreening, and aggression behaviors than the conventional breed and *S.* Typhimurium challenge reduced sham foraging in the slow-growing breed, but not the conventional breed. The *S.* Typhimurium challenge impaired intestinal morphology 10 days post-challenge and elevated IgA concentrations 7 days post-challenge in both breeds. Delineating the differences in basal and *Salmonella*-challenged phenotypes of broilers with divergent growth rates provides useful information for genetic, nutritional, and management decisions. Further research is needed to understand the extent of the differences between conventional and slow-growing broiler immune function, gut development, sickness behavior, and resistance to foodborne pathogens.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Maryland Animal Care and Use Committee (IACUC#: R-NOV-19-55).

AUTHOR CONTRIBUTIONS

SW, DK and TJ contributed to the conception and design of the study. SR, CR, and CW assisted AS with laboratory procedures and CR assisted AS with statistical analysis. AS and SW led writing the manuscript. All authors approve the submitted version.

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OPN5 Regulating Mechanism of Follicle Development Through the TSH-DIO2/DIO3 Pathway in Mountain Ducks Under Different Photoperiods

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Abstract: Photoperiod is an important environmental factor that influence seasonal reproduction behavior in bird. Birds translates photoperiodic information into neuroendocrine signals through deep brain photoreceptors (DBPs). OPN5 has been considered as candidate DBPs involving in regulation of seasonal reproduction in birds. However, little is known about the effect of OPN5 in non-seasonal breeding birds. Thus, we pondered on whether OPN5 regulating follicular development through TSH-DIO2/DIO3 system responds to different photoperiods in non-seasonal laying ducks. As an ideal non-seasonal breeding bird, a total of 120 mountain ducks were randomly divided into three groups and treated respectively to a different photoperiod: group S (8 L: 16D), group C (17 L:7D), and group L (24 L:0D). The ducks were caged in a fully enclosed shelter with the same feeding conditions for each group, free water and limited feeding (150 g per duck each day). Samples were collected from each group at d 0, d 5, d 8, d 20, and d 35 ($n = 8$). The ducks in 24 h photoperiod had the highest laying rate and the lowest feed-to-egg ratio, while the ducks in 8 h photoperiod had the lowest laying rate and the highest feed-to-egg ratio. Long-day photoperiod for 24 h significantly increased the ovarian index and GnRH, LH, E2, and P4 levels in serum; short-day photoperiod for 8 h increased testosterone levels in serum. Compared with 8 h photoperiod, long-day photoperiod significantly or highly significantly increased the mRNA level and protein expression of OPN5 in the hypothalamus of long-day photoperiod on d 35 ($p < 0.05$). The gene or protein expression patterns of GnRH, TRH, TSHβ, DIO2, THRβ, VIP, and PRL were positively correlated with OPN5, whereas the gene expression patterns of *GnIH* and *DI O 3* were negatively correlated with *OPN5*. The results revealed that OPN5 mediated the effect of light on follicular development through the TSH-DIO2/DIO3 pathway, the expression of OPN5 increased with light duration and improved the efficiency of the HPG axis to promote follicular development in mountain ducks.

Keywords: Opn5, TSH-DIO2/DIO3 pathway, photoperiod, Follicular development, mountain duck

1 INTRODUCTION

Opsin 5(OPN5) is a novel G protein-coupled receptor (GPCR), firstly identified in mammalian neural tissue in 2003 (Tarttelin et al., 2003; Nakane and Yoshimua, 2019), and mainly expressed in the retina, hypothalamic paraventricular organ (PVO), and gonads (Kojima et al., 2011; Stevenson and Ball, 2012). OPN5 is the main deep-brain photoreceptor (DBP) in birds (Nakane and Yoshimua, 2019), which is involved in regulating reproductive functions (Volle, 2021). Three main DBPs have been identified, including melanopsin (OPN4), neurophotoprotein (OPN5), and vertebra ancient opsin (VAOpn) (Kang and Kuenzel, 2015; Beaudry et al., 2017). OPN5 is a short-wavelength sensitive photopigment that absorbs at 360–474 nm and is thought to be a UV-sensitive serine protease capable of mediating light signaling (Kojima et al., 2011). It has been confirmed that OPN5 can affect bird reproduction through the hypothalamic-pituitary-gonadal axis (HPG axis) (Zhu et al., 2019a; Zhu et al., 2019b). A large number of studies (Kuenzel et al., 2015; Mishra et al., 2017; Mishra et al., 2018) have shown that OPN5 in the paraventricular nucleus of birds could transmit light signals to the pituitary nodule (PT) in order to initiate the thyroid hormone response (TH-responsive) signaling pathway, which has effects on the production and secretion of Gonadotropin-releasing hormone (GnRH) by regulating the secretion of triiodothyronine (T3) (Saelim et al., 2004). Therefore, OPN5 plays an important role in the light regulation of bird reproductive activities.

Reproduction in birds is regulated by the hypothalamic-pituitary-gonadal axis, and the HPG axis is a complete feedback system formed by the hypothalamus, pituitary, and gonads under central nervous regulation (Rose et al., 2022). Hypothalamus secretes GnRH and Gonadotropin-inhibitory hormone (GnIH) to regulate gonadotropin secretion at the pituitary level (Nabi et al., 2020). Gonadotropins regulate the secretion of gonadotropic hormones through blood circulation, which in turn promotes gonadal development and regulates animal reproduction (Zhu et al., 2019a; Zhu et al., 2019b). In addition, in seasonally breeding avian species, nesting behavior associated with reproduction is an important biological feature and is related to neurotransmitters produced by the hypothalamus, pituitary gland and hypothalamus-pituitary-Gonadal axis (HPG)reproductive hormones (Saelim et al., 2004; Gumułka et al., 2020). The HPG axis secretes a variety of hormones, including GnRH hormone at the hypothalamic level, prolactin (PRL),follicle-stimulating hormone (FSH), and luteinizing hormone (LH) hormones at the pituitary level, and E2 and P4 at the gonadal level and the nesting behavior is mediated by the action of ovarian E2 on the hypothalamus during egg laying, and that the enhanced activity of hypothalamic dopamine and pentraxin promotes the synthesis and release of Vasoactive Intestinal peptide (VIP) (Gumułka et al., 2020), which in turn promotes the secretion of PRL through feedback (Avital-Cohen et al., 2012).

Under natural conditions, most poultry (birds) are seasonal breeding animals, and breeding status is divided into breeding and non-breeding seasons due to changes in sunlight throughout the year. However, domesticated poultry, such as chickens and ducks, can breed throughout the year (Kuenzel et al., 2015; Mishra et al., 2017; Mishra et al., 2018). In both the production of breeders and laying poultry, light management is very important and directly affects laying performance (Chew et al., 2021; House et al., 2021). In practical production, the light duration during the egg-laying photoperiod is generally maintained at 16–18 h in non-seasonal breeding birds, and feed intake was limited every day (Cui et al., 2021; Ouyang et al., 2021). Female mountain ducks start laying eggs at 100-day-old of age, laying 280 to 300 eggs per year, no nesting characteristics (Cao et al., 2020). There are still no systematic studies reporting a light duration that what is the underlying regulatory mechanism in too long-day photoperiod or too short-day photoperiod for optimal follicle development and laying performance of non-seasonal breeding birds. Many scholars have reported that OPN5-mediated light regulation of avian reproduction mostly focuses on OPN5 regulation of GnRH secretion through the TSH pathway (Kuenzel et al., 2015; Mishra et al., 2017; Mishra et al., 2018). In the pathway of regulating avian reproductive activities by light, OPN5 and TSH, as two important mediators, are in the upstream and downstream between light and reproductive axis, respectively, and they show consistent changes in the regulation of avian reproductive activities by light (Zhu et al., 2019a; Zhu et al., 2019b). OPN5, as a photoreceptor, is upstream in the pathway of light-regulated reproductive activity and is the main factor mediating light changes (Nakane and Yoshimua, 2019). There is the evidence that the knockdown of OPN5 *via* small interfering RNA antisense in the MBH revealed that there is an inhibitory input in the photoinduced regulation of *TSHβ* mRNA expression (Stevenson and Ball, 2012). In the previous work we have carried out, some direct results on the association between OPN5 and TSH have been obtained but not published, which was that active immunization of ducks with OPN5 immunogen was found to promote OPN5 expression and upregulate TSHβ and DIO2 expression, which in turn regulate HPG axis-related genes to promote follicle development in mountain ducks. However, there are no reports on whether the TSH-DIO2/DIO3 pathway regulates follicle development through the production of key regulators of the avian reproductive axis including GnRH, GnIH, VIP, and PRL, and whether different light durations are associated with the expression of these key regulators. The existence of a dose-dependent relationship between different light durations and the expression of key regulators in these regulatory pathways has not been reported. What is the regulatory pathway difference between seasonal breeding avian species (including short-day breeding avian species and long-day breeding avian species) and non-seasonal breeding avian species? These questions will not only help to elucidate the mechanism of OPN5 in regulating breeding activities of birds but also provide important guidance for light management in the production of breeding and laying poultry. In our study, we examined the egg laying rate, feed return, ovarian performance, reproductive hormone levels, and reproductive gene and protein expressions of mountain ducks under different light treatments to understand the relationship between follicular development,

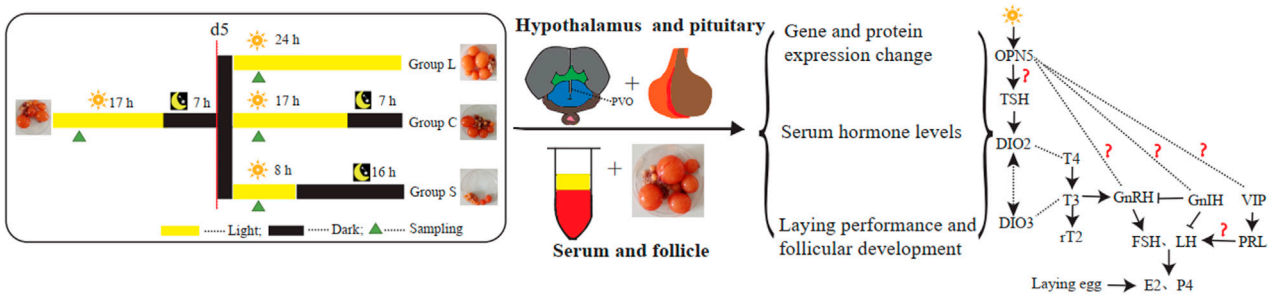

FIGURE 1 | Experimental design All mountain ducks were randomly divided into three types of group. All samples were collected at d0, d5, d8, d20, d35. Serum samples in the three types of group were collected for analysis of serum hormone levels. Hypothalamic and pituitary tissue in the in the three types of group were collected for related genes and proteins analysis.

OPN5 expression patterns, reproductive axis hormone secretion, and gene expression in ducks under different light regimes. Moreover, we revealed that the OPN5 regulating mechanism of follicular development occurs through the TSH-DIO2/DIO3 pathway.

2 MATERIALS AND METHODS

2.1 Ethics Statement

All experimental procedures in this study were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (State Science and Technology Commission in China, 1988) and EU Directive 2010/63/EU for animal experiments. All animal experiments were approved by the Animal Care and Use Committee of Zhongkai University of Agriculture and Engineering (Guangzhou, China).

2.2 Animals and Experimental Design

The trial was undertaken at the Institute of Animal Health, Guangdong Academy of Agriculture Sciences, Guangzhou, Guangdong Province, China. One hundred and twenty normal laying mountain ducks were pre-fed for 15 d in 150-day-old with limited food (150 g per duck each day) and unlimited water at three constant rooms temperature of 21°C and light intensity of 400 lux under a 17 h light/7 h dark cycle. At 165-day-old of age, all ducks were randomly divided into three groups, with 40 birds in each group kept in cages, among them two ducks in a cage (**Figure 1**). They were initially under 17 L:7D light conditions at light intensity of 400 lux for 5 days. Then, all ducks in the group receiving the long-day photoperiod of light (group L), the group receiving the 17 L:7D photoperiod of light (group C), and the group receiving the short-day photoperiod (group S), were maintained under 24 L:0D, 17 L:7D, and 8 L:16D light conditions for 30 days, respectively. All samples were collected at 2 h after lights-on zeitgeber time 6 (ZT6), which for group S birds was 6 h after dark onset, and for group C birds 15 h before dark onset, and for group L birds 24 h (lights-on was the same for group S and group C). The number of eggs laid in each group was recorded each day and blood samples were collected at d 0, 5, 8, 20, and 35 ($n = 5$ or $n = 8$), five samples per group for qPCR, five or eight for hormone assays (including five samples per group for d0 and d20, eight for d5, d8 and d35), five for western blots and three for immunohistochemistry, in which d 0, 5, 8, 20, and 35 were August 6, August 11, August 14, August 25, and 10 September 2020, respectively, China. The serum was separated and reserved for analysis of serum hormone levels, follicular development was examined, gonadal index was calculated, and hypothalamic and pituitary tissue samples were collected and reserved (−80°C) for tests of expression of related genes and proteins.

2.3 Detection of Serum Reproductive Hormones

The determination of E2/P4/GnRH/T/PRL/LH in serum was performed according to the kit instructions. These kits were: Duck Estradiol (E2) ELISA KIT (CUSABIO, Shanghai, China), Duck Progesterone (P4)/GnRH/Testosterone ELISA KIT (Elabscience, Shanghai, China), Duck Prolactin (PRL) ELISA KIT, and Chicken Luteinzing hormone (LH) ELISA KIT (SAB, Shanghai, China). Among them, the detection range of E2 is 40–1000 pg/ml, and the coefficient of variation CV is less than 10%; the detection range of P4 is 0.31–20 ng/ml, and the coefficient of variation CV is less than 10%; the detection range of GnRH is 15.63–1000 pg/ml, and the coefficient of variation CV is less than 10%; the detection range of T is 0.31–20 ng/ml, and the coefficient of variation CV is less than 10%; the detection range of PRL is 40–1000 μ IU/ml, coefficient of variation CV less than 8%; the detection range of LH is 0.5–100miu/ml, and the coefficient of variation CV is less than 8%.

2.4 Detection of Gene Expression Levels

Tissue RNA was extracted by trizol conventional method and cDNA was obtained by reverse transcription of RNA with PrimeScript^{TM}RT reagent Kit with gDNA Eraser (TaKaRa, Tokyo, Japan). Real-time quantitative polymerase chain reaction (qRT-PCR) was used to quantify the expression of *OPN5*, *TSHβ*, *TRH*, *TSHR*, *THRB*, *DIO2*, *DIO3*, *GnRH*, *GnIH*, *PRL*, *GnRHR*, *GnIHR*, *FSH*, and *LH* mRNA expression levels in

TABLE 1 | Primers for real-time fluorescent quantitative PCR

Gene	Primer Sequence (5′-3′)	Annealing temperature (°C)	Product size (bp)	GenBank No.
OPN5	F: ACCAGGATCCAGAACAGCCA R: GCAATGAGGAATCCGGCACA	56	80	XM_019613613.2
GnRH	F: CTGGGACCCTTGCTGTTTTG R: AGGGGACTTCCAACCATCAC	59	209	XM_013176792.1
GnIH	F: AAAGTGCCAAATTCAGTTGCT R: GCTCTCTCCAAAAGCTCTTCC	58	128	XM_015853673.2
VIP	F: TCAAACGCCACTCTGATGCT R: GAGGGGTTTAGCTCTTCCTGG	60	124	XM_035538857.1
DIO2	F: GACGCCTACAAGCAGGTCAA R: GTTCCACACTTGCCACCAAC	57	119	XM_013094234.4
DIO3	F: AGATGCTACTGATGCCCACG R: CCGAAGTTGAGGATGAGGGG	57	271	XM_013199473.1
TRH	F: TGGTGAAGTAAATTACCAGAACAC R: CCTAAATGGGGACACTCACTCAC	60	98	XM_013182272.1
TSHβ	F: CGTGTGCACATACAAAGAGAT R: GCAATAGTTTGGCCTAACCTT	56	162	NM_001310425.1
THRβ	F: GCTTATCTCTGGGCAATGTGAC R: TTGAAGCGACATTCCTGGCA	60	299	XM_038174583.1
TSHR	F:CCCCAACATCTCTAGGATTGAA R: CTGAAGTCATGAAAGGATTATCTGC	60	200	XM_009646634.2
GnRHR	F: TCTGCTGGACCCCCTACTAC R: TCCAGGCAGGCATTGAAGAG	62	127	NM_001012609.1
GnIHR	F: CATCCTGGTGTGCTTCATCG R: ACATGGTGTTGTCAAAGGGC	56	164	XM-005028365.3
PRL	F: ACCTCCTTGCCTATCTGCCC R: TTGTAATGAAACCCCGACCC	60	180	NM_001310372.1
FSH	F: GTGGTGCTCAGGATACTGCTTCA R: GTGCAGTTCAGTGCTATCAGTGTCA	60	209	XM_031607398.1
LH	F: CCAGGCCTCCTGCACCTAC R: GGCGCAGCGGCAGCTCAG	60	115	MK820637.1
β-actin	F: CCTCTTCCAGCCATCTTTCTT R: TGTTGGCATACAGGTCCTTAC	60	110	XM_035563367.1

hypothalamic or pituitary tissue. The qRT-PCR analysis was performed using the Applied Biosystems Quant Studio seven Flex Real-Time PCR System (Thermo Fisher, United States). Based on the reference sequence on NCBI, the fluorescent quantitative primers for the above genes and the primers for the β-actin gene as an internal reference were designed using Primer 5.0 (**Table 1**) and synthesized by Sangon Biotech Co, Ltd., Using cDNA as a template, a 10 μL reaction system was prepared: PowerUPTM SYBR™ Green Master Mix (Thermo Fisher, United States) 5 μL, upstream and downstream primers 0.1 μL each, ddH2O 3.8 μL, cDNA template 1 μL. Reaction conditions were set: 50°C pre-denaturation for 2 min, 95°C for 10 min1 cycle; 95°C for 15 s, T_m°C for 1 min, 40 cycles. Three replicates of each sample were normalized with β-actin as an internal reference gene using the $2^{-\Delta\Delta CT}$ method.

2.5 Protein Extraction and Western Blot Analysis

The total protein of the hypothalamus was extracted and total protein concentration was determined by BCA (Beyotime, Shanghai, China). After denaturation by SDS (Beyotime, Shanghai, China), the protein was sampled using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 10–15%, and electrophoresis was performed at 80 V for 15 min and at 120 V for 60 min. Upon completion of electrophoresis, the protein was transferred to polyvinylidene fluoride (PVDF) membranes at 200 mA and after blocking with phosphate-buffered saline with Tween 20 (PBST) containing 5% fat-free milk, PVDF membranes were co-incubated with the antibodies, anti-β-actin (1:5,000, Proteintech, Wuhan, China), anti-OPN5 (1:1000, self-made antibody), anti-THRA/THRB (C3) (1:1000, Invitrogen, United States), anti-GnRH-1-Ab (1:1000, Affinity Biosciences, OH, United States), anti-GnIH(1:1000, self-made antibody)and anti-DIO2 (1:1000, Affinity Biosciences, OH, United States). They were then incubated overnight at 4°C and washed five times at TBST each for 3 min. The secondary antibodies, goat anti-rabbit (1:10000, Affinity Biosciences, OH, United States) and goat anti-mouse (1:10000, Abcam, Cambridge, United Kingdom), were incubated for 1 h at room temperature and washed three times at TBST for 10 min each. Proteins were detected using the ECL kit (Beyotime, Shanghai, China), and visualized using a Tanon-5200Multi device (Tanon, Shanghai, China), photographed and stored. Densitometry analysis was performing using ImageJ software.

2.6 Detection of Immunohistochemistry

Deparaffinizing and rehydrating the paraffin section: the sections were placed into xylene I for 15 min, xylene II for 15 min, xylene III for 15 min, absolute ethanol I for 5 min, absolute ethanol II for

5 min, 85% alcohol for 5 min, 75% alcohol for 5 min, and then rinsed in distilled water. Antigen retrieval: The tissue sections were placed in a repair box filled with citric acid (pH 6.0) antigen retrieval buffer for antigen retrieval, within a microwave oven, heated on medium power for 8 min until boiling, kept warm for 8 min, and then heated again, this time on medium-low power, for 7 min. During this process, excessive evaporation of buffer should be prevented and the sections should not be allowed to dry. To cool the sections to room temperature before proceeding, the sections were placed in PBS (pH 7.4) and shaken on the decolorization shaker three times for 5 min each. Blocking endogenous peroxidase activity: the sections were placed in 3% hydrogen peroxide and incubated at room temperature in darkness for 25 min. The sections were placed in PBS (pH 7.4) and shaken on a decolorizing shaper three times for 5 min each. Serum sealing: 3% BSA was added to the circle to evenly cover the tissue, and the tissues were sealed for 30 min at room temperature (the primary antibody was sealed with normal rabbit serum from a goat source while other sources are sealed with BSA). Primary antibody incubation: the sealing solution was gently removed, the primary antibody prepared with PBS (pH 7.4) and then a known proportion was added to the sections and the sections placed flat in a wet box to be incubated overnight at 4°C (a small amount of water was added to the wet box to prevent evaporation of antibodies). Secondary antibody incubation: the sections were placed in PBS (pH 7.4) and washed by shaking on the decolorizing shaker three times for 5 min each. After the sections were slightly dried, the tissues were covered with a secondary antibody (HRP labeled) from the corresponding species of primary antibody and incubated at room temperature for 50 min. DAB chromogenic reaction: the sections were placed in PBS (pH 7.4) and shaken on the decoloring shaker three times for 5 min each. DAB color developing solution, newly prepared, was added to the circle after the sections had slightly dried. The color developing time was controlled under the microscope, until the optimal color of brownish yellow was reached. Subsequently, the sections were rinsed with tap water to stop the reaction continuing. Nucleus counterstaining: the sections were counterstained with hematoxylin stain solution for about 3 min, washed with tap water, differentiated with hematoxylin differentiation solution for several seconds, then washed again with tap water, treated with hematoxylin returning blue solution; and washed once more with tap water. Dehydration and mounting: The sections were placed in 75% alcohol for 5 min, then 85% alcohol for 5 min, absolute ethanolIfor 5 min, anhydrous ethanol IIfor 5 min, n-butanol for 5 min, and then xyleneIfor 5 min. Now dehydrated and transparent, they were then removed from the xylene and allowed to slightly dry, and then mounted with neutral gum. Staining of tissue was visualized using 3D Histech Quant Center 2.1 (3D Histech, Hungary).

The nucleus of hematoxylin stained is blue, and the positive expression of DAB is brownish yellow.

2.7 Statistical Analysis

Statistical analyses were performed using Prism 7 (Graphpad Sofware Inc., La Jolla, CA, United States). Multiple comparison analysis was performed using a two-way ANOVA followed by Tukey's post-hoc correction for multiple comparisons. All experimental data were analyzed using the means or the means ± standard error of mean (S.E.M.). and differences were considered to be significant at $p < 0.05$. Graphics were plotted using the ggplot2 package in R.

3 RESULTS

3.1 Effects of Different Photoperiods on Laying Performance and Follicular Development

The results showed no difference in laying rate, feed-to-egg ratio, and ovarian index in the three groups under the same light conditions (17 L:7D). After experiencing different light conditions, the average cumulative number of eggs laid per duck was 26.0, 22.0, and 19.6 in group L, C, and S, respectively. Prolonged light exposure increased laying performance and reduced light exposure suppressed laying performance, with the highest laying performance exhibited by group L (**Figure 2B**). The trend of feed-to-egg ratio (**Figure 2C**) was opposite to laying performance, with the highest feed-to-egg ratio in group S, which was significantly higher than group C and group L ($p < 0.05$), while group C was significantly higher than group L ($p < 0.05$). Ovarian development results (**Figures 2D–G**) showed that longer light exposure significantly increased the follicular ovarian index ($p < 0.05$) and shortened light exposure significantly decreased the ovarian index ($p < 0.05$). There was no significant difference in the number of LYF (Large Yellow Follicle) in the three groups under different photoperiods. However, the number of SYF (Small Yellow Follicle) and LWF (Large White Follicle) was highest in group L and significantly higher ($p < 0.05$) at d 8, d 20, and d 35 than in group C, and significantly higher ($p < 0.05$) in group C than in group S.

3.2 Serum Hormone Levels of the HPG Axis

Duck serum hormones showed no significant differences ($p > 0.05$) in the GnRH, P4, LH, T, PRL, and E2 levels in serum under the same light condition (17 L:7D; **Figures 3A–F**). After treatment with different light conditions (**Figures 3A–F**), the levels of GnRH, P4, LH, and E2 in serum were significantly increased ($p < 0.05$) and the level of testosterone was significantly decreased ($p < 0.05$) as the duration of light exposure increased. In contrast, the levels of GnRH, P4, LH, E2, and PRL in serum were highly significantly inhibited ($p < 0.05$), while levels of testosterone were highly significantly increased ($p < 0.05$) after the short-day photoperiod treatment.

3.3 OPN5 Expression Levels Under Different Photoperiods

Detection of the expression pattern of hypothalamic OPN5 revealed (**Figures 4A–C**) that OPN5 in mRNA and protein expression were both at stable levels under 17-h stable light

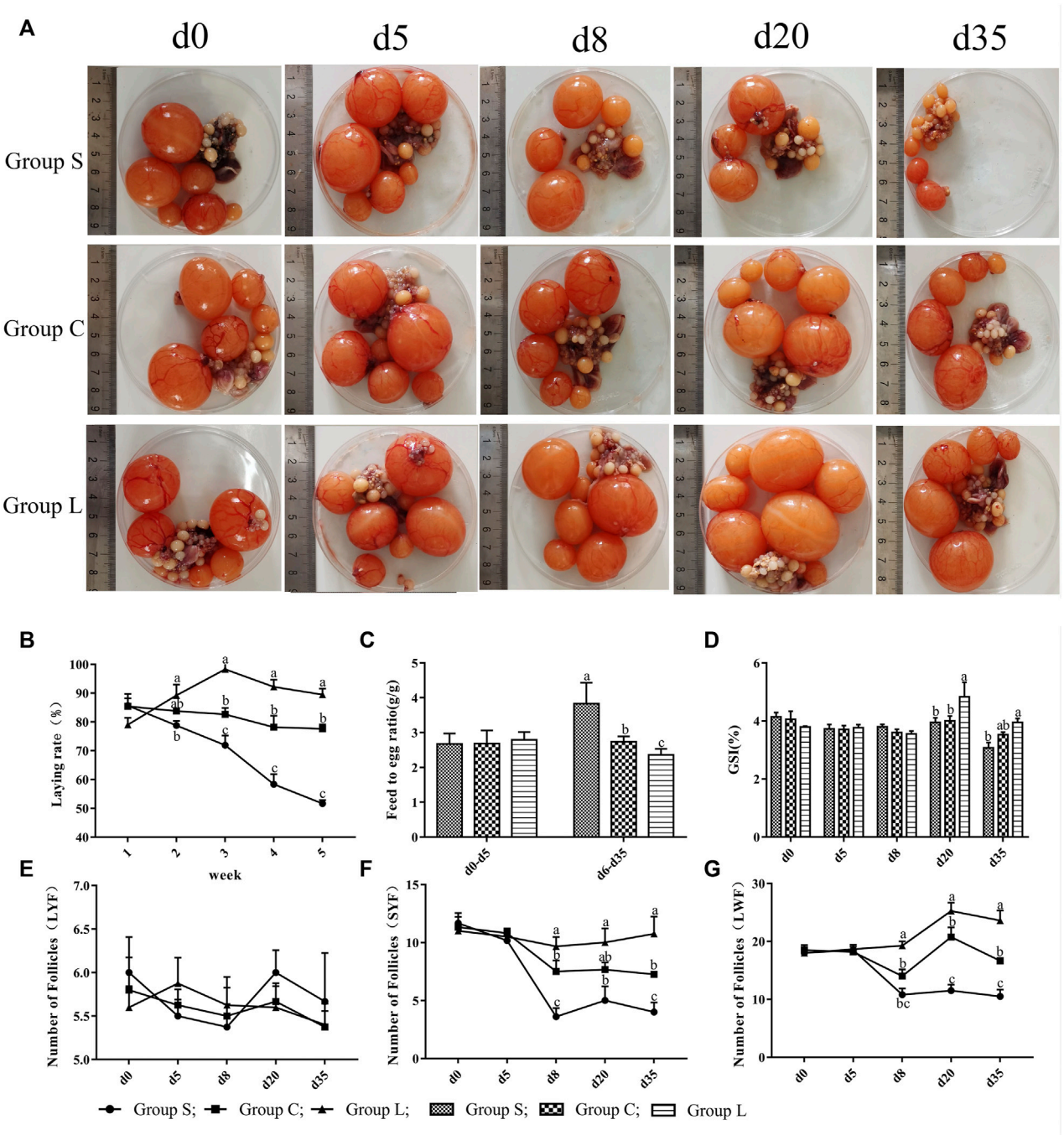

FIGURE 2 | Laying performance and follicular development of mountain ducks under different photoperiods. 1**(A)**, pictures of the follicles of the mountain ducks; 1**(B)**, egg production of mountain ducks; 1**(C)**, feed-to-egg ratio of mountain ducks; 1**(D)**, ovarian index of mountain ducks; 1**(E)**,1**(F)**,1**(G)**, number of follicles of mountain ducks. Consecutive letters **(A,B)** indicate significant differences ($p < 0.05$), and discrete letters **(A,C)** indicate highly significant differences ($p < 0.01$), the same as below.

conditions. Both gene and protein expression of OPN5 changed when light exposure was changed. OPN5 mRNA expression in group L was significantly higher than that in group C at d eight and d 20 ($p < 0.05$), and d 35 expression was reduced compared with d eight and d 20, but still higher than the control level ($p > 0.05$). These results were consistent with the results of western blots (**Figures 4B,C**) and immunohistochemistry (**Figures 4D–H**). The expression of both the OPN5 gene and protein decreased gradually in group S after the short-day photoperiod treatment and was significantly lower than that in group C at d 35 ($p < 0.05$).

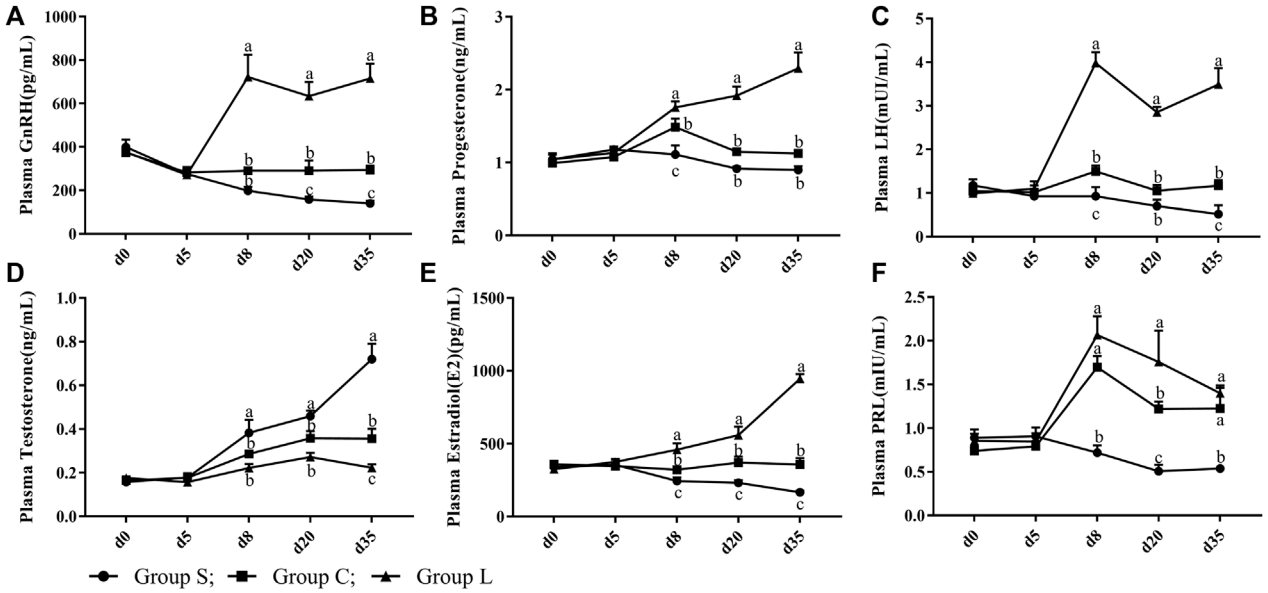

FIGURE 3 | Serum reproductive hormone levels of mountain ducks under different photoperiods. **(A– F)** indicated the levels of GnRH, Progesterone, LH, Testosterone, Estradiol, PRL reproductive hormones, respectively.

3.4 Expression Levels of Genes and Proteins in the TSH-DIO2/DIO3 Pathway

Detection of genes in the TSH-DIO2/DIO3 pathway revealed that the gene expression patterns of *TRH*, *TSHβ*, *TSHR*, *DIO2*, and *THRβ* in the pathway were basically consistent with the expression pattern of *OPN5*, which gradually increased under long-day photoperiod and decreased under short-day photoperiod. While the expression pattern of *DI O 3* was inversely correlated with *OPN5*, *DIO3* in group S was significantly higher than was *DIO3* in group C ($p < 0.05$) (**Figures 5A–F**). Detection of TSH-DIO2/DIO3 pathway proteins revealed that the results of THR and DIO2 western blots (**Figures 5G–I**) and the immunohistochemical results of TSHβ (**Figure 5J–M**) demonstrated an increasing trend under long-day photoperiod and a down-regulation of TSH, THR, and DIO2 protein expression under short-day photoperiod, which were positively correlated with the trend of OPN5 protein expression.

3.5 Expression Levels of the Main Factors of the HPG Axis

The results of the expression of genes related to the HPG axis found that the relative gene expression of *GnRH*, *GnRHR*, *FSH*, *LH*, *VIP*, and *PRL* gradually increased in group L, and the relative gene expression of *GnIH* and *GnIHR* gradually decreased; the opposite of group S (**Figures 6A–H**). This was consistent with the reproductive status of the ducks. Moreover, the gene and protein expression patterns of GnRH were consistent with those of OPN5 and THRβ, and the gene and protein expression patterns of GnIH were opposite to that of OPN5, GnRH, and THRβ. Therefore, this study suggests that OPN5 may affect follicle development by regulating the TSH-DIO2/DIO3 pathway in order to impact the production of key regulators of the avian reproductive axis, including GnRH, GnIH, VIP, and PRL. (**Figure 6**).

3.6 Expression Correlation Analysis Between Positive and Negative Regulatory Factors

At the gene level, OPN5 was positively correlated with GnRH, TRH, TSHβ, THRβ, DIO2, VIP, and PRL, and was negatively correlated with DIO3 and GnIH. The results of QPCR showed that the top seven most positively correlated gene pairs were, in ascending order: OPN5 and TSHβ (r = 0.63), TSHβ and PRL (r = 0.63), TRH and THRβ (r = 0.63), TSHβ and THRβ (r = 0.63), TSHβ and TRH (r = 0.67), VIP and TSHR (r = 0.7), and GnIH and DIO3 (r = 0.83). The top three most negatively correlated pairs of genes were TRH and PRL (r = -0.75), GnIH and THRβ (r = -0.75), and GnIH and TSHβ (r = -0.73). (**Figure 7A**).

At the protein level, OPN5 was positively correlated with GnRH, THR, DIO2, and was negatively correlated with GnIH. Western blot results showed that the three groups of proteins with the highest correlation were GnRH and GnIH (r = -0.85), OPN5 and THR (r = 0.69), and GnRH and THR (r = 0.61). (**Figure 7B**).

4 DISCUSSION

OPN5 is a deep brain photoreceptor that mediates the regulation of light on avian reproductive activity (Kang and

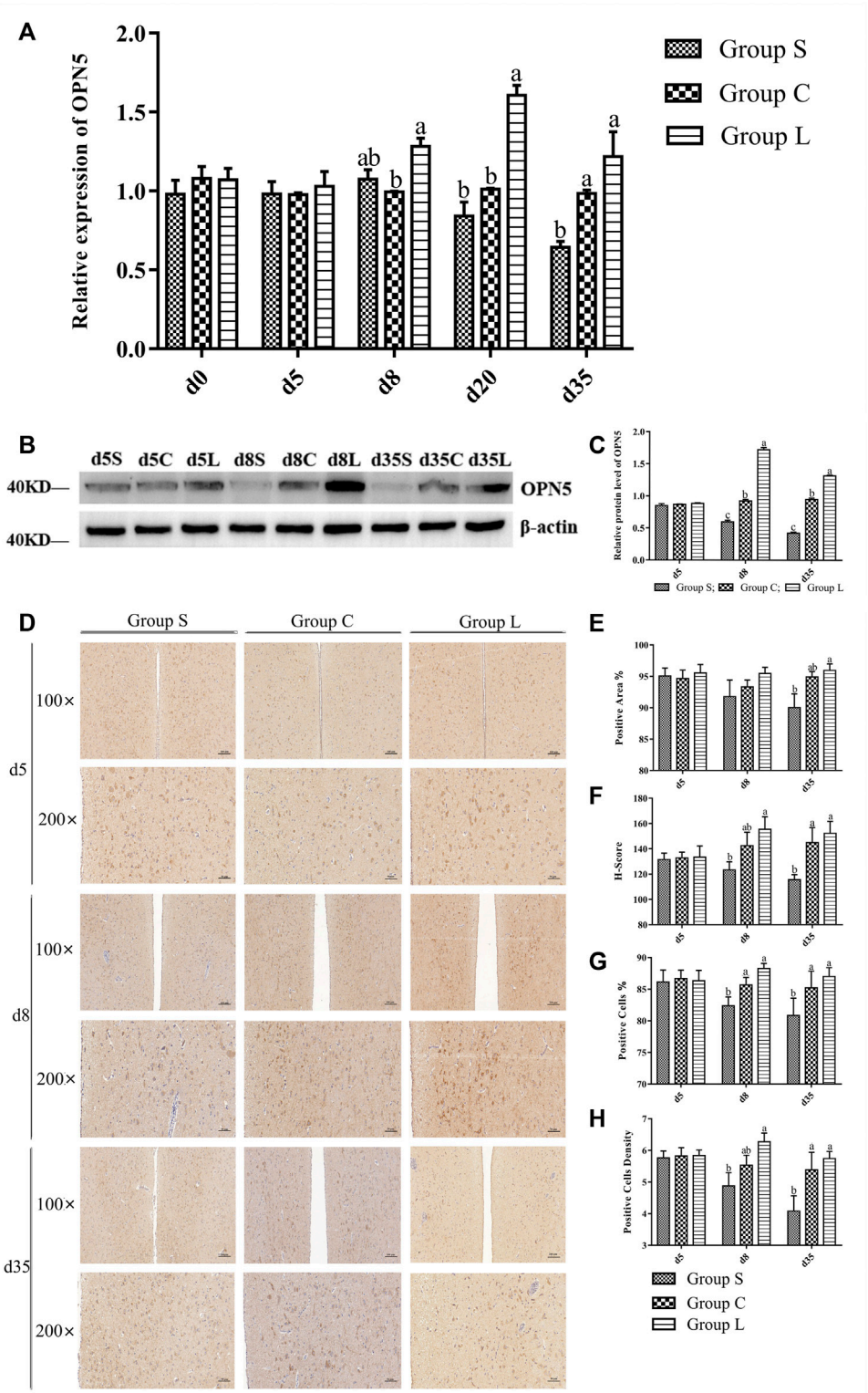

FIGURE 4 | OPN5 expression levels of protein and gene of mountain ducks under different photoperiods. **(A)**, the gene expression of OPN5; **(B)**, Western Blot results of relative protein levels of OPN5; **(C)**, changes in the protein expression of OPN5; **(D)**, the immunohistochemical staining images of OPN5; **(E–H)** were quantitative analysis of the immunohistochemical results of OPN5, indicating the analysis results of positive area, H-Score, positive cells, and positive cells density, respectively

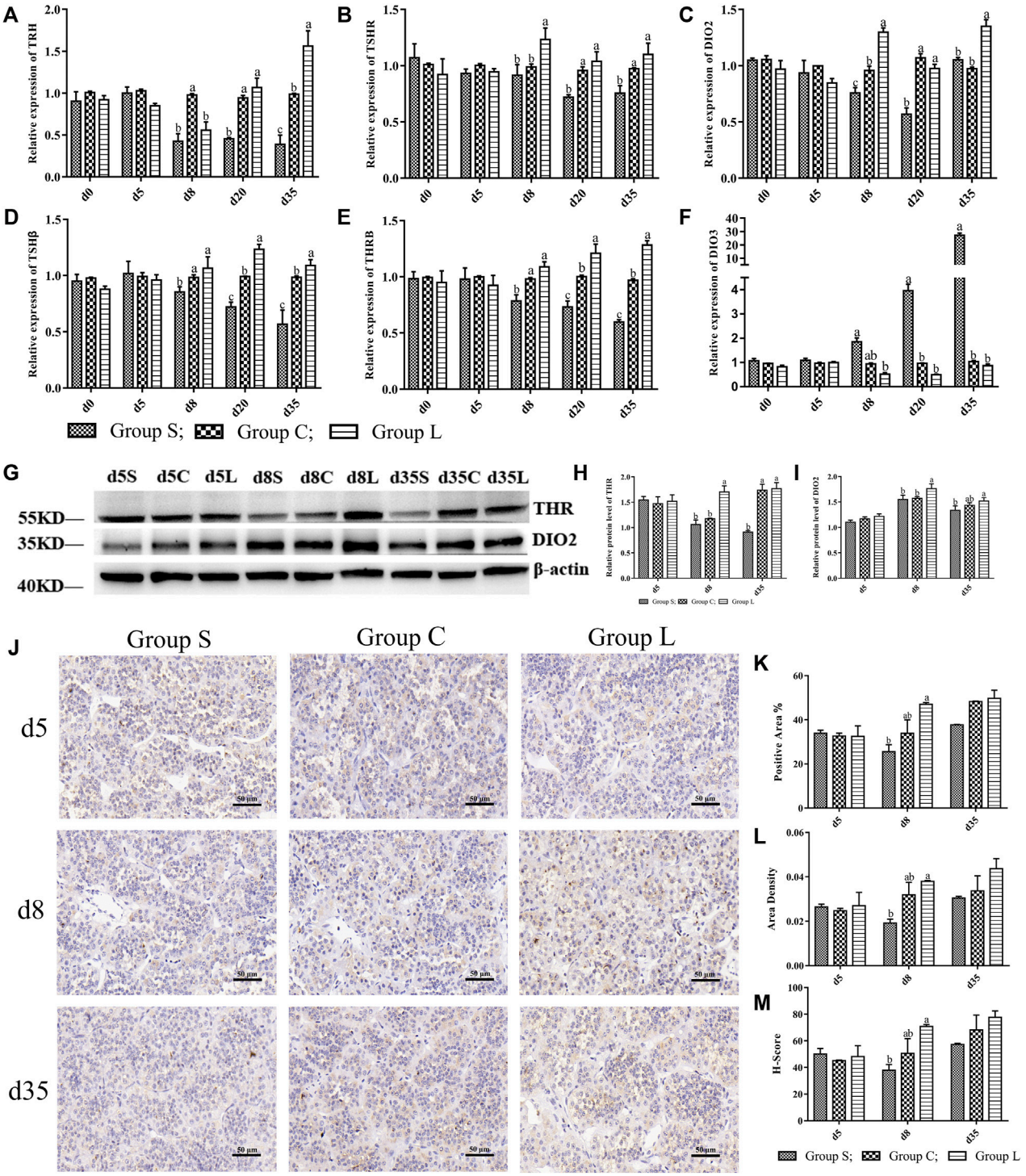

FIGURE 5 | Expression levels of genes and proteins in TSH-DIO2/DIO3 pathway under different photoperiods Expression of genes and proteins related to the TSH-DIO2/DIO3 pathway. **(A–F)** indicated the gene expression levels of TRH, TSHR, DIO2, TSHβ, THRβ, and DIO3, respectively; **(G)**, Western Blot results of relative protein levels of THR and DIO2; **(H)** and **(I)**, changes in the protein expression of THR and DIO2; **(J)**, the results of immunohistochemical staining images of TSH (400 ×); **(K–M)** were the results of TSH immunohistochemical quantification, indicating the results of positive area, Area density, H-Score, respectively.

Kuenzel, 2015; Beaudry et al., 2017). To date, studies on OPN5 have mainly focused on seasonally breeding birds, and a large number of studies have shown that their light perception can regulate the secretion of TSHβ through the mediation of OPN5, which in turn feeds back to the HPG axis to cause the release or inhibition of the corresponding hormones, thus

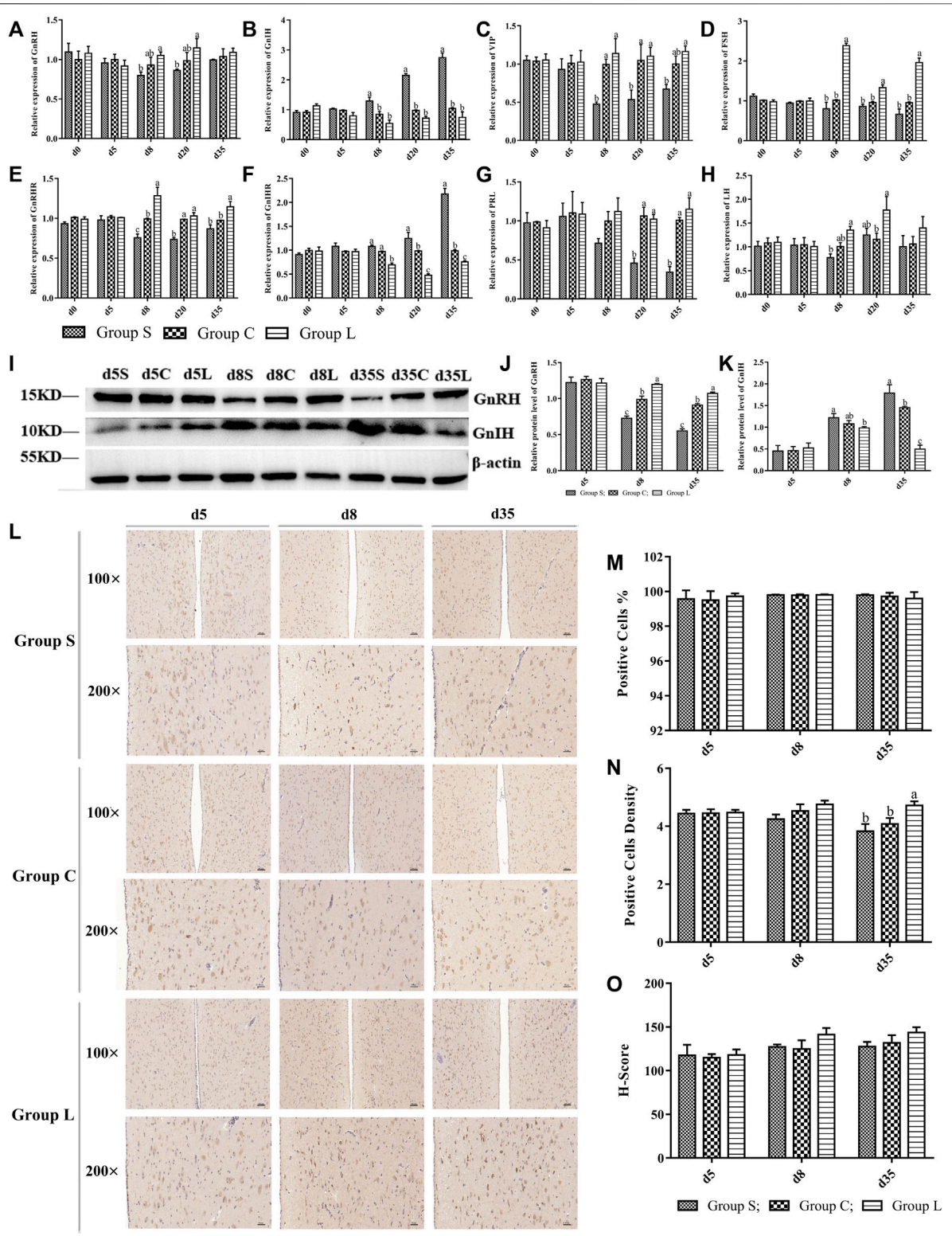

FIGURE 6 | Expression levels of main factors of HPG axis in mountain ducks under different photoperiods. Expression of HPG axis-related genes and proteins. **(A–H)** indicated the expression levels of the genes of GnRH、GnIH、VIP、FSH、GnRHR、GnIHR、PRL and LH, respectively; **(I)**, Western Blot results of relative protein levels of GnRH and GnIH; **(J)** and **(K)**, changes in the protein expression of GnRH and GnIH; **(L)**, the immunohistochemical staining images of GnRH; **(M–O)** were quantitative analyses of the immunohistochemical results of GnRH, indicating positive cells, positive cells density, and H-Score, respectively.

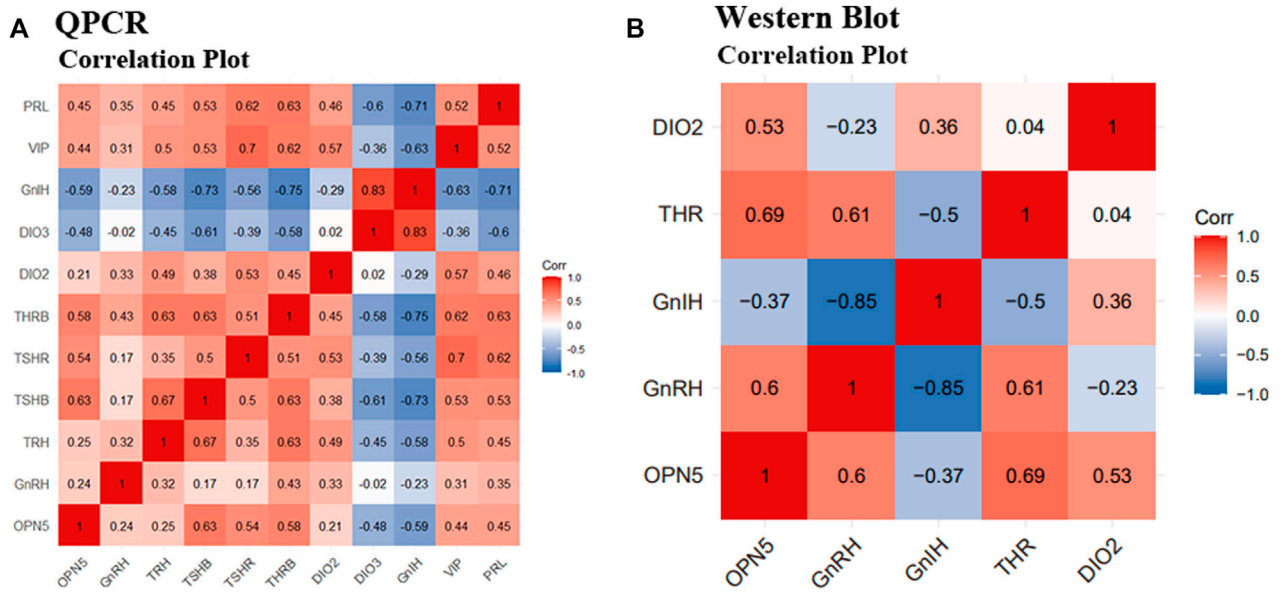

FIGURE 7 | Correlation analysis between positive and negative regulatory factors of reproduction activities under different photoperiods. Correlation analysis of QPCR results and Western Blot results. **(A)** indicates the correlation analysis of the gene results of OPN5, GnRH, TRH, TSHβ, TSHR, THRβ, DIO2, DIO3, GnIH, VIP, and PRL, respectively; **(B)** indicates the correlation analysis of the protein results of OPN5, GnRH, GnIH, THR, and DIO2, respectively.

participating in the regulation of gonadal development and the reproductive regulation process of animals (Zhu et al., 2019a; Zhu et al., 2019b). It is unclear that whether OPN5 regulating follicular development through TSH-DIO2/DIO3 system responds to different photoperiods in non-seasonal laying ducks.

In the present study, we observed that the morphology and function of the three groups of follicles were dramatically changed by altering the photoperiod. Compared to 17 h light time, 24 h light time showed higher egg production and lower egg ratio, while 8 h light time suppressed egg production and increased egg ratio in the mountain ducks. GnRH, P4, E2, LH and PRL levels in serum increased with longer light time, which increased the amount of LYF, SYF and LWF. Our results confirmed previous observations that male Japanese quail transferred to different photoperiods undergo a rapid change in plasma reproductive hormones and gonad weight (Oishi and Konishi, 1978; Wada, 1993; Henare et al., 2011). The results of the study showed that plasma levels of GnRH, E2, P4, LH, PRL and T dramatically changed by altering the photoperiod after 3 d. Compared to the 17 h photoperiod, longer light time promoted follicular development and plasma levels of GnRH, E2, P4, LH and PRL hormone in mountain ducks were significantly increased in the 24 h photoperiod, whereas the 8 h photoperiod inhibited follicular development and promoted plasma levels of testosterone hormone in mountain ducks. Studies have shown that longer light exposure elevates the gene level of GnRH (Zhu et al., 2017), while GnRH promotes the secretion of FSH and LH, and then LH promotes secretion levels of P4 and E2, which ultimately induce gonadal development and enhance reproductive activity (Hanlon et al., 2021). In quail, transfer from short-day photoperiod to long-day photoperiod causes a significant increase in serum LH, FSH and other reproductive hormone levels and gonad weight (Henare et al., 2011). Meanwhile, E2 and P4 are the main indicators of reproduction (Porcu et al., 2018), and LH and PRL are also potential key indicators of reproduction in poultry (Etches RJ Gordon Memorial Lecture, 1998; Tan et al., 2021a; Geng et al., 2022). Elevated plasma levels of PRL and LH in breeding ducks can promote egg production (Cui et al., 2021).

The link between OPN5 and TSH is very strongly in the hypothalamus and pituitary gland (Nakane and Yoshimura, 2014; Kuenzel et al., 2015; Mishra et al., 2017). In terms of the experiment, we investigated the relationship between OPN5 and TSH under different photoperiods. We observed that prolonged light time elevated the expression levels of OPN5 on proteins and genes, and there was a significant positive correlation between OPN5 and TSH under different photoperiods, which was inextricably linked to the expression of reproduction-related genes. In seasonally breeding avian species, seasonal changes in reproductive activity are mainly controlled by light, and different light durations or wavelengths may lead to an increase or decrease in OPN5 expression, which in turn regulates the animal's reproductive activity. Prolonging the light time promotes the expression of OPN5 and TSHβ, enhancing egg production in the Hungarian white geese as well (Kuenzel et al., 2015). The expression of OPN5 is higher under white and red light conditions than under blue and green light conditions, and enhancing the egg production performance in the Yangzhou geese (Zhu et al., 2019b). *OPN5* mRNA expression and testicular development are promoted by prolonging light time in the *Gallus* (Kang and Kuenzel, 2015). In quail, it is found that reducing OPN5

expression significantly suppressed TSHβ expression (Nakane et al., 2014). The results of the present study suggested that OPN5 played an important role in the regulation of avian reproductive activity by mediating photoperiod in non-seasonal breeding avian. OPN5 affects TSH, which activates the DIO2/DIO3 conversion system (Ono et al., 2008), which in turn affects the GnRH/GnIH system and the GnRHR/GnIHR system, altering the expression of related reproductive genes and causing changes in the corresponding reproductive hormones, thus regulating the ovarian functional system (Banerjee and Chaturvedi, 2018). Furthermore, OPN5 has been shown to mediate light signaling and cause a drift in rhythmic phase, which in turn is involved in the regulation of animal biological rhythms and reproductive functions, such as the association of altered sexual behavior and GnRH release (Prasad et al., 2015; Han et al., 2017). Non-seasonal reproductive activity can be driven by light stimulation and is dependent on neuroendocrine regulation, whereby hormones in the HPG axis are altered to initiate and maintain gonadal development (Mauro et al., 1989; El Halawani and Rozenboim, 1993; Dunn et al., 2004; Johnson, 2015). In birds, the activity of the HPG axis is strictly controlled by the level of GnRH. GnRH neurons in the hypothalamus have dynamic morphological plasticity in response to photoperiodic changes that regulate the seasonal secretion of GnRH in the hypothalamus (Jansen et al., 2003; Yamamura et al., 2004; Lehman et al., 2010). Whereas TSH is thought to be a key signaling molecule regulating reproductive seasonality in birds (Nicholls et al., 1988; Yoshimura et al., 2003; Nakao et al., 2008), TSH changes markedly from the nonreproductive active phase to the active phase (Donham, 1979) and T3 controls the seasonal pulse of GnRH release (Hanon et al., 2008; Nakane and Yoshimura, 20102008). The reproductive activity of the HPG axis with the release of GnRH/GnIH and the negative feedback of gonadal reproductive hormones are topical studies (Kriegsfeld et al., 2015).

We found that prolonging light time elevated OPN5 expression levels in genes and proteins, while shortening light time down-regulated OPN5 expression levels in genes and proteins. The expression patterns of *GnRH*, *TRH*, *TSHβ*, *TSHR*, *DIO2*, *THRB*, *VIP* and *PRL* mRNA showed positive correlation with *OPN5* mRNA expression, while the expression patterns of *GnIH* mRNA was inversely correlated with *OPN5* mRNA expression by prolonging light time in mountain ducks. The protein expression of GnRH、TSHβ、DIO2 and THR was positively correlated with that of OPN5, while the protein expression of GnIH was inversely correlated with that of OPN5. These results suggested that OPN5 might affect the reproductive activity of the mountain ducks by modulating theTSH-DIO2/DIO3 pathway and thus the HPG axis. It has been reported that exposing *Gallus* to long-day photoperiod conditions for 3 days results in a significant increase in the number of cells positive for OPN5 in the brain, which is induced to promote *FSHβ* And *TSHβ* mRNA expression, thereby promoting the function of the reproductive system (Kang and Kuenzel, 2015). In quail, knockdown of siRNA-OPN5 can significantly inhibit *TSHβ* mRNA expression in long-day photoperiod (Nakane et al., 2014). In sparrows, prolonged light stimulates the synthesis of TSHβ and promotes the expression of *DIO2* mRNA, and it promotes the secretion of GnRH-I (Anand and Dixit, 2018). In Yangzhou geese, *OPN5* mRNA is upregulated by prolonged light, while the expression of OPN5 and TSH and DIO2 are positively correlated (Zhu et al., 2019b). In Hungarian white geese, prolonged light exposure increases the *OPN5* mRNA expression and *TSHβ* mRNA expression, and contributes to the upregulation of *VIP* and *PRL* gene expression in hypothalamic and pituitary tissues (Zhu et al., 2019a). The results of the present study were consistent with the above results. Meanwhile, network interactions between OPN5 and TSH, GnRH, GnIH, VIP and PRL regulate the reproductive activity of laying ducks.

In addition, PRL plays an important regulatory role in avian reproduction. In the present study, PRL was also significantly increased with long-day photoperiod treatment to promote reproductive performance of the mountain ducks, while under short-day photoperiod, PRL and VIP were significantly decreased, and follicular development and egg laying performance of the mountain ducks were also significantly reduced. Numerous studies have shown that high levels of PRL are key for the development and maintenance of bird nests, and that high levels of PRL could inhibit the secretion of the gonadotropic hormones of GnRH, FSH, and LH in the reproductive axis (Takeshi, 2017). However, it has also been shown that moderate PRL is necessary for follicle development in birds and that too low PRL under short-day photoperiod could inhibit follicle development and laying performance in birds (Li et al., 2020; Tan et al., 2021a; Tan et al., 2021b). Moderately long light exposure will preserve better egg laying performance in breeding birds (Chew et al., 2021; Cui et al., 2021), and reduction of endogenous PRL levels by immunization with PRL and VIP inhibits avian follicle development. When using long-day photoperiod exposure to promote Magang geese from the breeding stage to the resting stage, long-day photoperiod treatment at the early stage could promote PRL secretion while at the same time also promote follicle development and egg laying performance (Huang et al., 2008). These studies suggest that light, along with PRL, are important factors in the regulation of avian reproductive activity. This is consistent with the results of related studies, but why did high levels of PRL not inhibit follicle development under 24-h light conditions? Is it because PRL levels are not high enough to inhibit follicle development in mountain ducks? Does it correlate with the fact that nesting is no longer present in mountain ducks due to their artificial selection? These questions deserve further study. At the same time, what is the relationship between PRL—an important regulator involved in the regulation of follicle development and egg production performance ultimately influenced by light exposure—and the OPN5 and TSH-DIO2/DIO3 pathways in mountain ducks? Again, further studies are needed.

5 CONCLUSION

Long light exposure (17–24 h) promoted the follicular development in the ovary of mountain ducks and improved egg-laying performance. Long-day photoperiod might have affected the expression of OPN5, TSH, DIO2, VIP and PRL, decreased the expression of DIO3, and promoted follicle development. While short-day photoperiod down-regulated the expression of OPN5, TSH, DIO2, VIP and PRL, elevated the expression of DIO3 and inhibited follicle development and egg production performance. The results suggested that OPN5 might affect the expression and secretion of reproduction-related genes in the gonadal axis through the TSH-DIO2/DIO3 pathway under different photoperiods, which in turn regulated follicular development and egg-laying performance in mountain ducks.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Zhongkai University of Agriculture and Engineering (Guangzhou, China).

AUTHOR CONTRIBUTIONS

SL, JP, XS, YT, and YH, were responsible for the project design, sample collection, and data analysis. SL, JP, and JS coordinated the sample collection. SL, JP, XS, and YH revised the manuscript. SL, DJ, HO, and DX coordinated the experimental design. SL, JP, and YH were responsible for the experimental design, data analysis, and manuscript preparation and revision. All authors approved the final manuscript.

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Differences in fear response strategy and stress susceptibility amongst four different commercial layer strains reared cage free

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Different commercial lines of laying hens may show varying levels of fearfulness in response to stressful events or situations. It is important to understand the differences in fear response and stress susceptibility. In this study, four commercial laying hen lines reared from hatch to 32 weeks of age in a cage free system Strains consisted of a brown egg laying line (Hyline Brown; HB) and three white egg laying lines (W36, W80, and LSL). Sixty hens from each strain were used. Each hen was assessed for fearfulness using the following tests: isolation (ISO), emergence (EMG), inversion (INV), and tonic immobility (TI). Stress was assessed based on physical asymmetry (ASYM), corticosterone (CORT) concentrations, and heterophil:lymphocyte ratio (HL). At 3 weeks of age, the W80 birds exhibited more vocalizations during ISO and a shorter duration to emerge than other lines except the HB birds during EMG. Conversely the W36 birds had fewer vocalizations during ISO and emerged quicker than other birds except the LSL during EMG. At 16 weeks of age, the LSL and the W36 bird demonstrated greater fear in TI than the HB. At 30 weeks of age, the observed fear response strategies of each strain changed from previous age and differences were observed between lines ($p < 0.05$). At both 16 and 30 weeks of age the HB birds had the highest ($p < 0.05$) stress indicators (CORT, HL, and ASYM). Furthermore, they had a higher CORT after acute stressor ($p < 0.05$). Commercial lines of laying hens show clear variation in their stress response strategy and stress susceptibility. Brown egg laying hens tend to actively avoid perceived threats whereas white egg laying hens use passive avoidance. Brown egg laying hens also have higher levels in the measures of stress susceptibility than white egg laying hens. Understanding of individual strain response to fearful stimuli and other stressors is important knowledge to appropriately determine welfare differences between strains of layers as the baseline measures are often different.

KEYWORDS

fear, stress, layer, cage free, variation

1 Introduction

One of the primary principles of optimum animal welfare is minimal fear and stress. One way to insure these are minimized is to select the appropriate animals for the housing system that they will be reared in. With the current transition from traditional cage housing towards alternative cage free housing worldwide in the laying hen industry this is becoming a huge concern. Many commercial strains were selected to cope with living in cages and simply switching them to a cage free system may not be optimal for their welfare as doing so could result in excessive fear and stress. As excessive or prolonged fear in animals can result in wasted energy, injuries, behavioral inhibition, reduced ability to adapt to change, delayed maturation, decreased growth and reproduction, and death (Jones, 1996) it should be avoided. Furthermore, fearfulness in laying hens can even result in severe feather pecking behavior (Agnvall et al., 2012; de Hass et al., 2013) and broken keel bones (Harlander-Matauschek et al., 2015) further decreasing their welfare.

Fear responses can be classified as either passive avoidance (freezing, tonic immobility), or active avoidance (withdrawal, fighting, and vigorous escape) (Jones, 1987). The freezing and tonic immobility associated with passive avoidance behavior and the fighting and fleeing associated with active avoidance behavior have been classified as the main four types of anti-predator fear related responses (Ratner, 1967). These anti-predator behaviors have been demonstrated to be the most reliable when evaluating fear (Miller et al., 2006). Previous research has demonstrated fear responses in laying hens have heritability ranging from very low at 0.07 to moderate at 0.49 (Uitdehaag et al., 2011; de Haas et al., 2013; Grams et al., 2015), consequently making fear-related behavioral responses a selectable trait in poultry.

Stress susceptibility is closely related to fear responsiveness, meaning that lower physiological indicators of stress susceptibility are also reliable indicators of overall animal welfare. When a bird becomes stressed, the hypothalamic-pituitary-adrenal axis is activated, ultimately resulting in the secretion of corticosterone (CORT) into the bloodstream (Mormede et al., 2007; Virden and Kidd, 2009). As the primary stress hormone in birds, excessive CORT threatens bird health by suppressing immune responses (Beard and Mitchell, 1987), altering metabolism to increase readily available energy (Mormede et al., 2007), slowing growth rate (McFarlane et al., 1989), and disrupting cecal microflora (Burkholder et al., 2008). Therefore, the most common way to measuring stress is by measures corticosterone levels in the blood (Mormede et al., 2007). Excessive corticosterone concentrations lead to hypotrophy of the lymphoid organs which results in a higher heterophil/lymphocyte ratio, which can be used as an indicator of long-term stress (Gross and Siegel, 1983). Physical asymmetry, which is a simple comparison of the growth of bilateral structures on a bird (Campo et al., 2008), has been strongly correlated to stress susceptibility (Graham et al., 1993; Knierim et al., 2007; Archer and Mench, 2013) and can be used in determining a bird's ability to cope with stressors to that point of life (Kellner and Alford, 2003).

Breed differences in fearfulness and stress susceptibility have been reported in previous research (Albentosa et al., 2003; Welfare Quality, 2009; Abe et al., 2013; Ferrante et al., 2016; Giersberg et al., 2020; Peixoto et al., 2020; Wei et al., 2022). However, Anderson and Jones (2012) observed no differences in tonic immobility among four genetic lines of White Leghorn hens although basal plasma corticosterone concentration differed between lines. Complicating the matter even more, it has been demonstrated that differences may be seen in one fear test but not in others (Albentosa et al., 2003). While Archer (2018) observed differences between different strains of fowl it was not always consistent across type of fear test. Archer (2018) did, however, observe that even within White Leghorns fear responses differed between different genetic lines selected for characteristics not related to fear and stress. Nelson et al. (2020) observed that brown egg layers and white egg layers differed in fear and stress responses over a large variety of measures. They concluded that brown egg laying hens tended to actively and passively avoid predators or threats. It should be noted that many of these previous research projects did not document or did not rear or test the strains in the same environment. Furthermore, many studies tested the birds either when they were housed in cages during production or in mismatch production systems.

The objective of this study was to determine how different commercial layers responded during fear tests and stress measures at different ages while being reared in a cage free system. Based on previous literature it was hypothesized that there would be differences in fear and stress measures between white and brown egg layers and possibly between white leghorn varieties. Evaluation and understanding of these differences will allow for the selection of the optimal commercial birds for different housing systems or at least for producers to be aware of the challenges some strains may have compared to others.

2 Materials and methods

2.1 Animals and husbandry

Sixty birds from each of four different genetic strains—three White Leghorn strains (Hyline W36, Hyline W80, and Lohmann LSL) and one brown layer strain (Hyline Brown)—were used in this study. All birds were grown from day of hatch until 32 weeks of age. All birds were obtained from the same commercial hatchery and were not beak treated. Each strain was reared in one of four floor pens (3.05 × 4.57 m) on wood shaving. All pens were adjacent and all environmental conditions were managed equally according to the guidelines set forth in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) and all procedures were approved by the Texas

A&M university animal care and use committee (IACUC 2017-0259). Nest boxes and perches were provided to all pens. Fear tests and physiological stress was measured at several time points during the 32-week experiment as described below. All birds were tested in all measures collected. Testing occurred in the space between animal home pens.

2.2 Fear measurements

2.2.1 Isolation (ISO)

This test was modified from methods outlined in (Archer and Mench, 2014). The isolation tests were performed at 3 weeks of age. Prior to testing 15 chicks were caught from a strain, placed in a holding area and then tested. This was repeated for each strain and the process was repeated until all birds had been tested. Order of strain was randomized for each testing block. The birds were individually placed in an unlidded 19 L bucket. A timer was set for 3 min, and the number of vocalizations produced by the bird during this time was counted. More vocalizations were considered to indicate more fearfulness (Forkman et al., 2007).

2.2.2 Emergence (EMG)

The emergence test was conducted at 3 weeks of age, modified from methods found in Archer and Mench (2014). A lidded 19 L bucket was modified to have a sliding door in the side, and the person performing the test was seated behind the door and not visible to the bird. A video camera and monitor were used to see when the bird emerged from the container. Prior to testing 15 chicks were caught from a strain, placed in a holding area and then tested. This was repeated for each strain and the process was repeated until all birds had been tested. Order of strain was randomized for each testing block. The birds were individually placed in the bucket with the door and lid closed. After 20 s, the door was slid open and a timer was started. The timer was stopped when the bird first stepped out of the container into the shaving lined testing pen, or at a maximum of 3 min. Longer latency to emerge was considered to indicate more fearfulness (Archer and Mench, 2014).

2.2.3 Tonic immobility (TI)

The test was carried out on all birds as described by Archer and Mench (2014) at 16 and 30 weeks of age. Prior to testing 15 chicks were caught from a strain, placed in a holding area and then tested. This was repeated for each strain and the process was repeated until all birds had been tested. Order of strain was randomized for each testing block. Each block was tested on 1 day at the same time of day for each testing period. In brief, each bird was placed in a u-shaped cradle on its back and head covered for 10 s then released and time to first head movement were recorded by an observer that sat 1 m away. If the bird could not be induced in three attempts it was scored as zero. Latency to first head movement, latency to right and number of induction attempts was recorded. The test was terminated in 600 s if a bird failed to right, and that bird was scored as 600. Longer latency to right indicated greater level of fear.

2.2.4 Inversion (INV)

At 16 and 30 weeks of age all birds were also subjected to (INV), as described by Newberry and Blair (1993) and Archer and Mench (2014). Prior to testing 15 chicks were caught from a strain, placed in a holding area and then tested. This was repeated for each strain and the process was repeated until all birds had been tested. Order of strain was randomized for each testing block. Each block was tested on 1 day at the same time of day for each testing period. Each bird was removed from its crate and then inverted by its legs using one hand until the bird ceased to flap its wings, or for a maximum of 30 s. Flapping intensity was determined using the number and duration of wing flaps, and a higher intensity indicated greater level of fear.

2.3 Stress measures

2.3.1 Plasma corticosterone (CORT)

Basal corticosterone was determined at both 16 and 31 weeks of age. Acute stress response was determined at 30 weeks of age. All blood samples were taken between 0800-1,000. To do this all birds were placed in transport crates in groups of 20 for 1 h then blood was collected procedures described by Archer and Mench (2013). At each time point approximately 1–2 ml of blood was collected from each bird *via* the wing vein within 1 min of being caught from home pen. Plasma was then stored and analyzed as described in Nelson et al. (2018). Total plasma corticosterone concentrations were determined using a 96-well commercial ELISA kit (ADI-901-097, Enzo Life Sciences, Inc., Farmingdale, NY). Absorbance was read at 450 nm using a microplate absorbance reader (Tecan Sunrise, Tecan Trading AG, Switzerland). Intra and inter plate %CV was less that 5%.

2.3.2 Heterophil to lymphocyte ratio (HL)

The heterophil to lymphocyte ratio was conducted using the method described in Nelson et al. (2018) at 16 and 30 weeks of age. Dry blood smear slides were stained with a neat stain hematology stain kit (Cat. #25034, Poly Sciences, Inc., Warrington, PA), and used to determine H/L ratio at 40x magnification using an oil immersion lens under microscopy (89404-886, VWR International, Radnor, PA).

2.3.3 Physical asymmetry (ASYM)

Physical asymmetry score for all birds was assessed following the protocol outlined in Archer and Mench (2013) at 16 and 30 weeks of age. Using a calibrated Craftsman IP54 Digital Caliper (Sears Holdings, Hoffman Estates, IL), the middle toe length, metatarsal length, and metatarsal width were measured for both the right and left legs. Composite asymmetry score was

calculated by taking the sum of the absolute value of left minus right of each trait, then dividing by the total number of traits. Thus, the formula for this trial would be: $\textbf{\textit{Composite Asymmetry Score}} = (|L - R| |MTL + |L - R|ML + |L - R|MW) \div 3$

2.4 Statistical analysis

All data was analyzed using the GLM procedure as used with strain, age, and strain × age interaction as the model apart from ISO, EMRG, and acute stress CORT data which were analyzed using ANOVA for strain effects only. The assumptions for ANOVA were tested using Shapiro-Wilk test for normality and Levene's test for homogeneity of variance. All analyses were performed using SAS 9.3 for Windows (SAS Institute Inc., Cary, NC). Data that did not meet the assumptions for ANOVA was analyzed using the Kruskal–Wallis test on the equality of the medians, adjusted for ties. When significant differences were found, the Dwass-Steel-Critchlow-Fligner method (Hollander and East, 1999) was used to test for all possible comparisons. Significant differences were defined as $p < 0.05$.

3 Results

3.1 Fear measures

As shown in Table 1, differences among strains were observed for all measures of fear. At 3 weeks of age, the W80 birds vocalized more (59.00 ± 7.94) than all other lines of layers ($p < 0.05$) and the W36 birds vocalized the least (12.25 ± 3.39) compared to all other lines ($p < 0.05$) during ISO. The LSL (37.28 ± 6.58) and HB (31.27 ± 4.40) birds were intermediate. Both the W80 (15.37 ± 3.98 s) and the HB (13.65 ± 2.55 s) birds emerged faster ($p < 0.05$) during the EMG than the W36 (40.67 ± 6.83 s) and LSL (39.12 ± 7.29 s) birds.

There was a strain effect observed in latency to first head movement during TI ($p = 0.01$). The HB strain had the shortest time to first head movement with the LSL having the longest latency and the other two strains being intermediate. No effect of age nor an interaction was observed in latency to first head movement during TI ($p > 0.05$). No overall difference was observed between strains ($p = 0.20$) or age ($p = 0.35$) in latency to right during TI. However, there was an interaction observed in latency to right ($p < 0.001$). No overall strain effect was observed in the difference in time from first head movement to righting ($p = 0.33$). An age ($p = 0.002$) and interaction ($p = 0.001$) effect was observed in the difference in time from first head movement to righting. No differences were observed between strains, age nor interaction of the two in number of attempts to induce TI ($p > 0.05$).

There was no strain effect observed in number of flaps during INV ($p = 0.19$). There was an age effect ($p < 0.001$) and interaction ($p < 0.001$) in number of flaps during INV. More flaps during INV were observed at 16 weeks than at 30 weeks. There was no strain ($p = 0.63$) or interaction ($p = 0.24$) effect observed in duration of flapping during INV. There was an effect of age on duration of flapping during INV ($p = 0.01$) with durations being longer at 16 weeks of age compared to 30 weeks. There was an effect of strain ($p < 0.001$), age ($p < 0.001$) and an interaction ($p < 0.001$) on intensity of flapping during INV. The HB strain had greater flapping intensity during INV than all other treatments. Intensity was observed to greater at 16 weeks compared to 30 weeks.

At 16 weeks of age, the HB had the shortest latency to first head movement during TI (5.80 ± 2.80 s, $p < 0.05$) compared to all other lines. The LSL had the longest latency to first head movement during TI (108.60 ± 16.10 s, $p < 0.05$) compared to all other lines ($p < 0.05$). W36 and W80 were intermediate in latency to first head movement during TI. The W80 (240.8 ± 24.4 s) and HB (176.0 ± 22.3 s) birds had shorter latency to right during TI compared to both the W36 and LSL birds (333.3 ± 26.4 s and 318.5 ± 26.2 s, respectively, $p < 0.05$). The W36 birds had a greater difference from first head movement to righting than all other lines (296.2 ± 27.8 s, $p < 0.05$). No differences were observed in number of attempts to induce TI ($p > 0.05$). The W36 birds flapped more (69.53 ± 4.66, $p < 0.05$) than the W80 and LSL birds during INV (55.80 ± 2.74and 56.18 ± 3.17, respectively) with the HB birds (61.93 ± 2.18) being intermediate. No differences were observed in duration of flapping during INV ($p > 0.05$). The W36 and HB birds flapped more intensely (6.14 ± 0.12 flaps/sec and 6.15 ± 0.09 flaps/sec, respectively, $p < 0.05$) than the W80 and LSL birds (5.56 ± 0.13 flaps/sec and 5.60 ± 0.31 flaps/sec, respectively) during INV.

At 30 weeks of age, the W36 had a longer latency to first head movement during TI (14.23 ± 4.12 s, $p < 0.05$) than the HB birds (4.22 ± 1.82 s) with the W80 and LSL being intermediate. No differences in time to right were observed at this time point ($p > 0.05$). The HB birds had a greater difference from first head movement to righting (332.3 ± 29.9 s, $p < 0.05$) than W36 birds (248.4 ± 26.1 s) with the W80 and LSL birds being intermediate. No differences in attempts to induce TI were observed ($p > 0.05$). The LSL and HB birds flapped more (55.30 ± 3.47 and 56.97 ± 2.60, respectively, $p < 0.05$) than the W36 birds (41.50 ± 4.12) during INV with the W80 being intermediate. No differences were observed in duration of flapping during INV ($p > 0.05$). All lines differed from each other in intensity of flapping during INV ($p < 0.05$) with the order going from least to most intense as follows: W36, W80, LSL, HB.

Additionally, to the treatment differences observed within the age timepoints there were some differences within strains at the two ages. The HB strain increased in righting time during TI

TABLE 1 Fear response of four strains of commercial layers (W36, W80, LSL, and Hy-Line Brown (HB)) at 3, 16, and 30 weeks of age.

Test	Time point	Measurement	W36	W80	LSL	HB	SEM
Isolation	3 weeks of age	Vocalizations	12.25^{c}	59.00^{a}	37.28^{b}	31.27^{b}	3.10
Emergence	3 weeks of age	Time (sec)	40.67^{a}	15.37^{b}	39.12^{a}	13.65^{b}	2.87
Tonic Immobility	16 weeks of age	Head (sec)	37.10^{b}	37.00^{b}	108.60^{a}	5.80^{c}	6.12
		Right (sec)	333.3^{a}	240.8^{b}	318.6^{a}	176.0^{b}	13.0
		Difference (sec)	296.2^{a}	203.8^{b}	210.0^{b}	170.2^{b}	12.2
		Attempts	1.15	1.17	1.12	1.33	0.03
	30 weeks of age	Head (sec)	14.23^{a}	6.97^{ab}	8.32^{ab}	4.22^{b}	1.50
		Right (sec)	262.6	270.9	264.0	336.5	13.9
		Difference (sec)	248.4^{b}	263.9^{ab}	255.7^{ab}	332.3^{a}	13.9
		Attempts	1.15	1.30	1.13	1.28	0.03
Inversion	16 weeks of age	Flaps	69.53^{a}	55.80^{b}	56.18^{b}	61.93^{ab}	1.67
		Time (sec)	11.08	9.98	10.57	10.15	0.26
		Intensity (flaps/sec)	6.14^{a}	5.56^{b}	5.60^{b}	6.15^{a}	0.09
	30 weeks of age	Flaps	41.50^{b}	48.15^{ab}	55.30^{a}	56.97^{a}	1.79
		Time (sec)	8.53	9.57	10.17	9.12	0.33
		Intensity (flaps/sec)	4.45^{days}	5.10^{c}	5.67^{b}	6.28^{a}	0.10

Different superscripts within row indicate differences between layer lines ($p < 0.05$).

from 16 to 30 weeks of age, while the other strains showed no differences between ages. The difference between first head movement and righting time was greater in HB birds at 30 weeks compared to 16 weeks as well while the W36 strain had the opposite trend, and the other two strains did not differ between time points. Only the W36 strain demonstrated an increase in flapping number from 16 to 30 weeks of age, all others were equal. Both the W36 and W80 strains demonstrated an increase in flapping intensity from 16 to 30 weeks of age, the other two strains were equal.

3.2 Stress measures

Data for plasma CORT, HL and ASYM, is shown in Table 2. There was no age effect on corticosterone concentrations over all ($p = 0.337$). An effect of strain was observed with HB having higher overall CORT concentrations than all other strains ($p < 0.001$). There was also an interaction effect observed between strain and age ($p = 0.008$). There was an effect of strain ($p < 0.001$) on HL with the HB strain having the highest HL and the having the W80 strain having the lowest HL and the other two strains being intermediate. There was also an effect of age on HL with the 30 week sampling having higher ($p < 0.001$) HL than the 16 week sampling. An interaction of strain and age was also observed in HL ($p < 0.001$). There was an effect of strain on ASYM ($p = 0.048$) with the HB strain having higher scores than the W80 and the LSL strains and the W36s being intermediate. There was no effect or age nor an interaction effect on ASYM ($p > 0.05$).

At 16 weeks of age, the HB birds had the highest plasma CORT concentrations (23,340 ± 1924 pg/dl) compared to all other lines (average 12,695 ± 2,177 pg/dl, $p < 0.05$). The W80 had the lowest HL ratio (0.062 ± 0.011) compared to W36 (0.121 ± 0.019, $p = 0.02$) and HB birds (0.178 ± 0.020, $p = 0.001$) with the LSL (0.108 ± 0.020) being intermediate. The HB birds also had the highest composite ASYM (2.071 ± 0.450) compared to both the W80 (1.353 ± 0.144, $p = 0.04$) and LSL (1.196 ± 0.096, $p = 0.01$) with the W36 (1.533 ± 0.142) being intermediate.

At 30 weeks of age, the HB (17,509 ± 1,216 pg/dl, $p = 0.03$) and LSL birds (17,508 ± 980 pg/dl, $p = 0.03$) had higher plasma CORT compared to W80 birds (14,092 ± 1,279 pg/dl) with the W36 birds (16,903 ± 696 pg/dl) being intermediate. The HB birds had the highest HL ratio compared to all other lines ($p < 0.05$). No differences in composite ASYM were observed at this time point between any of the layer lines.

The HB birds CORT concentrations decreased from the 16 week sampling when compared to the 30 week sampling.

TABLE 2 Stress susceptibility measures of four commercial strains of layer chickens (W36, W80, LSL, and Hy-Line Brown (HB)) at 16 and 30 weeks of age and after an acute stress test.

Measure	Time point	W36	W80	LSL	HB	SEM
Plasma Corticosterone (ng/dl)	16 weeks of age	11.65^{b}	11.72^{b}	14.71^{b}	23.34^{a}	1.17
	30 weeks of age	16.90^{ab}	14.09^{b}	17.51^{a}	17.51^{a}	0.55
	31 weeks of age acute stressor	16.67^{b}	16.09^{b}	21.36^{a}	19.06^{a}	0.77
Heterophil: Lymphocyte Ratio	16 weeks of age	0.121^{b}	0.062^{c}	0.108^{bc}	0.178^{a}	0.001
	30 weeks of age	0.318^{b}	0.194^{b}	0.272^{b}	0.588^{a}	0.028
Composite Asymmetry Score	16 weeks of age	1.533^{ab}	1.353^{b}	1.196^{b}	2.071^{a}	0.126
	30 weeks of age	1.468	1.474	1.394	1.636	0.079

Different superscripts within row indicate differences between layer lines ($p < 0.05$).

While the W36 birds CORT concentrations increased from 16 to 30 weeks and the other two strains had no differences between time points. All strains had an increase in HL from 16 to 30 weeks of age. No difference was observed in ASYM from 16 to 30 weeks of age.

Following the acute stressor at 31 weeks of age, the LSL birds had higher plasma CORT concentrations (21,358 ± 1,367 pg/dl) than both the W36 (16,668 ± 1,254 pg/dl, $p = 0.03$) and W80 birds (16,092 ± 1982 pg/dl, $p = 0.02$) with the HB birds (19,059 ± 1,226 pg/dl) being intermediate.

4 Discussion

All layer lines in this current study were reared from day of hatch until the end of the study in a cage free system. Not only did different lines show differing levels of fear during the fear tests in the current study, they also exhibited what could be concluded as differing fear response personality strategies. Jones et al. (1987) stated that fear responses could be classified as either passive avoidance (freezing, tonic immobility), or active avoidance (withdrawal, fighting, and vigorous escape). Furthermore, fear has been suggested as a personality trait in a variety of animal species (Gosling, 2001). At 3 weeks of age, W80 birds exhibited more vocalizations during ISO and a shorter duration to emerge than other lines except the HB birds during EMG. Conversely, the W36 birds had fewer vocalizations during ISO and emerged quicker than other birds except LSL birds during EMG. These results indicate that W80 birds at this age were more active avoiders while the W36 were more passive avoiders. The W80 birds by vocalizing and not freezing demonstrated that they were actively avoiding. By not vocalizing or freezing longer, the W36s demonstrated a more passive avoidance fear response. The LSL birds, while not as evident as the W36 birds, also took more of passive response while the HB birds took a more active response at this age. At 16 weeks of age the results of this current study indicated similar trends in fear response strategies for each line of hen. The LSL birds at this age demonstrated a more passive avoidance strategy in both TI and INV than other lines. The W36 birds had a more mixed fear response with more active avoidance during INV but more passive avoidance during TI. The W80 birds were more intermediate, not appearing to be more active or passive than other lines. The HB birds at this age were generally more active avoiders than other strains. At 30 weeks of age, the observed strategies of each strain when compared to the others changed from previous ages for most of lines. The W36 birds remained somewhat mixed in their fear strategy response. At this age, W36 birds did not flap the most as they did at 16 weeks of age, but they still flapped the most intensely. Similarly, W36 birds at 30 weeks of age had the shortest difference from first head movement until righting rather than the shortest difference for this measure. The W80 birds remained intermediate with their fear response strategy, while LSL birds shifted to a more active strategy at 30 weeks as indicated by more flapping and increased flapping intensity compared to other lines in INV.

Similar to this current study Nelson et al. (2020) observed that brown egg laying lines demonstrated more active fear strategies than white egg laying lines. In the current study birds were housed cage free while in Nelson et al. (2020) birds were housed in cages, making the consistent results found in each in fear strategies profound. Generally, the brown egg layers were active fear responders in both studies demonstrating possibly that system does not affect their strategy. While white egg layers may be affected by their rearing environment as the birds in this current study did exhibit some more active strategies, although further investigation is required. In the current study, W36 and HB birds flapped more

intensely than W80 and LSL birds at 16 weeks of age; however, at 30 weeks of age, W36 birds flapped the least intensely of all the strains while HB flapped the most intensely of all strains. The results for W36 birds coincide with previous results by Albentosa et al. (2003), which reported a reduction in fearfulness between 4 and 12 weeks of age for various strains of laying hens. Although the fear responses for the HB birds in this current study do not follow the results of Albentosa et al. (2003), it does support the idea that strains differ in their responsiveness. As demonstrated in previous research (Albentosa et al., 2003; Welfare Quality, 2009; Abe et al., 2013; Ferrante et al., 2016; Archer, 2018; Nelson et al., 2020) even layer hen lines within a breed like white leghorns can differ in their fear response.

At both 16 and 30 weeks of age the HB birds had the highest stress indicators (CORT, HL, and ASYM). Furthermore, they had a higher CORT after acute stressor. The white egg laying hens didn't differ from each other in stress measures generally with the exception of the W80 birds having the lowest HL at 16 weeks of age of all the strains in this current study and the LSL birds having higher CORT at 30 weeks of age and after acute stressor than the other two white egg layer strains. These results are consistent with results observed by Nelson et al. (2020) where brown egg layers had higher stress indicators than white egg layers. These results could mean that brown egg layers either have higher stress susceptibility or that their basal levels of corticosterone are higher than white egg layers. Both conclusions could greatly impact the selection of what line to house in a certain system or even how comparing strains is possible. Determining which is the case requires future research. Though it has previously been demonstrated that different strains chickens have different basal corticosterone concentrations (Decuypere et al., 1989) therefore comparing direct CORT concentrations between strains may not be as useful as the change in response to stressors. Pusch et al. (2018) found some contradictory findings to this current study. However, their study birds were housed individually in cages prior to and during testing which likely greatly affected the birds' responses. Furthermore, the stress measures seem to be more stable over time than fear responsiveness which may indicate as the birds age and experience more they may change their fear response strategy. It has also been demonstrated that environmental effects like lighting or maternal stress (Peixoto et al., 2020; Peixoto et al., 2021) can cause differential effects in fear and response of offspring of those birds in different layer strains.

Selecting the appropriate animals for specific housing types is paramount to ensuring optimal welfare. Fear and distress are two major factors that can be detrimental to animal welfare. Fearful animals tend to be less productive (Lyons, 1989; Hemsworth et al., 1990; Hemsworth et al., 1994; Voisinet et al., 1997; Minvielle et al., 2002) in addition to having compromised welfare. Both genetics (Craig et al., 1983; Jones et al., 1991; Mills and Faure, 2000) and developmental experiences appear to determine the propensity for fearfulness within individual birds (Boissy, 1995). Fear and stress are not synonymous; however, fear encompasses many biological systems that mediate the physiological stress response in poultry (Jones, 1986). Prolonged, severe stress causes significant biological damage *via* a cascade of behavioral, physiological, and immunological actions that divert energy away from normal biological functions (Cockrem, 2007; Lambert, 2009).

Often poultry and other livestock are selected for production characteristics without any emphasis or consideration of behavioral characteristics. Although modern domestic flocks do not experience predation from the same threats their wild predecessors, the innate emotion of fear still persists in poultry, and are often redirected from classic predators, such as hawks, to human handlers and environmental changes (Boissy, 1995). Selective breeding of less fearful individuals has been suggested as a means for improving both animal welfare and, in turn, productivity (Fordyce et al., 1988; Hemsworth et al., 1990; Mills and Faure, 1990; Manteca and Deag, 1993; Jones and Hocking, 1999). Historically, white egg laying lines have been housed in cages and brown egg layers in cage free housing. However, currently there is pressure to transition to predominately cage free housing, making it important to understand the differences in fear responsiveness of various layer lines in order to select the best lines to rear in each setting and also optimize the management of all layer lines.

5 Conclusion

In conclusion, this study confirmed that different lines of laying hens have varied fear responses and stress susceptibly. With the shift of the predominant commercial housing system to cage free it will become more important to understand how existing and future commercial lines of laying hen respond to fearful situations and to stressors. Selecting for these parameters in addition to production parameters will be paramount to ensure optimal bird welfare. Furthermore, the current study demonstrated that while stress and fear measures are useful tools for welfare assessment, a comparison of birds across genetic strains may be confounded by differences in CORT basal concentrations or innate fear response strategy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Texas A&M IACUC.

Author contributions

GA collected all the data, performed the analyses, and drafted the manuscript. GA, AB, ES, and JN contributed to the chicken raising, sample and data collection. GA and AB designed the research and contributed to data collection, data analysis and interpretation, and revise the manuscript. All authors submitted comments on the draft and approved the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chronic dietary exposure to a glyphosate-based herbicide results in total or partial reversibility of plasma oxidative stress, cecal microbiota abundance and short-chain fatty acid composition in broiler hens

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Glyphosate-based herbicides (GBHs) are massively used in agriculture. However, few studies have investigated the effects of glyphosate-based herbicides on avian species although they are largely exposed via their food. Here, we investigated the potential reversibility of the effects of chronic dietary exposure to glyphosate-based herbicides in broiler hens. For 42 days, we exposed 32-week-old hens to glyphosate-based herbicides via their food (47 mg/kg/day glyphosate equivalent, glyphosate-based herbicides, n = 75) corresponding to half glyphosate's no-observed-adverse-effect-level in birds. We compared their performance to that of 75 control animals (CT). Both groups (glyphosate-based herbicides and control animals) were then fed for 28 additional days without glyphosate-based herbicides exposure (Ex-glyphosate-based herbicides and Ex-control animals). Glyphosate-based herbicides temporarily increased the plasma glyphosate and AMPA (aminomethylphosphonic acid) concentrations. Glyphosate and aminomethylphosphonic acid mostly accumulated in the liver and to a lesser extent in the leg muscle and abdominal adipose tissue. Glyphosate-based herbicides also temporarily increased the gizzard weight and plasma oxidative stress monitored by TBARS (thiobarbituric acid reactive substances). Glyphosate-based herbicides temporarily decreased the cecal

concentrations of propionate, isobutyrate and propionate but acetate and valerate were durably reduced. The cecal microbiome was also durably affected since glyphosate-based herbicides inhibited *Barnesiella* and favored *Alloprevotella*. Body weight, fattening, food intake and feeding behavior as well as plasma lipid and uric acid were unaffected by glyphosate-based herbicides. Taken together, our results show possible disturbances of the cecal microbiota associated with plasma oxidative stress and accumulation of glyphosate in metabolic tissues in response to dietary glyphosate-based herbicides exposure in broiler hens. Luckily, glyphosate-based herbicides at this concentration does not hamper growth and most of the effects on the phenotypes are reversible.

KEYWORDS

birds, glyphosate, metabolism, cecal microbiome, oxidative stress

Introduction

Glyphosate (Gly) is the most widely used herbicide in agriculture worldwide. It is a broad-spectrum herbicide with a generalized effect on all types of crops. Since it is a non-selective product, its commercialization is often coupled with that of genetically modified crops designed to resist the action of the herbicide, enabling farmers to spread increasingly large amounts on their fields without destroying their own crops (Martins-Gomes et al., 2022), while wild plants start to develop natural resistances to it. In animals and plants, Gly is metabolized into CO_2 and aminomethylphosphonic acid (AMPA) by the enzyme glyphosate oxidoreductase (Mesnage et al., 2015). Gly's herbicidal effect is due to disruption of the shikimate pathway, which produces aromatic amino acids in plants and in some microorganisms (Schönbrunn et al., 2001). Since humans and animals do not use the shikimate pathway to produce amino acids, Gly is not supposed to have any adverse effect on their health. However, several studies have shown that glyphosate-based herbicide (GBH) formulations can induce tissue damage (Jasper et al., 2012; Larsen et al., 2014), act as endocrine disruptors in various models (Walsh et al., 2000; Romano et al., 2010; Gill et al., 2018), and induce developmental issues in rats brain (Cattani et al., 2021). The use of GBHs is therefore very controversial, since scientific organizations have drawn contrasting conclusions about its dangerousness and recent studies have shown that populations are widely exposed to it (Grau et al., 2022).

Beside their controversial hypothetic effects on human health, GBHs are suspected to have ecotoxicological effects and to be part of the numerous factors leading to the current biodiversity crash. The fate of bird populations is of particular concern. European wild birds are going through a massive decline (Inger et al., 2015) and pesticides (with Gly as the prime representative) are suspected to be major actors of this loss. Gly residues are detectable in soil, water and food (Gill et al., 2018; Fogliatto et al., 2020) which allows them to threaten non-target species. Moreover, it is now established that, like for many pesticides, GBH's toxicity is enhanced by (if not conditioned to) the presence of other components such as coformulants in herbicide formulations (Bradberry et al., 2004; Kim et al., 2013; Mesnage et al., 2019). Some of them are designed to reduce leaf surface tension and thus to allow penetration of the water-soluble Gly into the plant system first, and then into its cell's membranes (Mesnage et al., 2019). It is therefore more relevant to study the toxicity of GBHs, rather than that of Gly alone. Few studies of this type have been conducted on poultry, while these animals are frequently exposed to GBHs through their diet. Most studies indeed show that GBHs administered in good farming practice conditions do not have any adverse effect on birds themselves, but rather on their foods and habitats (Gill et al., 2018). However, a recent study shows that GBHs can decrease liver catalase activity and reduce testosterone levels in Japanese quail. The gut microbiome is also disrupted, with a possible suppression of beneficial microorganisms (Ruuskanen et al., 2020). The exposure doses used in this study (12–20 mg Gly/kg body weight/day) being at least five times lower than the NOAEL (no-observed-adverse-effect level; 100 mg Gly/kg body weight/day) reported by the European Food Safety Authority (EFSA), more investigations are needed to characterize the impact of Gly and GBHs on avian models. The host gut microbiome is involved in many processes other than digestion such as xenobiotic detoxication, immune system homeostasis and vitamin synthesis (Samsel and Seneff, 2013). It has been shown that diet composition can induce changes in the gut microbiome bacterial community, and that these changes could have drastic impacts on the host's biology (metabolism, immunity, behavior etc.) (Sommer and Bäckhed, 2013). Regarding GBHs, a 2013 study demonstrated a reduction of beneficial bacteria in the gastrointestinal tract microbiome after oral administration of GBHs in poultry, while highly pathogenic bacteria were found to be resistant (Shehata et al., 2013). They could therefore induce pathogenic dysbiosis in hens' gut microbiome.

Thus, the aim of our study was to investigate the effects of a GBH-enriched diet on adult hens' metabolism and on different

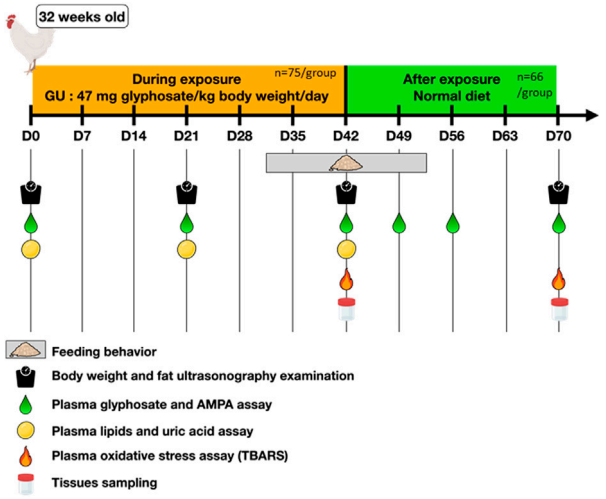

FIGURE 1
Experimental design applied to hens. Hens (32 weeks old; n = 75) were dietary exposed to GBH (Glyphosate-Based Herbicide, corresponding to a dose of 47 mg glyphosate/kg body weight/day, group named GBH) for 42 days straight, then they received a normal diet (not exposed to GBH, same diet, group named ex GBH) for the 28 following days. Their respective controls (CT, n = 75 and Ex-CT, n = 66) received a normal diet for the whole time. During these 70 days, hens were regularly weighed and ultrascanned to determine fat thickness, their feeding behavior was assayed by counting pecks at the diet and their blood was sampled for glyphosate, AMPA, lipids, uric acid and oxidative stress assays. Nine and twelve animals of both groups were euthanized at D42 and D70, respectively, for tissue sampling.

biomolecular stress markers, using a dose equivalent to 47 mg Gly/kg body weight/day, half EFSA's NOAEL, for 6 weeks. We also assessed the reversibility of the potential effects detected, by following the animals for 4 weeks after withdrawal of GBH from their diet. Meanwhile, Gly and AMPA were assayed in plasma and metabolic tissues (leg muscles, liver and abdominal adipose tissue). Detoxication processes were assayed by measuring mRNA expression of the enzymes cytochrome P450 (CYP) and GST (glutathione S-transferase) in animals' liver, which is the main tissue of biotransformation. We also studied the impact of this diet on gut microbiome composition and diversity. To our knowledge, it is the first study evaluating the potential reversibility of changes in metabolism-related parameters after chronic dietary exposure to a GBH in broiler hens.

Materials and methods

Ethical issues

All experimental procedures were performed in accordance with the French National Guidelines for the care and use of animals for research purposes (certificate of authorization to experiment on living animals APAFIS number 21549–2019071809504554v3, approval date: 6 November 2021, Ministry of Agriculture and Fish Products, and a notice of the ethics committee of Val de Loire No. 19).

Animals

All animals (150 female chicks of the commercial breed ROSS 308) were obtained at 1 day of age from a local hatchery (Boye Accouvage La Villonniere 79,310 La Boissière en Gatine, France) and reared at "Pôle Expérimental Avicole de Tours" (INRAE, Nouzilly, France) according to traditional breeding conditions. In our experiment, all 150 hens (32 weeks old) were used. They were divided into groups of five birds in 30 pens, each pen with an area of 3 m^2. The design of the experiment is summarized in Figure 1. The timeline is represented in days. Seventy-five hens (15 pens) were exposed for 42 days to GBH via their food (GBH hens, a GBH dose equivalent to 47 mg Gly/kg body weight/day), and 75 hens (15 pens) were fed with a regular diet without GBH (control animals, CT) (day 0 to day 42 of the protocol). After that, all animals were fed with a regular diet (day 43 to day 70 of the protocol, Ex-GBH and Ex-CT hens). During this protocol, blood samples were collected from hens to quantify Gly and its metabolite AMPA within their blood plasma. At day 42, exposure to GBH was stopped, and nine CT hens and nine GBH hens were slaughtered to recover biological samples. At day 70, 12 Ex-CT hens and 12 Ex-GBH hens were slaughtered to recover biological samples. All animals were killed by electrical stunning and bled out, as recommended by the ethical committee.

Diet composition

Hens (32 weeks old) received a restricted laying diet according to Hendrix Genetics recommendation. The composition of the diet is shown in Supplemental Table 1. For the GBH animals, this diet was mixed with Gallup super 360 in our laboratory in accordance with the directives of the Directions Départementales de la Protection des Populations (Departmental Directorate for the Protection of Populations). Mixing was carried out by a technician with "Certiphyto" certification for the handling of phytosanitary products, as recommended by French law. Gallup super 360, named GBH within the text, was obtained from Axereal (Monnaie, France); it contained 360 g/L Gly (485.8 g/L isopropylamine salt). Animals were fed with either feed containing GBH (n = 75) or control feed (n = 75) from the first week of the protocol to week 6 (Figure 1). The control feed contained low measurable Gly and AMPA concentrations (0.21 mg/kg feed for Gly and undetectable levels for AMPA as determined by Phytocontrol, Nimes,

France). The GBH feed contained 1,250 mg/kg feed of Gly and 0.30 mg/kg feed of AMPA, as determined by Phytocontrol. Hens were food-restricted as recommended by the provider, and their food consumption was 200 g/day. Thus, the concentration in the feed corresponded to a dose of 47 mg Gly equivalent/kg body weight/day. From day 43 to day 70, all hens were fed with control feed (Ex-GBH n = 63 and Ex-CT n = 63).

Count of pecks at the diet

Feeding behavior was quantified by counting pecks at the diet over the daily diet distribution. This count was carried out in three hens per pen during the 8 secondes on D31, D32, D35, D36 (during GBH exposure) and D49, D50, D51 and D52 (after exposure).

Biological samples

Blood samples from 10 hens were collected from the occipital sinus into heparin tubes on different days during the experiment (days 0, 21 and 42 during GBH exposure and days 49, 56 and 70 after exposure). Blood samples were centrifuged (5,000 × *g* for 10 min at 4°C) and stored at −20°C before use for Gly and AMPA assays (Phytocontrol, Nimes, France). Tissue samples were obtained at different points in the experiment [day 42 of the protocol during GBH exposure (n = 9 GBH and n = 9 CT) and day 70 after GBH exposure (n = 12 Ex-GBH and n = 12 Ex-CT)] by dissection after slaughtering.

Gly and aminomethylphosphonic acid assays in hen plasma

Gly and AMPA concentrations were measured in the blood plasma of hens after a derivatization reaction using FMOC-Cl (9-fluorenylmethyl chloroformate), in collaboration with Dr S El Balkhi (Service de Pharmacologie, Toxicologie et Pharmacovigilance, Limoges, France) as previously described (Serra et al., 2021).

Lipid and uric acid assays in hen plasma

Plasma concentrations of triglycerides, uric acid, phospholipids and cholesterol were determined by enzymatic assay using specific kits from Biolabo SAS (Maizy, France): triglycerides (reference: LP80519), uric acid (reference: 80,351), phospholipids (reference: 99,105) and cholesterol (reference: 80,106, Biolabo SAS, Maizy, France). The measurements were performed according to the manufacturer's protocol. For all these assays, the inter- and intra-assay coefficient variations were <15%.

Body weight gain and measurement of tissue index

Chickens were individually weighed on days 0, 21, 42 and 70. Body weight was recorded and, based on the differences, the body weight gain per day was calculated [(final body weight − initial body weight)/number of days)] for each period D0–D21, D21–D42 and D42–D70 (Figure 1). The weight of organs (liver, spleen, heart, kidney, brain, gizzard) and abdominal adipose tissue (AAT) collected on the 42nd and 70th days of the protocol was determined and the weight of the organs or AAT as a percentage of the body weight was calculated and an organ/tissues index was shown as described by Pandey et al. (2019).

Plasma thiobarbituric acid reactive substances (TBARS) assay

Lipid peroxidation, as determined through measuring the amount of MDA (malondialdehyde) that reacts with 2-thiobarbituric acid, was used to estimate oxidative stress (Armstrong and Browne, 1994). Blood samples were collected into EDTA-treated tubes, then gently shaken and kept and handled on wet ice. The plasma was separated by centrifuging the blood samples at 1,000 × *g* for 10 min at 4°C, then transferred to 1.5 ml microcentrifuge tubes and stored at −80°C. The TBARS values of the EDTA-treated plasma were measured using the modified method of Grotto et al. (2007). A standard curve for 1,1,3,3-tetramethoxypropane was used, and the concentration was expressed as nmol MDA/mL solution.

Measurement of liver ATP concentration

Liver total proteins from 9 CT, 9 GBH, 12 Ex-CT and Ex-12 GBH hens were extracted using lysis buffer (1 M Tris (pH 7.4), 0.15 M NaCl, 1.3 mM EDTA, 1 mM EGTA, 43–23 mM VO, 0.1 M NaF, 1% NH_2PO_4, 0.5% Triton) and an Ultra-Turrax instrument for grinding, according to the manufacturer's recommendations (Invitrogen by Life Technologies, Villebon-sur-Yvette, France). Lysates were centrifuged for 20 min at 16,000 × *g* and 4°C, and the supernatants collected. Lysate protein concentrations were then measured using the bicinchoninic acid (BCA) protein assay (Interchim, Montluçon, France). The ATP assay was performed using a Promega CellTiter® Luminescent Cell Viability Assay. Briefly, the assay buffer and the substrate were equilibrated to room temperature, then the buffer was transferred to the substrate and

TABLE 1 Oligonucleotide primer sequences used for RT-qPCR.

Tissue	Gene	Primer F	Primer R	References
	GAPDH	ACGGATTTGGTCGTATTGGG	TGATTTTGGAGGATCTCGC	Grandhaye et al. (2021)
	EEF1α	AGCAGACTTTGTGACCTTGCC	TCACATGAGACAGACGGTTGC	
	β-actin	ACGGAACCACAGTTTATCATC	GTCCCAGTCTTCAACTATACC	
Liver	*CYP1A1*	AATGCTCGTTTCAGTGCCTTC	CCTCCCCTGTCCTTTTCTCC	Cong et al. (2019)
	CYP1A2	AACCCAGAGCGTTTCCTCAA	CTCCCACTTGCCTATGTTTTCC	
	CYP2C	CAAAATGGAACAGGAGAAAGAGAAC	CCCGCAAGGAACAAGTCAA	
	CYP3A	CCAAGCTATGCTCTTCACCG	TCAGGCTCCACTTACGGTCT	
	CYP2A6	CTGCAGAGAATGGCATGAAG	CCTGCAAGACTGCAAGGAA	Wang et al. (2018)
	EPHX1	GAAGATGTCAGGCGGATGTT	CAGGAGAGTCATTCAAACCACA	
	GSTA3	AGACCAGAGCCATCCTCAAC	TGCCAGTCCTTCCACATACA	
	GSTA4	GCTACATCGCAGGGAAATACA	TGGAGAGAAAGGAAACACCAA	
	FXR	AAAGCCGTTCTGTGCGTT	GGATTGGTGGGGTTCCTG	Dai et al. (2020)
	CYP3A37	AAATCAGACAGCAATGGGAGC	GGTAAGCCAGGTAACCAAGTGT	
	BSEP	TGCAAAGCAAAGGAGACT	GCAATGGATAATGGAGGG	
	CYP1A5	CTCTGCTCTGTTCDCAAAGCGTCTC	GCTCGCCTGCDCACCDCATCDCACT	
	CYP1A4	CDCAGGACGGAGGCTGACDCAAGG	GCDCAGGATGGTGGTGAGGAAGA	
	Ces1	TGACCATTCAATATCGCC	ACACTTTCTCCTCCCGCT	
	SLC O 1B3	CAGGACTCTCGCTGGGTGG	TGGCTTTCAGGGGCTTTTT	
	CYP2H1	TCATCCACGAAATCCAAAG	GATGGGAGACAGCAAAGG	
	CYP2H2	GGCCCGGATGGAGCTATT	TTGCCGCCGAGGTGACTA	
Gizzard	*Hsp27*	ACACGAGGAGAAACAGGATGAG	ACTGGATGGCTGGCTTGG	Zhao et al. (2016)
	Hsp40	GGGCATTCAACAGCATAGA	TTCACATCCCCAAGTTTAGG	
	Hsp60	AGCCAAAGGGCAGAAATG	TACAGCAACAACCTGAAGACC	
	Hsp70	CGGGCAAGTTTGACCTAA	TTGGCTCCCACCCTATCTCT	
	Hsp90	TCCTGTCCTGGCTTTAGTTT	AGGTGGCATCTCCTCGGT	
	NF-κB	TCAACGCAGGACCTAAAGACAT	GCAGATAGCCAAGTTCAGGATG	Xing et al. (2015)
	iNOS	CCTGGAGGTCCTGGAAGAGT	CCTGGGTTTCAGAAGTGGC	
	COX-2	TGTCCTTTCACTGCTTTCCAT	TTCCATTGCTGTGTTTGAGGT	
	PTGES	GTTCCTGTCATTCGCCTTCTAC	CGCATCCTCTGGGTTAGCA	
	TNF-α	GCCCTTCCTGTAACCAGATG	ACACGACAGCCAAGTCAACG	
Proventriculus	*PGA5*	TCCGTCTACCTGAGCAAGGAT	AAGCAGGCGACGTACTTGTT	Al-Khalaifah et al. (2020)
	PGC	ATCGGGATTGAGGACTTCGC	TGAAGACCTGGTTGGGAACG	
Caecum	*Chemerin*	CGCGTGGTGAAGGATGTG	CGACTGCTCCCTAAAGAGGAACT	Estienne et al. (2020)
	CMKLR1	CGGTCAACGCCATTTGGT	GGGTAGGAAGATGTTGAAGGAA	
	IgA	GTCACCGTCACCTGGACTACA	ACCGATGGTCTCCTTCACATC	Grandhaye et al. (2020)
	IFNα	CAACGACACCATCCTGGACA	GGGCTGCTGAGGATTTTGAA	Garrido et al. (2018)
	IFNβ	TCCTGCAACCATCTTCGTCA	CACGTCTTGTTGTGGGCAAG	
	IL-1β	AGGCTCAACATTGCGCTGTA	CTTGTAGCCCTTGATGCCCA	Grandhaye et al. (2020)
	IL-6	GCTTCGACGAGGAGAAATGC	GCCAGGTGCTTTGTGCTGTA	Garrido et al. (2018)
	IL-8	CTGCGGTGCCAGTGCATTAG	AGCACACCTCTCTTCCATCC	
Spleen	*IFNα*	CAACGACACCATCCTGGACA	GGGCTGCTGAGGATTTTGAA	Garrido et al. (2018)
	IFNβ	TCCTGCAACCATCTTCGTCA	CACGTCTTGTTGTGGGCAAG	
	IL-1β	AGGCTCAACATTGCGCTGTA	CTTGTAGCCCTTGATGCCCA	Grandhaye et al. (2020)
	IL-8	CTGCGGTGCCAGTGCATTAG	AGCACACCTCTCTTCCATCC	Garrido et al. (2018)
	IL-22	TGTTGTTGCTGTTTCCCTCTTC	CACCCCTGTCCCTTTTGGA	

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gently mixed with it to obtain a homogeneous solution. After a 30 min equilibration of the cell plate to room temperature, protein lysates (100 µL) were put into a 96-well plate and CellTiter-Glo reagent (100 µL) was added to each well. The plate was orbitally mixed for 2 min and incubated at room temperature for 10 min. The ATP concentration was then measured using a luminometer. Luminescence at the integration time 1,000 (ms) was read using an Ascent Luminoskan Luminometer (Thermo Scientific, Illkirch, France). Lysates' ATP concentration was normalized with the previously determined total protein concentration.

Measurement of the gene expression in tissues

Total RNA from 9 CT, 9 GBH, 12 Ex-CT and 12 Ex-GBH hens was extracted from hens' liver, gizzard, proventriculus, cecum and spleen using TRIzol RNA Isolation Reagents and an Ultra-Turrax instrument for grinding, according to the manufacturer's recommendations (Invitrogen by Life Technologies, Villebon-sur-Yvette, France). The purity and concentrations of the obtained RNA were checked via their A260/A280 ratios using a Nanodrop machine. cDNA was obtained by reverse transcription of 2 µg of the total RNA in 20 µL of a mix containing each deoxyribonucleotide triphosphate (dATP, dTTP, dGTP, dCTP; 0.5 mM), 2 M RT Buffer, 15 µg/µL oligodT, 0.125 U of ribonuclease inhibitor and 0.05 U of Moloney murine leukemia virus reverse transcriptase (MMLV); the mixture was kept for 1 h at 37°C. Quantitative PCR was performed using a mix of 3 µL of cDNA and 8 µL of SYBR Green Supermix 1X Reagent (Bio-Rad, Marnes-la-Coquette, France) with 250 nM of specific primers (Invitrogen by Life Technologies, Villebon-sur-Yvette, France) given in Table 1. Samples were set up in duplicate in a 384-well plate and a MyiQ Cycle Device (Bio-Rad, Marnes-la-Coquette, France) was used to apply the following procedure: incubation (2 min at 50°C), denaturation (10 min at 95°C) and 40 PCR cycles (30 s at 95°C, 30 s at 60°C, 30 s at 72°C). Relative expression of genes was related to the geometric mean of the expression of three reference genes (*GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*), *ACTB* (*actin B*) and *EEF1α* (*eukaryotic elongation factor 1 alpha*)). For each target gene, expression was calculated according to primer efficiency (E) and quantification cycle (Cq), where expression = E − Cq. Then, relative expression of the target gene to the three reference genes was analyzed.

Nuclear magnetic resonance (NMR) metabolomics

^{1}H NMR spectra for the metabolic fingerprinting of plasma samples were obtained at 300 K on a Bruker Avance III HD 600 MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 600.13 MHz for ^{1}H resonance frequency using an inverse detection 5 mm ^{1}H-^{13}C-^{15}N-31P cryoprobe attached to a cryoplatform (the pre-amplifier cooling unit). Plasma samples were prepared, and the analyses performed as previously described (Chamorro-García et al., 2021), with slight modifications. For all spectra, a total of 128 transients were collected into 65,536 data points using a spectral width of 20 ppm, a relaxation delay of 5 s and an acquisition time of 2.72 s. All free induction decays were then multiplied by an exponential function with a line broadening factor of 0.3 Hz prior to Fourier transform. All spectra were manually phase- and baseline-corrected, and referenced to the chemical shift of glucose (δ 5.24 ppm).

Analysis of short-chain fatty acids (SCFAs) in cecal samples

Approximately 35 mg of cecal content was weighed, snap-frozen and stored at −80°C until analysis. The samples were extracted with water and proteins precipitated with phosphotungstic acid. 2-Ethylbutyrate was added to supernatants at a ratio of 1: 4 as an internal standard. The SCFA content was determined from a 0.3 µL volume of supernatant by gas chromatography (Agilent 7890B gas chromatograph, Agilent Technologies, Les Ulis, France) equipped with a split-splitless injector, a flame-ionization detector and a fused silica capillary column (15 m × 0.53 mm × 0.5 µm; Supelco, Saint-Quentin-Fallavier, France). The carrier gas (H_2) flow rate was 10 ml/min. The oven temperature was initially set at 100°C for 10 min, then increased from 100 to 180°C at a rate of 20 C/min and held for 2 min. The detector temperature was 240°C. Samples were analyzed in duplicate. The peaks obtained were integrated using OpenLAB Chemstation software (Agilent Technologies, Les Ulis, France).

Microbiome analysis

DNA from bacteria was extracted from cecal content [CT (n = 5), GBH (n = 9), Ex-GBH (n = 10), Ex-CT (n = 5)] using a G'NOME DNA isolation kit (MP Biomedicals, Strasbourg, France) (Furet et al., 2009). The V3–V4 region of the 16S rRNA genes was amplified using MolTaq (Molzym, Plaisir, France), 50 ng DNA and the primers V3F: TACGGRAGGCAGCAG and V4R: ATCTTACCAGGGTAT CTAATCCT (Kozich et al., 2013). Purified amplicons were sequenced using MiSeq sequencing technology (Illumina) on the GeT-PLaGe platform (Toulouse, France). The sequences were submitted to the Short-Read Archive with accession number PRJNA741111. Paired-end reads obtained from

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MiSeq sequencing were analyzed using the Galaxy-supported pipeline named FROGS (Find, Rapidly, OTUs (Operational Taxonomic Units) with Galaxy Solution) (Escudié et al., 2018). For the preprocessing, reads with a length ≥380 bp were kept. The clustering and chimera removal tools followed the guidelines of FROGS (Escudié et al., 2018). Assignation was performed using SILVA138 16S pintail100. OTUs with abundance lower than 0.005% were removed from the analysis (Bokulich et al., 2013).

Statistical analysis

GraphPad Prism® software (version 6) was used for all analyses (except for microbiome and NMR metabolomic analysis). All data are reported as means ± standard error of mean (SEM). Outliers were identified by the Rout method and removed. We performed t-tests to compare means at D42 and D70, or two-way ANOVA followed by Tukey's HSD tests. Regarding gut microbiome analysis, all statistical analyses were performed using R software (version 4.2.0; R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.). The microbiota composition analysis was performed using the mia package (Ernst et al., 2022). For the variability within bacterial communities (α diversity), Chao, Shannon and Faith indices were computed. The effect of exposure to GBH on α diversity was investigated using Kruskal–Wallis tests followed by Dunn's post-hoc tests and was considered significant if $p < 0.05$. For the differences between bacterial communities (β diversity), the Bray–Curtis matrix and Unifrac distance were computed and then visualized with non-metric multidimensional scaling (NMDS) plots.

For the differential analysis, OTUs with a prevalence below 0.05% were filtered out as well as those with a low number of reads (OTUs with sum counts of less than 0.05% of the sum of all counts). Then, OTU counts were agglomerated at the genus level and relative abundances were computed at each taxonomic level. As recommended by Nearing et al. (2022), three differential analysis methods were used (ALDEx2 (ANOVA-like differential expression 2) (Fernandes et al., 2014), ANCOM-BC (analysis of compositions of microbiomes with bias correction (Lin and Peddada, 2020) and DESeq2 (differential gene expression analysis based on the negative binomial distribution (Love et al., 2014)) and we focused on the common results. All p-values were adjusted with the Benjamini–Hochberg correction. A difference is significant if the adjusted p-value is below 0.05 and if the size effect is above 1 for ALDEx2 or if the logFC (log fold change) is above 2 for DESeq2. Finally, sparse partial least-squares discriminant analysis (sPLS-DA) was computed on the centered log ratio (CLR)-transformed data using the mixOmics package (Lê Cao et al., 2016; Rohart et al., 2017). The choice of optimal values for the sparsity parameters and the evaluation of classification was performed using a 5-fold cross-validation and 100 repeats.

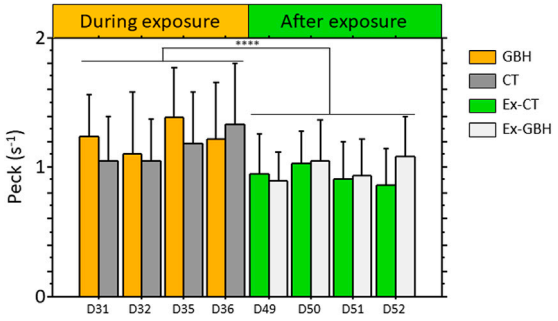

FIGURE 2
Frequency of pecks for feeding made by control hens (CT, n = 45) and those exposed to GBH (GBH, n = 45) at the manger during (D31, D32, D35 and D36) and after (D49, D50, D51 and D52; Ex-CT, n = 45; Ex-GBH, n = 45) GBH exposure. Results are presented as means ± SEM. ****p ≤ 0.0001 CT: Control, GBH: Glyphosate-Based Herbicide, Ex-CT: Ex-Control, Ex-GBH.

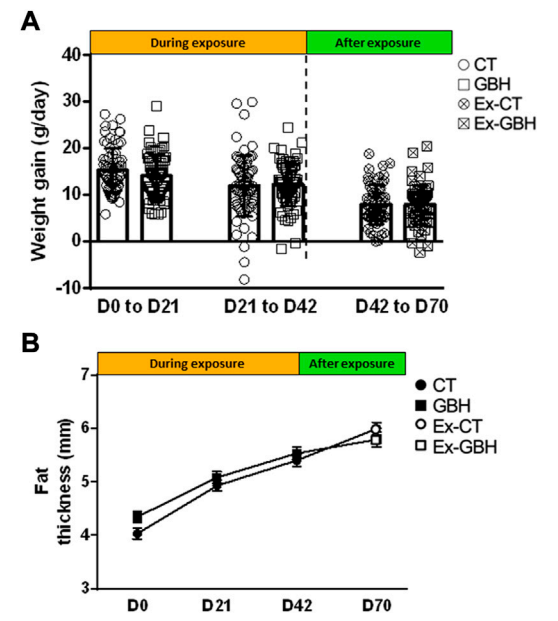

FIGURE 3
Body weight gained **(A)** and fat thickness **(B)** during dietary GBH exposure (D0 to D42; CT, n = 75; GBH, n = 75) and after (D42 to D70; Ex-CT, n = 66; Ex-GBH, n = 66) dietary GBH exposure. Results are presented as means ± SEM **(A)** and as means **(B)**. CT: Control, GBH: Glyphosate-Based Herbicide, Ex-CT: Ex-Control, Ex-GBH.

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TABLE 2 Hens organ index [Tissue weight (g)×100)/(Body weight (g)] in GBH exposed and control animals during the period of exposure (42nd day of protocol, D42, CT n = 9, GBH n = 9) and after GBH exposure (70th day of protocol, D70, Ex-CT, n = 12, Ex-GBH, n = 12).

	D42		*p* Value	D70		*p* Value
	CT	GBH		Ex-CT	Ex-GBH	
Liver	1.59	1.68	0.502	1.65	1.64	0.930
Spleen	0.069	0.062	0.318	0.059	0.075*	0.015
Heart	0.360	0.336	0.367	0.359	0.375	0.427
AAT	2.04	2.12	0.734	2.30	2.42	0.148
Kidney	0.154	0.151	0.902	0.377	0.324	0.064
Brain	0.092	0.097	0.097	0.091	0.088	0.667
Gizzard	0.737	0.888***	0.0003	0.812	0.842	0.408

CT, control; *GBH, Ex-CT, Ex-Control; Ex-GBH, animals that have been GBH, exposed for 42 days then non-exposed until D70*, Ex-*AAT*, abdominal adipose tissue, Index = (Tissue weight (g)×100)/(Body weight (g)). Results are presented as means. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. *CT, control; GBH, Ex-CT, Ex-Control, Ex-GBH.*

All analyses were first performed considering all four groups (CT, GBH, Ex-CT and Ex-GBH) separately. Because no significant difference was detected between CT and Ex-CT groups, they were combined and renamed CT + Ex-CT for the microbiome analysis.

Results

Chronic dietary glyphosate-based herbicide exposure did not change chickens' feeding behavior

One parameter of the hens' feeding behavior was estimated by counting the number of times hens pecked at the diet during and after GBH exposure. As shown in Figure 2, no significant difference was detected between GBH and CT hens and between Ex-GBH and Ex-CT hens. All hens made significantly fewer pecks at the diet after exposure than during GBH exposure ($p < 0.001$).

Chronic dietary glyphosate-based herbicide exposure did not affect chickens' weight gain but increased gizzard weight

Weight gain determined at D0–D21, D21–D42 and D42–D70 is presented in Figure 3A. No significant difference was detected, either during (GBH vs. CT hens) or after GBH exposure (Ex-GBH vs. Ex-CT animals). In addition, no significant effect of GBH on chickens' fat thickness was detected, either during or after GBH exposure (Figure 3B). Liver, spleen, heart, abdominal adipose tissue (AAT), kidney, brain and gizzard indices are presented in Table 2. During GBH exposure (D42), no significant effect was detected except for gizzards, which were significantly heavier in GBH as compared to CT hens ($p < 0.05$). Furthermore, after exposure (D70), spleens and kidneys were heavier and lighter ($p < 0.05$), respectively, in Ex-GBH hens compared to Ex-CT hens.

Gly and aminomethylphosphonic acid accumulated in plasma and tissues after chronic dietary glyphosate-based herbicide exposure in chickens

Plasma Gly and AMPA concentrations were assayed during GBH exposure and normal diet. The results are presented in Table 3. Plasma Gly and AMPA concentrations reached a peak between D0 and D21 before progressively decreasing to values approximatively twice as low at D42 (1.7- and 1.9-fold decrease, respectively) in GBH animals. Concentrations continued to decrease after GBH exposure. Gly and AMPA accumulations in tissues are presented in Figure 4. Both molecules were found in liver (Gly: 8.10 mg/kg and AMPA: 2.40 mg/kg), AAT (Gly: 2.00 mg/kg and AMPA: 0.13 mg/kg) and leg muscle (Gly: 0.86 mg/kg and AMPA: 0.09 mg/kg) in GBH hens at D42 and were observed in smaller amounts in Ex-GBH at D70 (after GBH exposure): 3.11 and 0.74 mg/kg, 0.80 and 0.04 mg/kg and 0.09 and 0.07 mg/kg in liver, AAT and leg muscle, respectively. In CT and Ex-CT animals, Gly and AMPA were almost undetectable in the three tissues at D42 and D70.

Chronic dietary glyphosate-based herbicide exposure reversibly increased plasma oxidative stress in chickens

Plasma oxidative stress was measured using the TBARS index, which quantifies the MDA concentration in plasma. The results are presented in Figure 5. At D42, the TBARS index was significantly ($p < 0.05$) higher in GBH hens than in CT hens. No significant difference was observed between Ex-GBH and Ex-CT hens after GBH exposure at D70.

Chronic dietary glyphosate-based herbicide exposure did not modulate plasma lipids and uric acid concentrations

Plasma triglyceride, cholesterol, phospholipid and uric acid concentrations were assayed at D0, D21 and D44 (Supplemental Figure S1A–D). No significant effect of GBH exposure on any of these parameters was detected.

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TABLE 3 Glyphosate and AMPA concentrations in hen's plasma during (n = 10) and after (n = 10) the dietary GBH exposure period.

	Time (day)	(Glyphosate] (ng/ml)	(AMPA] (ng/ml)
During exposure	D0	5.37 ± 0.60^{a}	0 ± 0^{a}
	D21	1,549.02 ± 89.37^{d}	19.82 ± 1.75^{d}
	D42	910.59 ± 117.29^{c}	10.15 ± 0.61^{c}
After exposure	D49	297.54 ± 7.96^{b}	6.24 ± 0.26^{b}
	D56	160.99 ± 9.12^{ab}	4.45 ± 1.03^{ab}
	D70	67.21 ± 6.87^{ab}	1.59 ± 0.52^{a}

Results are presented as means ± SEM; letters indicate significant differences detected by Two-way ANOVA, and Tukey HSD's test for pair-wise comparisons ($p < 0.05$).

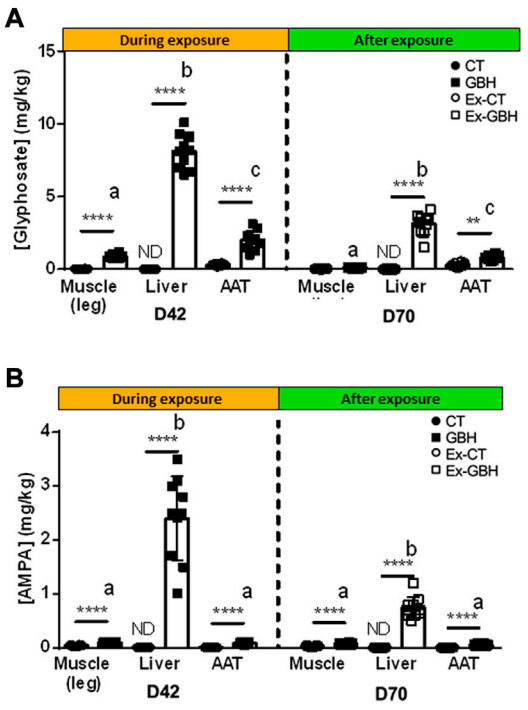

FIGURE 4
Glyphosate **(A)** and AMPA **(B)** concentrations in hens' leg muscles, liver and abdominal adipose tissue (AAT) during (D42) and after (D70) GBH exposure. Results are presented as means ± SEM. p-values express mean differences between CT (n = 10) and GBH (n = 10) groups, and Ex-CT (n = 10) and Ex-GBH (n = 10); **p ≤ 0.01, ****p ≤ 0.0001; letters indicate significant differences detected by two-way ANOVA ($p < 0.05$) followed by Tukey's HSD test for comparing mean tissue concentrations at D42 and D70 separately. CT: control, GBH: Glyphosate-Based Herbicide, Ex-CT: Ex-Control, Ex-GBH.

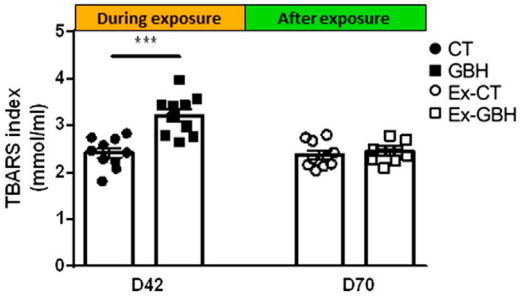

FIGURE 5
TBARS index in hens' plasma during (D42; CT, n = 10 and GBH, n = 10) and after (D70; Ex-CT, n = 10 and Ex-GBH, n = 10) GBH exposure. Results are presented as means ± SEM. ***p ≤ 0.001. CT: Control, GBH: Glyphosate-Based Herbicide, Ex-CT: Ex-Control, Ex-GBH.

Chronic dietary glyphosate-based herbicide exposure did not trigger biotransformation enzyme transcription in liver but enhanced IgA transcript levels in chickens

The mRNA expression of liver biotransformation enzymes was measured by RT-qPCR. The results are presented in Table 4.

No significant effect was detected on any of the transcripts of which we measured the level, either during exposure (CT vs. GBH hens) or after exposure (Ex-CT vs. Ex-GBH). In the cecum, expression of the immunoglobulin A gene (*IgA*) was significantly ($p < 0.05$) enhanced by GBH treatment after exposure in Ex-GBH hens as compared to Ex-CT hens whereas no significant effect was detected during exposure (CT vs. GBH hens). We next determined the expression levels of genes involved in the inflammatory and immune responses. No significant effect was detected for *chemerin*, *CMKLR1*, *IFNα*, *IFNβ*, *IL-1β*, *IL-6* and *IL-8* (Table 4). mRNA expression of stress-related genes in the gizzard was also measured and is shown in Table 4 *iNOS* expression was significantly ($p < 0.05$) higher in the Ex-GBH group than in the Ex-CT group after exposure and that of *HSP70* was significantly ($p < 0.05$) higher in the Ex-CT group than in the Ex-GBH group after exposure. No significant effect on any of these genes was detected during exposure (CT vs. GBH hens). No significant effect on the mRNA expression of some digestive genes (*PGA5* and *PGC*) was detected in the proventriculus (Table 4). The TNF-γ protein concentration in hens' plasma was assayed and the results are presented in Supplemental Figure 2A. No significant effect of GBH exposure was detected, either at D42 or D70.

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TABLE 4 Relative mRNA expression of various genes in hen's organs at the end of GBH exposure (D42, CT, n = 9 and GBH, n = 9) hens) and after dietary GBH exposure (D70, Ex-CT, n = 12 and Ex-GBH, n = 12 hens).

Organ		D42			D70		
		CT	GBH	*p*.value	Ex-CT	Ex-GBH	*p*.value
Liver	*CYP1A2*	10.47	8.74	0.578	16.42	12.31	0.210
	CYP1A4	0.110	0.130	0.598	0.170	0.190	0.835
	CYP2A6	20.0	18.4	0.696	15.1	16.28	0.741
	CYP2C	11.4	9.86	0.498	12.4	11.40	0.793
	CYP3A37	6.87	6.78	0.956	5.11	5.16	0.966
	CYP3A4	0.300	0.35	0.401	0.35	0.310	0.425
	CYP3A80	0.460	0.42	0.681	1.98	0.740*	0.027
	CYP2H1	22.8	22.0	0.854	16.7	18.4	0.661
	CYP2H2	13.0	13.44	0.905	10.2	12.4	0.432
	GSTA3	3.10	2.92	0.700	5.65	3.86	0.200
	GSTA4	4.71	3.50	0.054	4.43	4.32	0.861
	EPHX1	0.500	0.45	0.541	0.40	0.44	0.763
	FXR	0.760	0.78	0.881	1.49	1.24	0.461
	SL O 1B3	0.710	0.71	0.972	0.71	0.77	0.759
Gizzard	*COX2*	0.201	0.132	0.188	0.188	0.142	0.194
	HSP27	39.9	33.8	0.637	64.1	53.4	0.360
	HSP40	94.1	93.1	0.835	91.3	93.3	0.582
	HSP60	0.533	0.361	0.113	0.707	0.628	0.310
	HSP70	3.18	2.48	0.443	4.31	2.29*	0.015
	HSP90	2.24	1.49	0.249	1.87	1.55	0.544
	iNOS	0.028	0.022	0.412	0.023	0.042**	0.003
	NFκB	0.079	0.059	0.417	0.104	0.099	0.760
	PTGES	0.087	0.054	0.168	0.074	0.055	0.245
	TNFa	0.036	0.029	0.154	0.023	0.033	0.052
Proventriculus	*PGA5*	32,266	38,158	0.330	26,653	27,223	0.899
	PGC	5,916	7,495	0.114	6,874	6,838	0.971
Caecum	*Chemerin*	0.336	0.337	0.986	0.272	0.257	0.731
	CMKLR1	0.017	0.017	0.984	0.065	0.048	0.301
	IgA	12.3	8.49	0.364	4.46	6.70*	0.041
	IFNα	0.071	0.086	0.620	0.434	0.290	0.230
	IFNβ	0.002	0.005	0.179	0.024	0.011	0.100
	IL-1β	0.013	0.007	0.095	0.020	0.016	0.338
	IL-6	0.001	0.001	0.895	0.004	0.002	0.077
	IL-8	0.040	0.025	0.185	0.049	0.032	0.242
Spleen	*IFNα*	0.031	0.025	0.607	0.112	0.061	0.108
	IFNβ	3.50	2.18	0.301	7.90	6.00	0.414
	IL-1β	0.006	0.004	0.182	0.010	0.014	0.279
	IL-8	0.076	0.075	0.964	0.065	0.038*	0.036
	IL-22	0.035	0.021	0.273	0.093	0.024**	0.003

Results are presented as mean of the pltarget gene mRNA, expression relative to the geometric mean of three housekeeping genes expression (*GAPDH, EEF1α* and *β-actin*); *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. *CT, control; GBH, Ex-CT, Ex-Control, Ex-GBH* .

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TABLE 5 Significant plasma metabolites identified in GBH dietary exposed (GBH, n = 10) as compared to control hens (CT, n = 10) at day 42 (D42).

Metabolites	FC	Pvalue
Glycerol	1.05	0.024
Glycine	0.941	0.045
Isoleucine	1.11	0.025
Leucine	0.926	0.024
Lysine	1.53	0.028
Methionine	1.18	0.028
Tyrosine	1.42	0.049

FC (Fold Change) = GBH/CT (*CT*, control, *GBH*). Results are presented as means.

Chronic dietary glyphosate-based herbicide modified the plasma concentration of glycerol and six amino acids

Metabolites whose concentrations in hens' plasma were significantly affected by GBH exposure are mentioned in Table 5. Plasma glycerol, isoleucine, lysine, methionine and tyrosine concentrations were significantly increased ($p < 0.05$), while plasma glycine and leucine concentrations were significantly reduced in GBH as compared to CT animals ($p < 0.05$).

Chronic dietary glyphosate-based herbicide exposure changed SCFA concentrations in chickens' cecum

Cecal contents were subjected to gas chromatography to measure the cecal SCFA concentration. SCFAs in hens' cecum were assayed and the results are shown in Table 6. Cecal acetate, propionate, isobutyrate, isovalerate and valerate concentrations were significantly ($p < 0.05$) reduced during exposure in GBH as compared to control animals (CT). All of them were restored in Ex-GBH chickens' cecum after GBH exposure, as compared to their respective controls (Ex-CT), except for acetate and valerate, which were still significantly ($p < 0.05$) reduced.

Chronic dietary glyphosate-based herbicide exposure increased gut microbiome diversity

GBH durably changed the composition of the microbiota, which was not completely resilient (Figures 6A,B). Surprisingly, GBH increased the diversity of the microbiota in a delayed manner. Indeed, the diversity indices (Chao, Shannon and Faith indices) became different at the final time point even though they were similar at the end of the GBH exposure (Figures 6C–E). The delayed impact of GBH was reflected in eight individual taxa that were different from the control at the end of the experiment but not just after the GBH exposure (Figure 7). For example, the Bacteroidales F082 family was not immediately affected by GBH administration but was significantly enhanced in the Ex-GBH group. Similarly, the *Synergistes* (family Synergistaceae) were favored by GBH at the final time point only. The other groups that showed a delayed impact were: *Akkermansia* (family Akkermansiaceae), *DTU089* (family Clostridiaceae), *Paraprevotella* (family *Paraprevotella*), *S50* (family Rikenellaceae) and *Treponema* (family Treponemataceae). Perhaps unsurprisingly, nine taxa were immediately impacted by GBH: the amounts of Muribaculaceae family, *Alloprevotella* (family Prevotellaceae), Porphyromonadaceae and *Candidatus Vestibaculum* were significantly higher after exposure in

TABLE 6 Short-chain fatty acid concentration in cecal content (µmol/g) during dietary GBH (GBH, D42, n = 8) exposure in control (CT, n = 8) and after dietary GBH exposure (Ex-CT, D70, n = 12 and Ex-GBH, n = 11 hens).

	D42			D70		
	CT	GBH	*p*.value	Ex-CT	Ex-GBH	*p*.value
Acetate	28.0	18.0**	0.009	25.8	16.6*	0.022
Propionate	8.36	5.43*	0.022	8.02	5.63	0.126
Isobutyrate	0.770	0.510**	0.004	0.730	0.59	0.194
Butyrate	2.08	1.64	0.293	1.85	1.30	0.166
Isovalerate	0.530	0.389*	0.041	0.510	0.450	0.377
Valerate	0.710	0.516*	0.044	0.620	0.390*	0.033
Caproate	0.000	0.000		0.020	0.000	0.068
Total isoAGCC	1.30	0.900**	0.006	1.24	1.04	0.233
Total AGCC	41.5	26.4*	0.015	38.0	25.2*	0.045

Results are presented as means; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. *CT, control; GBH, Ex-CT, Ex-Control, Ex-GBH.*

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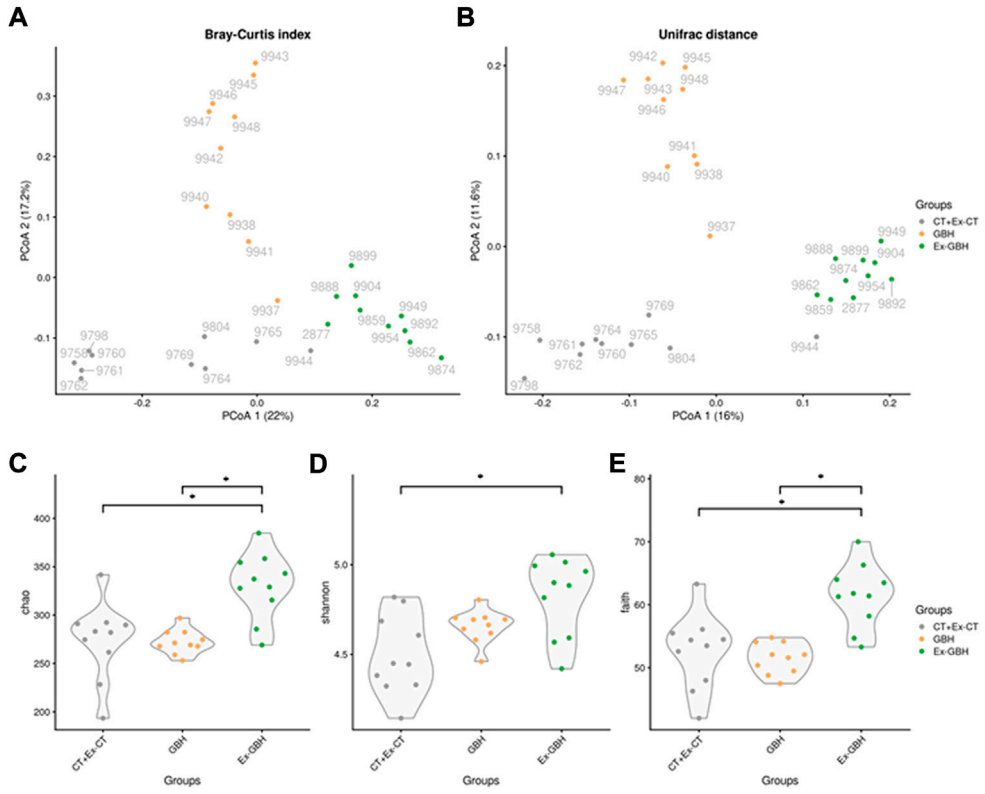

FIGURE 6
Microbiome β-diversity in cecal content of hens exposed to GBH for 42 days (GBH, n = 9), then non-exposed until D70 (Ex-GBH, n = 12) and controls at D42 and D70 taken together (CT + Ex-CT, n = 10), collected during (D42) and after GBH exposure (D70) measured by principal coordinates analysis (PCoA) on Bray–Curtis index **(A)** and Unifrac distance **(B)**. Results are presented as individual values. Microbiome α-diversity in the very same groups measured by Chao **(C)**, Shannon **(D)** and Faith indices **(E)**. *p ≤ 0.05. CT + Ex-CT: Control and ex-Control taken together, GBH: Glyphosate-Based Herbicide, Ex-GBH.

GBH and Ex-GBH groups than in the CT + Ex-CT group. The abundance of *Barnesiella* was significantly decreased whereas that of *Colidextribacter* was significantly higher in the GBH group than in the CT + Ex-CT group. The abundance of *Coprobacter* was different in all three groups: it was significantly lower in the GBH group than in the CT + Ex-CT group and was significantly higher in Ex-GBH than in the other groups. *DTU089* abundance was significantly lower in the Ex-GBH group than in the CT + Ex-CT group. *GCA-900066575* abundance was significantly higher in the Ex-GBH group than in GBH hens. *Ruminococcus* abundance was significantly higher in Ex-GBH and CT + Ex-GBH groups than in the GBH group. Abundance of the *S50* wastewater-sludge group was significantly higher in Ex-GBH than in the GBH group. *Synergistes* abundance was significantly higher in Ex-GBH than in the CT + Ex-GBH group. *Clostridia* vadin BB60 abundance was significantly higher in the CT + Ex-CT group than in the GBH group. sPLS-DA analysis was performed to explain group variations. Two components have been identified. Most of the differential taxa identified by sPLS-DA analysis was already identified by the previous analysis. The first component (X-variate 1) explains 16% of the variations and allows to separate GBH and CT + Ex-CT hens from Ex-GBH hens. Second component (X-variate 2) explains 13% of the variations and allows to separate GBH hens from CT + Ex-CT hens. For each component, most contributing taxa were identified based on their abundance. The first component has 5 most contributing taxa. Its most contributing taxon is *Treponema*, more abundant in Ex-GBH group, followed by Prevotellaceae, more abundant in GBH group, *Paraprevotella*, a non-identified *F082* family bacterium, more abundant in Ex-GBH group and *Butyricicoccus* which is more abundant in CT + Ex-CT group. The second component has 5 most contributing taxa. Its most contributing taxon is Muribaculaceae, more abundant in GBH group, followed by *Bacteroidales*, more abundant in GBH group too, itself followed by *Flavobacteriales*, more abundant in CT + Ex-CT group and *Candidatus Vestibculum* and *Synergistes*, more abundant in GBH and CT + Ex-CT group.

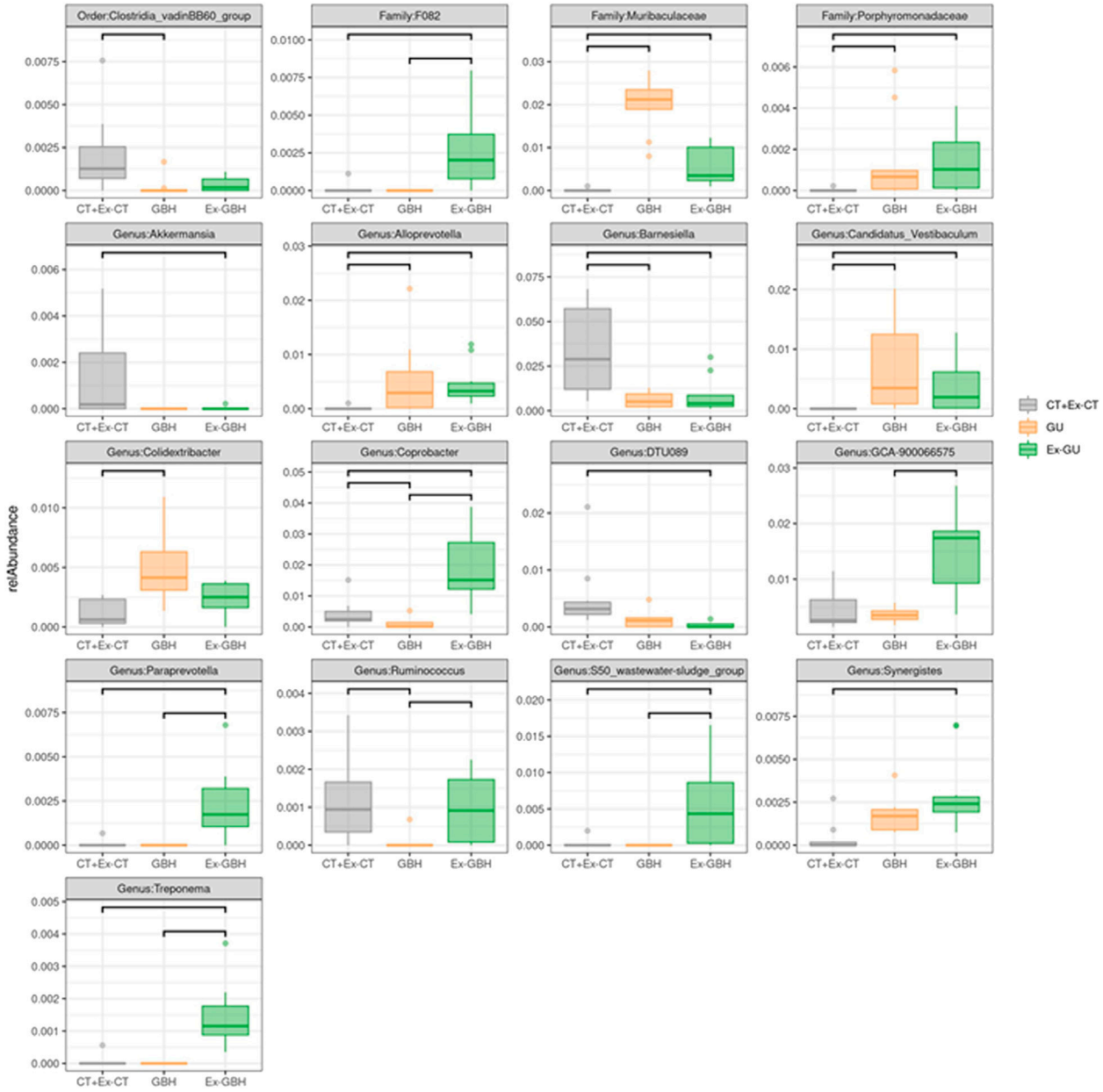

FIGURE 7
Relative abundance of differential taxa in cecal content of hens exposed to GBH for 42 days (GBH, n = 9), exposed for 42 days then non-exposed until D70 (Ex-GBH, n = 12) and controls at D42 and D70 taken together (CT + Ex-CT, n = 10), commonly identified by ALDEx2, DESeq2 and ANCOM-BC methods. A bar between two groups indicates that the relative abundance is significant between these two groups. Taxa are identified at order, family and genus levels Results are presented as means ± SEM. *p ≤ 0.05. CT + Ex-CT: Control and ex-Control taken together, GBH: Glyphosate-Based Herbicide, Ex-GBH.

Discussion

Our results show for the first time possible disturbances of the cecal microbiota associated with plasma oxidative stress and the accumulation of Gly in metabolic tissues in response to chronic dietary GBH exposure in breeder broiler hens (Figure 8). Furthermore, some of these alterations are potentially reversible and were observed without any variation of growth performance (Figure 8).

Indeed, when we monitored hens' body weight and fat thickness during and after chronic dietary GBH exposure, we did not observe any differences in either of these two parameters

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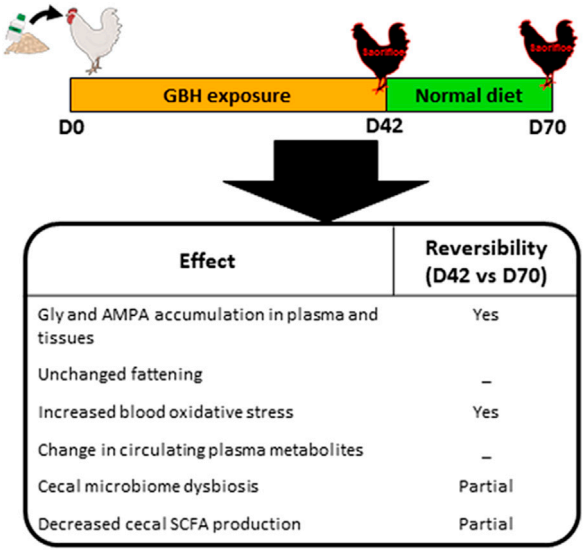

Effect	Reversibility (D42 vs D70)
Gly and AMPA accumulation in plasma and tissues	Yes
Unchanged fattening	–
Increased blood oxidative stress	Yes
Change in circulating plasma metabolites	–
Cecal microbiome dysbiosis	Partial
Decreased cecal SCFA production	Partial

FIGURE 8
Schematic representation of effects of dietary GBH exposure in hens and their potential reversibility on glyphosate and AMPA accumulation in plasma and tissues, fattening, plasma oxidative stress, plasma metabolites, cecal microbiome dysbiosis and cecal SCFA production.

between the hens fed with GBH and those fed without GBH. These results are in accordance with those obtained after a 13-weeks study performed on rats (Panzacchi et al., 2018). Moreover, triglyceride, cholesterol and phospholipid concentrations in plasma were not affected and the uric acid assay did not show any difference with the control either, suggesting that fatty acid and purine metabolism is not affected by the GBH diet. However, metabolomic analysis revealed that the concentrations of some serum metabolites were altered. A recent study shows that in Gly exposed humans, several metabolites levels in serum can be altered (Zhang et al., 2022). These alterations could be linked to metabolism dysfunctions, including fatty acids metabolism and purine biosynthesis. The authors also found that TCA cycle intermediates were altered following to the exposition. In our study, all altered serum metabolites were amino acids (except for glycerol). More specifically, five out of eight of the significantly affected metabolites were ketogenic amino acids, including two glucoformers. Also, all the metabolites we identified were reported several times as potential biomarkers for human colorectal cancer detection (Ni et al., 2014). Among them, leucine, lysine and tyrosine can be oxidized to generate acetyl-CoA for ketone body synthesis; methionine and isoleucine are also convertible into propionyl-CoA, then into succinyl-CoA to enter the TCA cycle (Newsholme et al., 2011; Chiang, 2014; Kumari, 2018). This suggests a potential disruption of mitochondrial activity. However, when we determined the ATP concentration in hens' livers, we observed no significant effect of the treatment. The effect of such variations in amino acid circulation therefore remains unclear.

Still, it is worth noticing that Gly and AMPA mainly accumulated in the liver and, to a lesser extent, in leg muscles and AAT. However, no significant effect on liver and AAT weight was detected. Gly and AMPA were also detectable in plasma at both D42 (during exposure) and D70 (after exposure), where they were accompanied by an increase in oxidative stress only at D42. Oxidative stress induction has widely been demonstrated in mammals exposed to Gly and AMPA (Kwiatkowska et al., 2014; Owagboriaye et al., 2019; Turkmen et al., 2019). This effect is reversible since plasma TBARS levels drop back to CT values after exposure. The maximum Gly and AMPA concentrations were measured on the 21st day of the diet, and the values measured later on the 42nd day were lower. This suggests that their degradation/excretion rate is lower than their intake rate before D21 but becomes greater during the following days. In order to clarify this observation, we quantified transcript levels of biotransformation enzymes. A recent study has shown that *in ovo* injections of Gly and GBH trigger modulations in the mRNA expression of cytochromes and other biotransformation-related genes in chick embryos (Fathi et al., 2020). Yet, in adult hens, we did not detect any significant effect on the expression of biotransformation-related genes, whether they encode for Phase I (cytochromes) or Phase II enzymes (GSTs). CYP enzymes enable detoxication of xenobiotics by catalyzing redox and hydrolysis reactions, modifying their chemical properties and thus their toxicity. GSTs catalyze the conjugation reaction of GSH to xenobiotics, which enhances their hydrosolubility and thus their elimination in urine and feces. It has been shown that in rats, 98% of administered Gly is excreted as the unchanged parent compound (Panzacchi et al., 2018), which could explain the lack of action of Phase I and Phase II enzymes on it, which would have turned it into modified compounds. Moreover, a 2000 risk assessment reported that orally administered Gly and AMPA are weakly biotransformed in animals (Williams et al., 2000). However, Gly concentrations in the liver do not quickly drop to negligible values after exposure. Yet, as previously mentioned, GBH's suspected toxicity is not due to Gly alone but also to the surfactants contained in GBH formulations (Bradberry et al., 2004; Kim et al., 2013; Mesnage et al., 2019). Studies have shown that some surfactants are even more toxic than the active ingredient. One example is lipid-based POEAs (polyethoxylated tallow amines) (Martins-Gomes et al., 2022) which are now banned in the EU but still allowed in the United States The presence of these coformulants could explain the high and sustainable accumulation of Gly in the liver, but we would have expected them to trigger the expression of biotransformation enzymes. The exact composition of most GBHs is, however, strictly confidential. We are therefore unable to determine which coformulants could be responsible for this phenomenon.

Considering that hens' spleens were heavier in the Ex-GBH group than in the Ex-CT group, we assumed a disruption at the immune system level. We therefore investigated immune system gene transcript levels in several immune tissues and observed an increase in IgA mRNA expression in the cecum of Ex-GBH animals compared to that in controls. Since IgA is known to be involved in gut microbiome dynamics (Shi et al., 2017), we suspected potential disruption to hens' gut microbiome. The main effects of GBH dietary exposure observed in the present study are actually at the gut microbiome level. It is now well established that GBH and Gly are able to induce changes in microbial communities, especially in the gut of exposed animals (Lozano et al., 2018; Mao et al., 2018). A recent study has shown that orally administered Gly and GBH trigger inhibition of the shikimate pathway in the gut microbiome of Sprague Dawley rats. This inhibition is coupled with increased levels of *Akkermansia muciniphila* (Mesnage et al., 2021). Interestingly, our results rather show a decrease in *Akkermansia* abundance, which is not restored after exposure. In humans, the shikimate pathway is mainly achieved by *A. muciniphila* (Mesnage and Antoniou, 2020), which, extrapolated to poultry, could make the crushing of its abundance expectable and consistent since it is the pathway targeted by Gly. Our results also show that *Barnesiella* and *Clostridia* vadin BB60 abundance follows the same scheme, as does that of *Ruminococcus* whose abundance follows a trend toward restoration after Gallup exposure, implicating various potential neuropsychiatric disorders (Barnett et al., 2022). On the other hand, Muribaculaceae, Porphyromonadaceae, *Alloprevotella*, *Candidatus Vestibaculum* and *Colidextribacter* abundances are significantly increased by GBH exposure and follow a trend toward restoration after GBH exposure. Interestingly, *Colidextribacter* species are known to be positively correlated with oxidative stress (Wang et al., 2021). The impact of GBH may be similar between rats and poultry, since GBH exposure also increases *Alloprevotella* in rats' microbiome (Dechartres et al., 2019). However, to our knowledge, no evident link between variations in the populations of other above-mentioned bacteria and GBH exposure has been established. It has nevertheless previously been shown that a decrease in one bacterial population can induce an increase in other populations (Ruuskanen et al., 2020). We therefore assume that some of the community variations observed here are rather due to inner ecological competition triggered by the reduction in abundance of some taxa than to a direct antibiotic effect of the herbicide. Moreover, Gly is not deleterious to all bacteria: some are able to degrade the molecule (Mesnage and Antoniou, 2020) for phosphorus supply or energy production (Hove-Jensen et al., 2014; Strilbyska et al., 2021). Several recent studies suggest an increase in α-diversity in various models when individuals are exposed to glyphosate or GBHs (Tang et al., 2020; Castelli et al., 2021; Mesnage et al., 2021, 2022). Our results are similar to these findings, except that α-diversity is not increased in exposed animals, but in formerly exposed animals. It is surprising that adding a perturbation does not have any effect on a system but the removal of this perturbation does produce some significant effects on it. To our knowledge, no previous study has reported this kind of observation following to the administration of dietary GBH. However, since we observe some effects on the gut microbiome at the family and genus levels, we can hypothesize that cecal bacteria quickly accustom to the GBH presence, probably by gaining genetic resistances to it. They could however not accustom to its withdrawal as quickly. Since a genetic resistance, for instance to an antibiotic, is often a disadvantage in absence of it, GBH withdrawal could free up an ecological niche for other bacteria, resulting in an increase in α-diversity.

SFCA concentrations in animal feces are also good indicators of potential disruptions in the gut microbiome. Dietary carbohydrates that reach the large intestine without having previously been lysed into smaller molecules in the small intestine are metabolized by bacteria into SCFAs. These molecules are critical in maintaining animals' health, as they are the primary energy source for colonocytes and are involved in the production of hormones which act on their metabolism (blood glucose regulation, fat and protein digestion, satiation promotion) (Barnett et al., 2022). *Ruminococcus* (Ruminococcaceae) is part of the gut bacteria able to produce SCFAs from dietary carbohydrates (Barnett et al., 2022) that we identified as reversibly diminished by GBH exposure. Our data are also in good agreement with those observed in rats (Dechartres et al., 2019) where Ruminococcaceae abundance was lowered by exposure to GBH. *Akkermansia* has also been reported to be an SCFA (propionate) producer in humans (Morrison and Preston, 2016). Our results show a global diminution of all cecal SCFAs during GBH administration, which are restored after exposure for acetate and valerate only. We can therefore hypothesize a link between the fall of these bacteria and that of cecal SFCA levels. Since SFCAs are also suspected to be involved in neuroendocrine regulation and gut microbiome–brain communication, disturbance in their dynamics suggests potential physiological and mental disorders (Silva et al., 2020). A 2017 study revealed depressive-like behaviors in young adult rats when exposed to a GBH (Cattani et al., 2017). However, we did not detect any effect on hens' feeding behavior. At this point, it therefore seems important to remember the correlational nature of such studies. Since correlation does not imply causation, more studies are needed to confirm direct guilt of GBH/Gly/surfactants in disruption of the gut microbiome and cecal SFCA production and on the disorders they trigger. Other effects of GBH have been demonstrated in other cells. For instance, it has been shown that low GBH levels could induce oxidative stress and impair Ca^{2+}-mediated functions in rat Sertoli cells, resulting in a reduced male fertility (de Liz Oliveira Cavalli et al., 2013). Future works could therefore focus on the potential effects of

GBH administration on male and female reproductive functions.

Conclusion

Here, we show that half the current NOAEL of dietary Gly does not induce a direct impact on hens' metabolism (at least on the various parameters determined in our 150 animals), though it reversibly triggers oxidative stress in blood plasma and impacts several gut microbes. Investigating the link between GBH and fertility is a direction that future works should probably take, in order to constitute a robust database allowing characterization of the actual impact that the molecule and its formulations have on animal and human health.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJNA741111.

Ethics statement

The animal study was reviewed and approved by ethics committee of Val de Loire No. 19.

Author contributions

MF, AE, CR, MC, PF, and JD. contributed to the overall approach and design of experiments. GL, MF. and JD performed statistical data analysis. CS and EV determined the fattening of animals by using ultrasound. CL and JL realized the analyses of food intake behavior and RG participated to the RTqPCR analysis. EM performed the analyses of SCFA concentrations in hen's caecum and AH and OZ realized the caecal microbiome analysis. MF and JD. wrote the manuscript. All authors critically revised the manuscript and approved the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.974688/full#supplementary-material

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Glossary

Actin B: Actin Beta ALDEx2: ANOVA-Like Differential Expression 2

AMPA: Amino-Methyl-Phosphonic Acid

ANCOM-BC: Analysis of Compositions of Microbiomes with Bias Correction

BCA: bicinchoninic acid

BSEP: ATP binding cassette subfamily B member 11

Ces1: Carboxylesterase 1

CMKLR1: Chemerin Chemokine-Like Receptor 1

COX-2: Cyclooxygenase 2

Cq: quantification cycle

CT: Control

CYP1A1: Cytochrome P450 Family 1 Subfamily A Member 1

CYP1A2: Cytochrome P450 Family 1 Subfamily A Member 2

CYP1A4: Cytochrome P450 Family 1 Subfamily A Member 4

CYP1A5: Cytochrome P450 Family 1 Subfamily A Member 5

CYP2A6: Cytochrome P450 family 2 subfamily A member 6

CYP2C: Cytochrome P450 Family 2 Subfamily C

CYP2H1: Cytochrome P4502H1

CYP2H2: Cytochrome P4502H2

CYP3A: Cytochrome P450, family 3, subfamily A

CYP3A37: Cytochrome P450, family 3, subfamily A member 37

EPHX1: Epoxide Hydrolase 1

FMOC-Cl: 9-fluorenylmethyl chloroformate

FROGS: Find, Rapidly, OTUs, Operational Taxonomic Units

FXR: Farnesoid X receptor

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GBH: Glyphosate-based Herbicides

Gly: Glyphosate

GSH: Glutathione

GSTA3: Glutathione S-Transferase Alpha 3

GSTA4: Glutathione S-Transferase Alpha 4

GSTs: Glutathione S-Transferase

Hsp27: Heat shock protein 27

Hsp40: Heat shock protein 40

Hsp60: Heat shock protein 60

Hsp70: Heat shock protein 70

Hsp90: Heat shock protein 90

IFNα: Interferon alpha

IFNβ: Interferon beta

IgA: Immunoglobulin A

IL-1β: Interleukin-1 beta

IL-22: Interleukin-22

IL-6: Interleukin-6

IL-8: Interleukin-8

iNOS: Inducible nitric oxide synthase

MDA: MalonDiAldehyde

MMLV: Moloney murine leukemia virus reverse transcriptase

NF-κB: Nuclear factor-kappa B

NMDS: non-metric multidimensional scaling

NMR: Nuclear magnetic resonance metabolomics

NOAEL: Non observed adverse effect level

OTUs: Operational Taxonomic Units

PGA5: Pepsinogen A5

PGC: Progastricsin

POEA: Polyethoxylated tallow amines

PTGES: Prostaglandin E Synthase

SCFA: Short-Chain Fatty Acid

TCA cycle: tricarboxylic acid cycle

TNF-α: Tumor Necrosis Factor-alpha

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Ileal and cecal microbiota response to *Salmonella* Typhimurium challenge in conventional and slow-growing broilers

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Despite the negative impacts of *Salmonella* intestinal colonization on human health, *Salmonella* is a natural colonizer of the gastrointestinal tract and is not overtly pathogenic to the avian host. It is of interest to understand the impacts and colonization rates of *Salmonella* across selected genetic lines such as slow-growing (SG) and conventional (CONV) broilers. The objective of this study was to characterize the relationship between *Salmonella enterica* serovar Typhimurium challenge and selected broiler genetic lines on the ileal and cecal microbiome. Male chicks of two broiler breeds (n = 156/breed) were cohoused in an open floor pen until day 7. On day 13, the chicks were then separated into 12 isolators per breed (4 rooms, 6 isolators/room, 11 chicks/isolator). On day 14, chicks in the 12 treatment isolators (6 isolators/breed, 108 total) were challenged with *Salmonella* Typhimurium (ST) (1×10^8 CFU/ml) via oral gavage while the remaining chicks (n = 108) were given an oral gavage of sterile tryptic soy broth control (C). Ileal and cecal contents were collected on day 7 from 24 chicks of each breed, and on days 13, 17, 21, and 24 from two chicks per isolator. Samples underwent DNA extraction and PCR amplification to obtain 16S rRNA amplicons that were sequenced with Illumina MiSeq. *Salmonella* Typhimurium colonization in the cecum was not different in the two broiler breeds. The main effect of breed had the greatest impact on the ileum microbiota of broilers 7 days of age where SG broilers had significantly lower diversity and richness compared to CONV broilers ($p < 0.05$). *Salmonella* Typhimurium challenge consistently caused a change in beta diversity. Regardless of day or intestinal location, challenged broilers had many amplicon sequence variants (ASVs) with decreased abundance of likely beneficial bacteria such as *Mollicutes* RF39, *Shuttleworthia*, *Flavonifractor*, and *Oscillibacter* compared to broilers that were unchallenged with *Salmonella* Typhimurium ($p < 0.05$). Additionally, there was a difference in the timing of when the microbiota alpha and beta diversity of each breed responded to *Salmonella* Typhimurium challenge. Thus, both broiler breed and *Salmonella* Typhimurium can impact the intestinal microbiota.

KEYWORDS
breed, poultry, 16S rRNA, slow-growing, colonization

Introduction

Salmonella is a prominent foodborne pathogen that is commonly present on broiler farms. Despite its negative public perception, *Salmonella* is a common gut colonizer in poultry, which may be why it is so ubiquitous in tainted meat products (Antunes et al., 2003). The strains that cause Salmonellosis in live broilers are different than the strains that are known to cause Salmonellosis in humans (Pieskus et al., 2006). However, since Salmonellosis is an important health concern, its effects are under constant surveillance and study to determine how *Salmonella* colonization can be limited in large broiler production systems (Finstad et al., 2012). The use of antimicrobials, the lack of genetic variability, and limited living space may all contribute to the colonization of poultry by *Salmonella* in commercial farms (Foley et al., 2011).

Concurrently, due to increased public concern for animal welfare, there is increased public interest in poultry products from slow-growing chickens. These broilers have been bred through less intensive genetic selection leading to slower growth and longer periods to reach market weight. Studies have shown that such breeds allow for better welfare outcomes, but as expected, there are differences in growth and efficiency when compared to conventional fast-growing broilers (Torrey et al., 2021). Further investigation is necessary to determine the response of a slow-growing line to pathogen exposure and colonization resistance. The role of broiler host genetic factors in resistance and immune response when exposed to *Salmonella* has been studied previously. For example, some breeds that were resistant to *S. enterica* serovar Typhimurium were also resistant to other serovars, including Gallinarum, Pullorum and Enteritidis (van Hemert et al., 2006). Additionally, different breeds allow varying levels of *Salmonella* colonization in the intestinal tract along with differing responses to vaccination (van Hemert et al., 2006). The intestinal microbiome of broilers may provide insight into colonization rate of *Salmonella* as well as the impact of host genetics on the composition of the microbiome. Recent advancements in bacterial identification and microbiome analysis by next generation sequencing (NGS) have determined that the development and microbial composition of the intestines are influenced by genetics (Zhao et al., 2013; Mignon-Grasteau et al., 2015). Microbiota are essential for maintaining a healthy gut and preventing colonization of pathogenic bacteria, while *Salmonella* colonization may lead to dysbiosis and increased susceptibility to disease. The microbiota most commonly found within the intestinal tract of broilers belong to the phyla Firmicutes, Bacteroidetes, and Proteobacteria, and consist of hundreds of genera responsible for aiding in absorption and digestion in the ileum and cecum, respectively (Oakley et al., 2014; Sergeant et al., 2014; Clavijo and Flórez, 2018). However, the gut microbiome is complex and variable due to differences in age, intestinal region, diet, and genetics. More research is needed to clearly identify the relationship between pathogen exposure, broiler genetics, and the resulting microbial community.

The purpose of this study was to evaluate differences between ileal and cecal microbiomes of conventional and slow-growing broilers when challenged with *Salmonella* Typhimurium. It was hypothesized that the microbiome would differ between breeds and challenge status, as selection for growth has been associated with lower resistance to *Salmonella* (Guillot et al., 1995; Kramer et al., 2003). Similar to other studies, it was also expected that broiler age would have a prominent effect on microbial community characteristics (Schokker et al., 2021). This work could aid in understanding how intensive selection has played a role in the development of the intestinal microbiome as well as identifying and managing the prevalence of *Salmonella* in broiler flocks.

Materials and methods

Animals and experimental design

All procedures were approved by the University of Maryland Animal Care and Use Committee (IACUC#: R-NOV-19-55). A 2 × 2 split plot design was utilized with 156 male slow-growing broilers (SG) and 156 male conventional broilers (CONV). On day 0, all 312 chicks were placed in an open floor pen for co-mingling to establish a baseline microbial community. On day 7, twenty-four chicks from each breed were euthanized to obtain ileal and cecal contents (N = 48 total birds). After sampling, the remaining chicks were transferred to an animal biosafety level (ABSL) 2 research facility and separated into six isolators in four rooms with 11 chicks in each isolator (N = 24, total isolators). *Salmonella* Typhimurium strain #289-1 (Cox and Blankenship, 1975) was utilized to challenge the selected broilers because it was nalidixic acid (NAL)-resistant to allow for its isolation from any naturally colonizing *Salmonella* spp. On day 14, two rooms were randomly selected, and all birds in those rooms were orally gavaged with 1 ml of a tryptic soy broth (TSB) culture containing 1.3×10^8 colony forming units (CFUs)/

ml *Salmonella* Typhimurium (ST) while birds in the other two rooms were orally gavaged with a saline control (C) of tryptic soy broth. Two chicks from each isolator were euthanized and their ileal and cecal contents were collected on days 13, 17, 21 and 24 (N = 48 samples per day). The contents for day 7 remained in one tube with no media to be used for DNA extraction. The contents for days 13–24 were divided between two tubes, one tube with glycerol to be used for bacterial culturing and one tube with no media to be used for DNA extraction. The tubes were shipped on dry ice to Purdue University where they remained at −20 °C until further processing.

Salmonella enumeration

All samples for enumeration of *Salmonella* Typhimurium were kept at −20°C in glycerol until used. Samples were plated in triplicate on Bismuth Sulfite agar, supplemented with 200 μg/ml nalidixic acid and incubated for 18–20 h at 37°F. To determine the initial dilution in 20% glycerol at the time of sample collection, the samples were centrifuged 4,000 rpm for 15 min to pellet the cecal contents and the volume of the pellet and solution was determined in milliliters. Samples were resuspended before returning to the freezer.

Microbiome library preparation and analysis

DNA was extracted from samples using the MagAttract PowerMicrobiome DNA/RNA Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The concentration of the extracted DNA was determined using the Quant-iT PicoGreen dsDNA Assay Qubit dsDNA Assay Kit (Thermofisher Scientific Waltham, MA, United States) and subsequently normalized to 10 ng/ul by dilution in DNA-free molecular grade water. Extracted DNA was used for the construction of a 16S rRNA gene library following a standardized protocol (Kozich et al., 2013). Briefly, Illumina indexed amplicons were created using PCR amplification of the V4 region of bacterial 16S rRNA gene using the 515R (GTGCCAGCMGCCGCGGTAA)/806R (GGACTACHVGGGTWTCTAAT) primers. PCR and sequencing quality were assessed by preparing 16S rRNA gene libraries for a known positive control mock community (20 Strain Even Mix Genomic Material; ATCC® MSA1002TM) and water as a negative control. Amplification products were visualized through gel electrophoresis. No bands were observed in the negative control samples. Amplified DNA was normalized using a SequalPrep Normalization Plate (Invitrogen) and pooled into libraries containing the amplification products from 94 samples, mock community, and water. These libraries were sequenced (Illumina, MiSeq v2 kit, 2 × 500 cycle) at the Purdue Genomics Core Facility.

16S rRNA amplicon sequence analysis

Raw reads (25,694,292 ileal read pairs and 24,069,503 cecal read pairs) were analyzed using Quantitative Insight into Microbial Ecology (QIIME2, v.2020.2) (Bolyen et al., 2019). The general pipeline for QIIME2 is as follows: demultiplex samples from raw reads, process sequences through quality control filtering (DADA2) to remove low quality reads (Callahan et al., 2016), construct a feature table from corrected sequence data, produce a phylogenetic tree (Price et al., 2010), subsample features (max depth of 15,000 sequences per sample), and calculate diversity metrics. For DADA2, the 5' end of the ileal forward and reverse sequences were not trimmed (--p-trim-left-f 0 and --p-trim-left-r 0) while they were truncated at position 251 (--p-trunc-len-f 251 and --p-trunc-len-r 251). For the cecal sequences, both forward and reverse reads were trimmed at position 5 (--p-trim-left-f 5 and --p-trim-left-r 5) and truncated at position 251. After removal and processing, a total of 18,173,050 high quality sequences were obtained from the ileum and 16,915,543 were obtained from the cecum for downstream analysis. Ileal data was subsampled to 32,448 sequences per sample, resulting in removal of four samples; one from day 7 and three from day 13. All were CONV broiler samples. Cecal data was subsampled to 20,689 sequences per sample, only removing one sample from a challenged CONV broiler on day 21.

Data organization

In order to account for the effect of environment and age, samples from each day were analyzed separately resulting in five data sets. 7 day old chicks were co-housed together to establish a community baseline within the gastrointestinal tract. On day 7, chick was the experimental unit. After sequence quality filtering, 48 cecal and 40 ileal samples remained in the dataset. After day 7, the isolator was used as the experimental unit at each time point, so the counts of identical sequence groups (amplicon sequence variants, ASVs) of the two birds from the same isolator were averaged so that each bird was equally represented in the isolator composite sample. If one of the two replicates was removed during sequence quality filtering and rarefaction, the sequences from the remaining animal represented the isolator. In this study, all isolators on all time points had at least one representative animal.

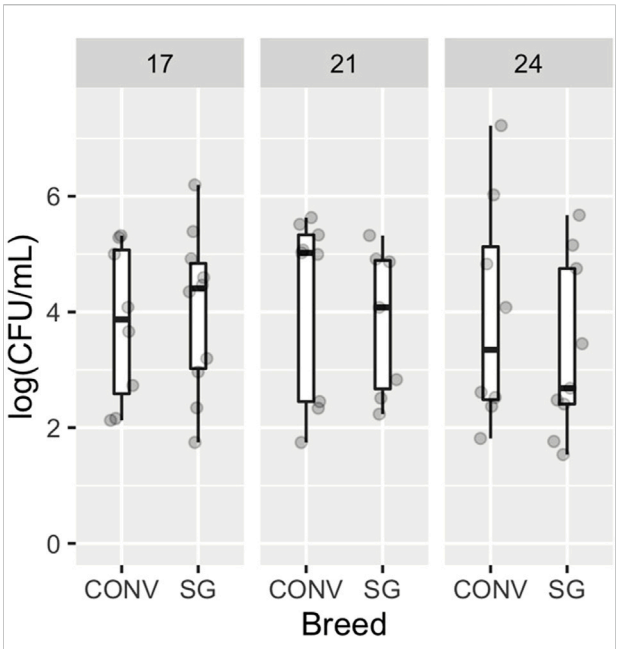

FIGURE 1
Enumeration of *Salmonella* Typhimurium from cecal contents of broilers. Box and whisker plots indicate the abundance of *Salmonella* Typhimurium in log colony forming units (CFU) per ml of cecal contents for conventional (CONV) and slow-growing (SG) broilers, separated by age. Points represent individual samples.

Statistical analysis

Statistics regarding *Salmonella* enumeration were completed using R software (v1.1.423). For each sample, the average colony forming unit (CFU) per milliliter cecal contents was calculated from the triplicate counts. A general linear model using ANOVA was created with the fixed effects of age and breed, and the random effect of isolator nested within room. Data were transformed using log10 to normalize the counts.

Alpha diversity metrics are used to describe community characteristics such as richness (observed ASVs), evenness (Pielou), and phylogenetic diversity (Faith) and biodiversity (Shannon). These were analyzed using a general linear model in R and a Type III Sum of Squares was utilized to account for unevenness between groups when running ANOVA. Assumptions for the normality of the residuals and homogeneity of variance were checked using the ggplot2 package and dependent variables not meeting these assumptions were log or square root transformed. Tukey's test of additivity was utilized to determine if the interaction between the effect of genetic line and *Salmonella* challenge was statistically significant. Statistical significance was defined as $p \leq 0.05$. Beta diversity (measure of dissimilarity between communities) was estimated using Bray Curtis, weighted Unifrac and unweighted Unifrac dissimilarities. Differences in beta diversity was determined using pairwise PERMANOVA tests. Differential abundance of genera was calculated with DESeq2 (v1.32.0) (Love et al., 2014). For purposes of reproducibility; metadata, scripts, and commands used in QIIME2 and R are available at https://github.com/sheets27/16SrRNABroilerSalmonella.

Results

Salmonella enumeration

All samples from unchallenged birds were determined by plate count to not have nalidixic acid resistant *Salmonella* Typhimurium and thus are not part of the following analyses (data not shown). There were no significant differences in log (CFU/mL) of *Salmonella* Typhimurium due to age ($p = 0.724$, $\omega^2 = -0.02$) nor breed ($p = 0.865$, $\omega^2 = -0.014$) as shown in Figure 1. It should be noted that the omega squared value (ω^2) indicates a small effect size. No interaction was found between age and breed.

The impact of breed on the intestinal microbiome

In the ileum on day 7, conventional broilers (CONV) had significantly greater Shannon diversity, phylogenetic diversity (Faith), and richness (observed ASVs) compared to slow-growing broilers (SG) (Figure 2, ANOVA, $p < 0.05$). In the cecal contents on day 7, phylogenetic diversity, richness, and evenness (Pielou) remained the same between the two breeds. After separation into isolators and sampling on day 13, there was no significant difference in community alpha diversity between CONV and SG broilers when evaluating both intestinal region microbiomes. On days 17 and 21, Shannon diversity in the cecum was found to be significantly higher in SG compared to CONV (Supplementary Figure S1, ANOVA, $p < 0.05$). On day 24, the main effect of breed did not significantly impact alpha diversity in the ileum or cecum of broilers.

Beta diversity showed a similar pattern as alpha diversity for 7-day-old broilers. In the ileum samples on day 7, Bray-Curtis, unweighted Unifrac, and weighted Unifrac were all significantly affected by breed (Supplementary Figure S2, PERMANOVA, $p < 0.05$). Also in the ileum on day 7, ASVs assigned to *Enterococcus*, *Lactobacillus*, and *Romboutsia* were enriched in CONV broilers while ASVs assigned to *Bacillales*, *Sporosarcina*, *Paenibacillus*, and *Planococcaceae* were enriched in SG broilers (Figure 3, $p < 0.05$). In the cecum on day 7, Bray-Curtis distances were also slightly different between breeds (Supplementary Figure S3,

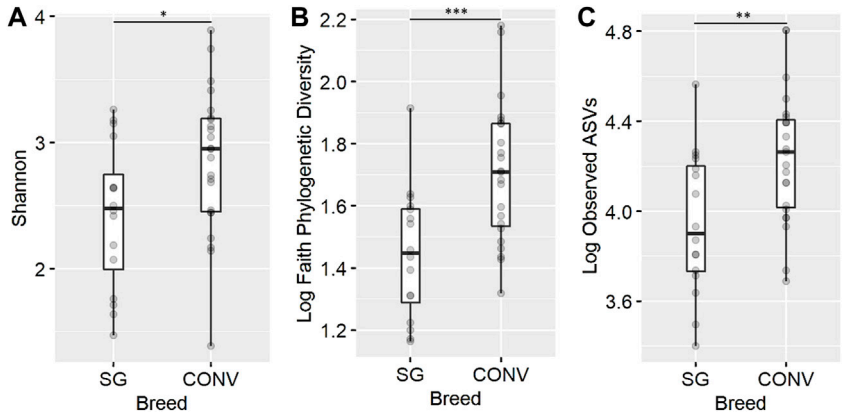

FIGURE 2
The effect of genetic line on the ileal Shannon diversity **(A)**, Faith phylogenetic diversity **(B)**, and observed ASV richness **(C)** for 7-day-old broilers. The presence of an asterisk indicates a significant difference between breeds in which * corresponds to $p < 0.05$, ** corresponds to $p < 0.01$, and *** corresponds to $p < 0.001$. Conventional (CONV) and slow-growing (SG) broilers.

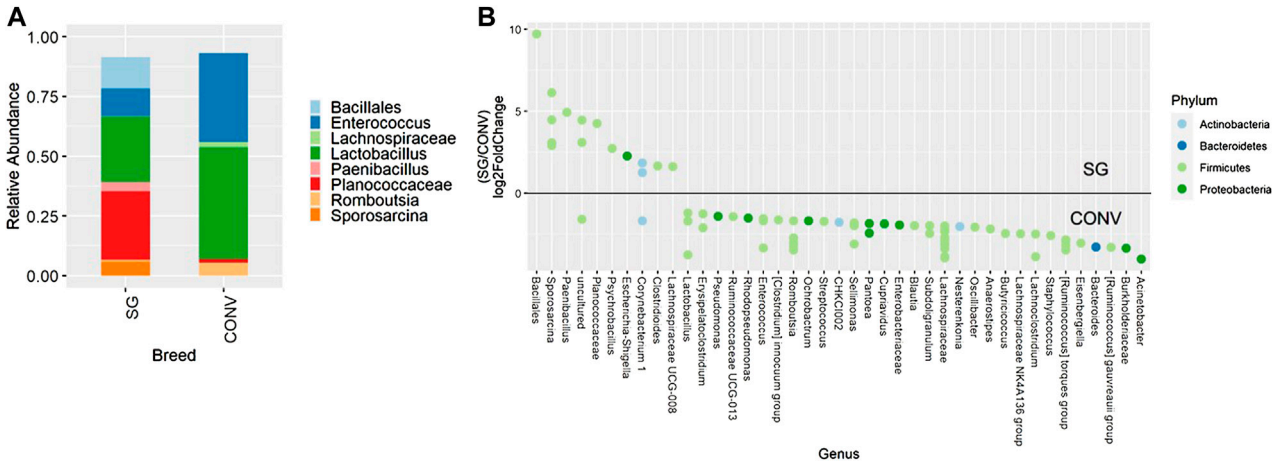

FIGURE 3
Relative abundance of genera in the ileum on day 7 that are present in greater than 2% of total taxa found in conventional (CONV) and slow-growing (SG) broilers **(A)**. Differentially abundant amplicon sequence variants (ASVs) between broiler genetic lines in the ileum on day 7 **(B)**. Significantly different ($p < 0.05$) ASVs are presented and organized by abundance within each breed. ASVs enriched in SG broilers are indicated with a log 2-fold change >0 while ASVs enriched in CONV broilers are indicated with a log 2-fold change of <0.

PERMANOVA, $p < 0.05$) while Unifrac measures were not different. On day 13, the cecal communities in CONV and SG broilers were different when using Bray-Curtis and weighted Unifrac distances (Supplementary Figure S4, PERMANOVA, $p < 0.05$). An ASV assigned to *Lachnospiraceae* NK4A136 as well as ASVs assigned to *Oscillibacter*, *Ruminiclostridium* 9, and *Mollicutes* RF39, were enriched in the cecum of CONV broilers on day 13 (Supplementary Figure S5, $p < 0.05$). Similarly, Bray-Curtis and weighted Unifrac measurements were also significantly different because of breed when evaluating the cecum of 21-day-old broilers (Supplementary Figure S6, PERMANOVA, $p < 0.05$) with *Lachnospiraceae* NK4A136 being abundant in CONV broilers at this time point as well (data not shown).

The impact of challenge on the intestinal microbiome

Several alpha diversity measures were affected by the main effect of *Salmonella* Typhimurium challenge. On day 17 of the experiment, 3 days after *Salmonella* gavage, challenged

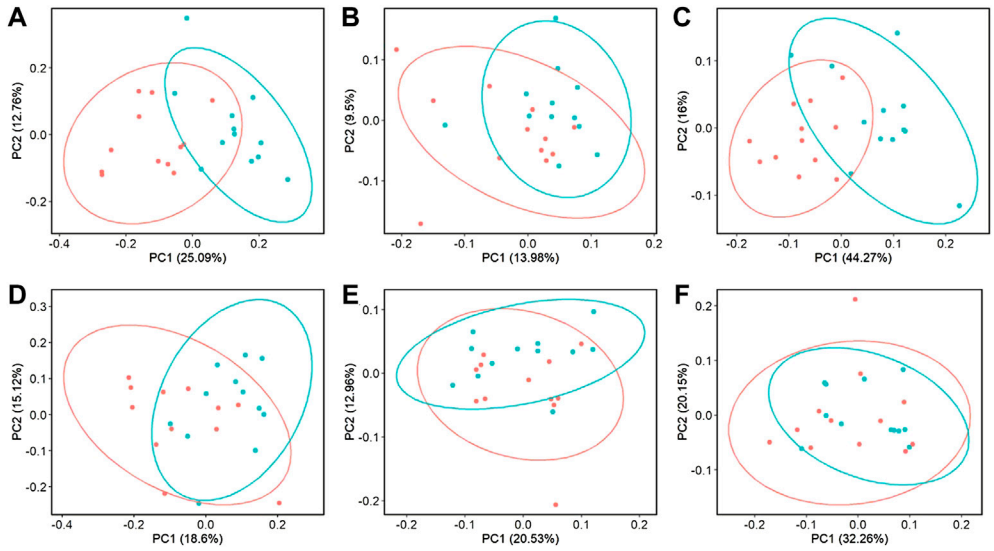

FIGURE 4
The effect of *Salmonella* challenge on beta diversity measures in the cecum. Significant dissimilarity was seen in Bray-Curtis **(A,D)**, unweighted Unifrac **(B,E)**, and weighted Unifrac **(C,F)** for 17 **(A–C)** and 24-day-old **(D–F)** broilers. Red represents control (C) broilers while blue represents *Salmonella* Typhimurium challenged (ST) broilers.

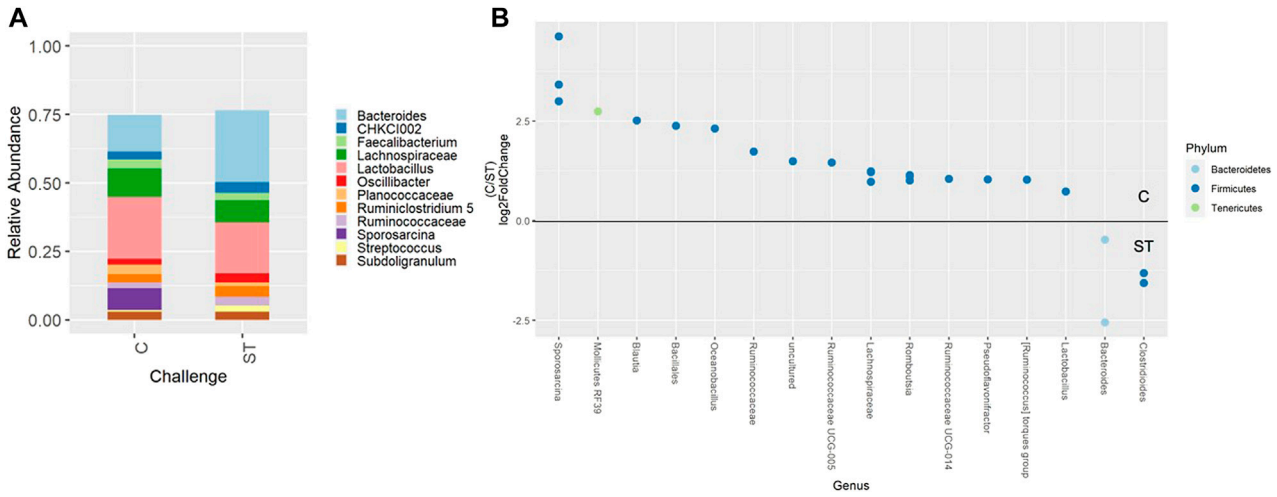

FIGURE 5
Relative abundance of genera in the cecum on day 17 that are present in greater than 2% of total taxa found within control (C) and *Salmonella* Typhimurium challenged (ST) broilers **(A)**. Differentially abundant amplicon sequence variants (ASVs) between broiler challenge groups in the cecum on day 17 **(B)**. Significantly different ($p < 0.05$) ASVs are presented and organized by abundance within each group. ASVs enriched in C broilers are indicated with a log 2-fold change >0 while ASVs enriched in ST broilers are indicated with a log 2-fold change of <0.

broilers (ST) had significantly lower Shannon diversity in the cecum compared to non-challenged, control broilers (C) (Supplementary Figure S7, ANOVA, $p < 0.05$). In regard to the ileum, ST broilers were found to have lower richness on day 21 but greater evenness on day 24 compared to controls (Supplementary Figure S8, ANOVA, $p < 0.05$).

Beta-diversity analyses showed consistent cecal dissimilarity throughout later time points between ST and C broilers. There was substantial overlap in Bray-Curtis as well as unweighted and weighted Unifrac ellipses, yet these measures significantly differed due to challenge at 17 and 24 days of age (Figure 4, PERMANOVA, $p < 0.05$). Many

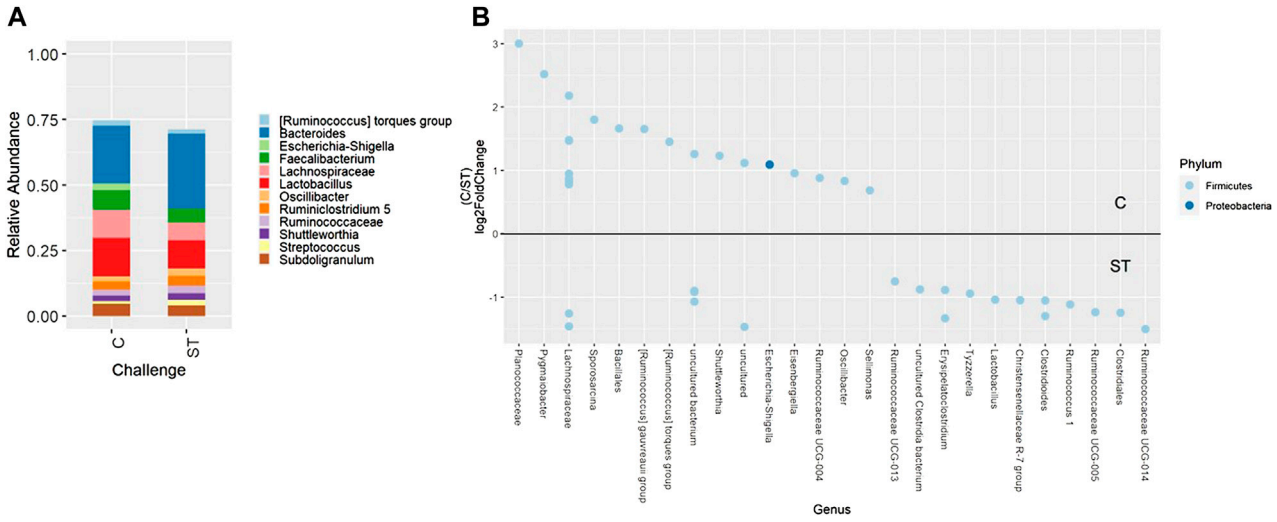

FIGURE 6
Relative abundance of genera in the cecum on day 24 that are present in greater than 2% of total taxa found within control (C) and *Salmonella* Typhimurium challenged (ST) broilers **(A)**. Differentially abundant amplicon sequence variants (ASVs) between broiler challenge groups in the cecum on day 24 **(B)**. Significantly different ($p < 0.05$) ASVs are presented and organized by abundance within each group. ASVs enriched in C broilers are indicated with a log 2-fold change >0 while ASVs enriched in ST broilers are indicated with a log 2-fold change of <0.

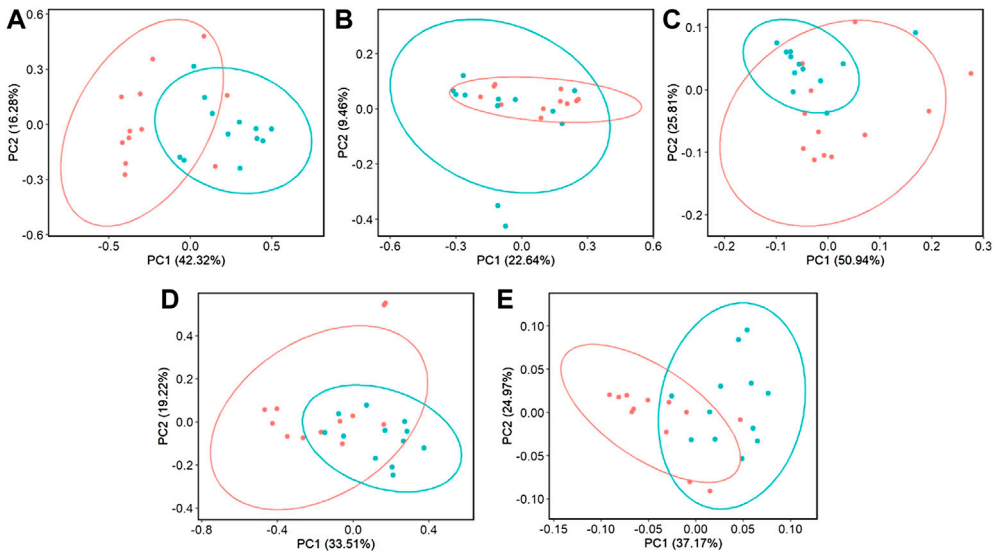

FIGURE 7
The effect of *Salmonella* challenge on beta diversity measures in the ileum. Significant dissimilarity was seen in Bray-Curtis **(A,D)**, unweighted Unifrac **(B)**, and weighted Unifrac **(C,E)** for 21 **(A–C)** and 24-day-old **(D,E)** broilers. Red represents control (C) broilers while blue represents *Salmonella* Typhimurium challenged (ST) broilers.

differentially abundant genera were found when comparing the cecal communities of non-challenged to challenged broilers on both day 17 and 24. In challenged broilers on day 17, three *Sporosarcina* ASVs were reduced about 8-fold and two ASVs of *Bacteriodes* were increased (Figure 5, $p <$ 0.05). On day 24, challenged broilers had decreased relative abundance of several ASVs including 5 *Lachnospiraceae* ASVs (decreased by 3-fold), and single ASVs of *Planococcaceae* (decreased by 8-fold), *Sporosarcina*, *Shuttleworthia*, *Ruminococcus gauvreauii* group, and *Oscillibacter* (Figure 6, $p < 0.05$).

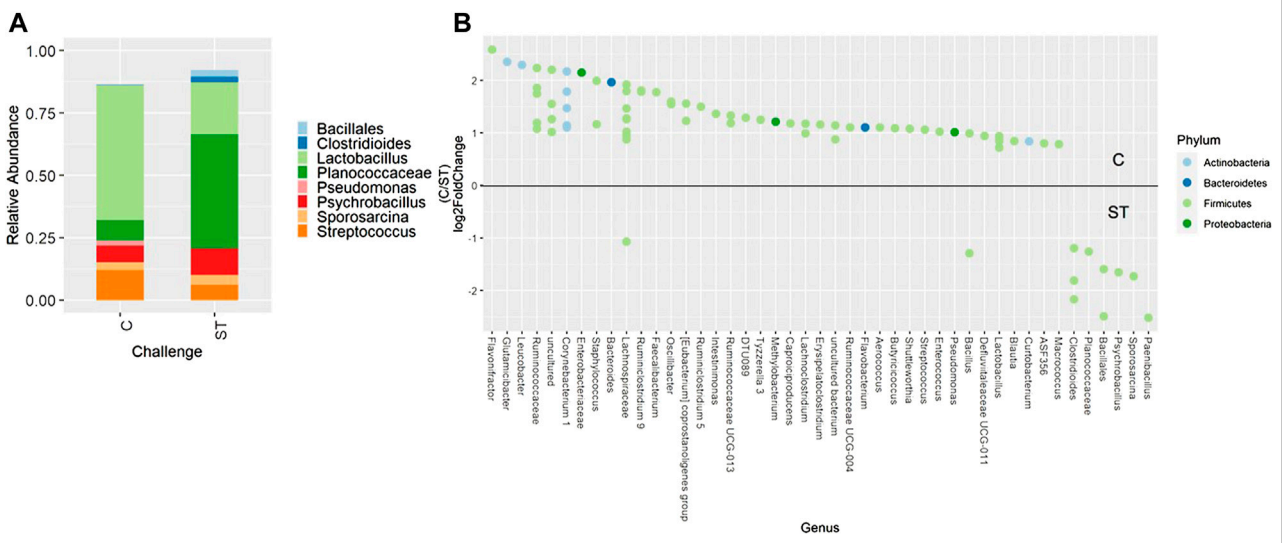

FIGURE 8
Relative abundance of genera in the ileum on day 21 that are present in greater than 2% of total taxa found within control (C) and *Salmonella* Typhimurium challenged (ST) broilers **(A)**. Differentially abundant amplicon sequence variants (ASVs) between broiler challenge groups in the ileum on day 21 **(B)**. Significantly different ($p < 0.05$) ASVs are presented and organized by abundance within each group. ASVs enriched in C broilers are indicated with a log 2-fold change >0 while ASVs enriched in ST broilers are indicated with a log 2-fold change of <0.

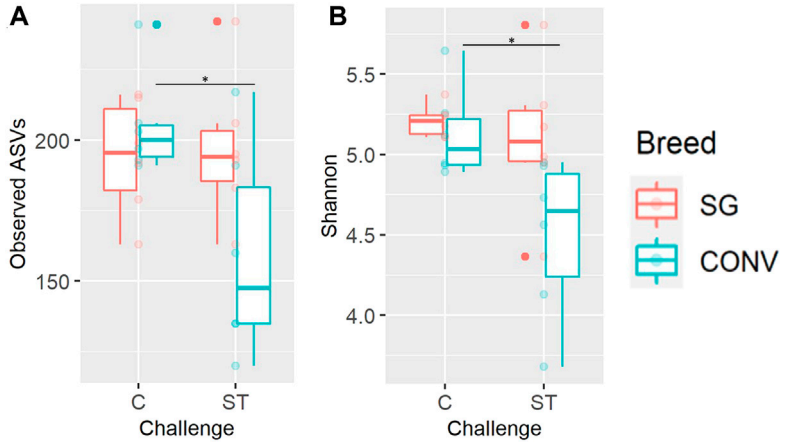

FIGURE 9
The interaction between of genetic line and *Salmonella* challenge on the cecal observed amplicon sequence variant (ASV) richness **(A)** and Shannon diversity **(B)** for 17-day-old broilers. The presence of an asterisk indicates a significant difference between pairwise groups in which * corresponds to $p < 0.05$, ** corresponds to $p < 0.01$, and *** corresponds to $p < 0.001$. Control (C) and *Salmonella* Typhimurium challenged (ST) broilers.

When evaluating ileal samples, all beta diversity measurements were found to be significantly different due to challenge on day 21 (Figures 7A–C, PERMANOVA, $p < 0.05$), and Bray-Curtis and weighted Unifrac distances were significantly different at day 24 (Figures 7D,E, PERMANOVA, $p < 0.05$). Along with community dissimilarity between broilers of different challenge states, many differentially abundant ASVs were identified. On day 21, ASVs from 37 genera were increased in non-challenged broilers, including *Flavonifractor*, *Ruminiclostridium* 9, *Oscillibacter*, and *Shuttleworthia* while challenged broilers were enriched in ASVs from only 6 genera including *Clostridioides*, *Bacillales*,

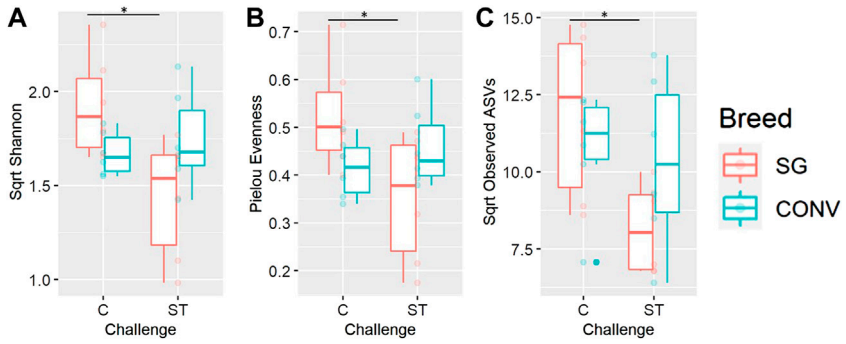

FIGURE 10
The interaction between of genetic line and *Salmonella* challenge on the ileal Shannon diversity **(A)**, Pielou evenness **(B)**, and observed amplicon sequence variant (ASV) richness **(C)** for 21-day-old broilers. The presence of an asterisk indicates a significant difference between pairwise groups in which * corresponds to $p < 0.05$, ** corresponds to $p < 0.01$, and *** corresponds to $p < 0.001$. Control (C) and *Salmonella* Typhimurium challenged (ST) broilers.

and *Paenibacillus* (Figure 8, $p < 0.05$). Fewer differentially abundant genera were found in the ileum of 24 day old broilers, but *Clostridioides* continued to be differentially abundant in ST broilers compared to C broilers (Supplementary Figure S9, $p < 0.05$).

The interaction effects between breed and *Salmonella* challenge on the intestinal microbiome

Alpha and beta diversity metrics of both the ileal and cecal microbiome were impacted by *Salmonella* challenge differently according to breed (interaction effect). In the ileum, there was a significant interaction between breed and challenge when measuring Shannon diversity, richness, and phylogenetic diversity of 7-day-old broilers, yet there were no significant pairwise comparisons when evaluating this interaction (Supplementary Figure S10, Tukey, $p < 0.05$). When evaluating the cecum on day 17, there was a significant breed and challenge interaction regarding richness (observed ASVs metric) with challenged CONV broilers having significantly lower richness compared to non-challenged CONV broilers (Figure 9A, Tukey, $p < 0.05$). No interaction was detected in Shannon diversity but challenged CONV broilers had significantly lower Shannon diversity compared to non-challenged CONV broilers in the cecum on day 17 (Figure 9B, Tukey, $p < 0.05$). On day 21 in the ileum, an interaction was detected where challenged SG broilers had significantly lower Shannon diversity, Pielou evenness, and observed ASV richness compared to non-challenged SG broilers (Figure 10, Tukey, $p < 0.05$). On day 21 and 24 in the cecum, only phylogenetic diversity exhibited a significant interaction between breed and challenge, with no significant pairwise comparisons for this alpha diversity measure (Supplementary Figure S11, Tukey, $p < 0.05$).

Regarding beta diversity, there was a significant interaction between breed and challenge on day 17 in the ileum when evaluating weighted Unifrac distances (Supplementary Figure S12, PERMANOVA, $p < 0.05$). Specifically, differences were found between CONV broilers of different challenge states and between challenged SG and challenged CONV broilers (Supplementary Figure S12, Pairwise PERMANOVA, $p < 0.05$). For the remaining days, no significant beta diversity interaction effects were found in ileal or cecal samples.

Discussion

Ileal and cecal microbiomes have been characterized and well-studied in conventional broiler populations to help identify how modulations of the gut microbial community could influence a variety of performance and disease responses (Oakley et al., 2013; Stanley et al., 2013; Clavijo and Flórez, 2018). This is because the microbial community of the gastrointestinal tract plays a role in extracting energy from nutrients as well as harboring potential pathogenic organisms such as *Campylobacter*, *Salmonella enterica*, *Escherichia coli*, and *Clostridium perfringens* that can colonize and cause illness to the avian host and humans (Clavijo and Flórez, 2018). The current body of literature includes many microbiome analyses regarding the effect of probiotics (Gao et al., 2017; Rodrigues et al., 2020; Gyawali et al., 2022) or pathogen exposure (Park et al., 2017; Latorre et al., 2018; Joat et al., 2021) on the broiler microbiome. However, it should be noted that many of these single-facet studies utilize conventional, fast-growing broilers such as the Ross or Cobb breeds. Moreover, the concern for improved animal welfare has allowed slow-growing commercial broiler

breeds to become recognized by well-informed consumers (Lusk, 2018). Previous research has compared characteristics between slow-growing and conventional broilers related to differences in behavior (Bokkers and Koene, 2003; Çavuşoğlu and Petek, 2019), gene profile (Cui et al., 2012; Hu et al., 2013), carcass traits (Mikulski et al., 2011; Singh et al., 2021), or immune response (Williams et al., 2013; Giles et al., 2019), while a limited number of studies have compared slow-growing and conventional breeds in their intestinal microbiota and the interaction of the microbiota with a separate factor such as the addition of a feed additive or pathogen challenge. Therefore, the purpose of this study was to investigate the ileal and cecal microbiota response to *Salmonella* challenge in both conventional and slow-growing broilers. The behavior and immune response results from the broilers in this study have been published previously (Snyder et al., 2022).

The impact of genetics on the development and composition of the intestinal microbiome is an active area of research in efforts to understand factors that control microbiome modulation. In the present study, there were significant differences in the alpha diversity of both the ileal and cecal microbiome of broilers, but these distinctions were variable across time and dependent on the intestinal location under study. The ileal microbial community alpha and beta diversities were affected by breed on day 7, while the effect of genetic line became prominent in the cecal microbial community beta diversity beginning on day 13. Differences in microbial composition appear to begin in the ileum and progress to the cecum due to differential development of the tissue between the two breeds during those times (Danzeisen et al., 2015). Strong selection for broilers with high digestive efficiency produced heritable microbial communities with specific ratios of bacteria such as *Lactobacillus crispatus*, *Clostridium leptum*, and *Clostridium coccoides*, and *Escherichia coli* (Mignon-Grasteau et al., 2015). The intimate relationship between bacteria and nutrient digestibility may allow for a clearer signal when evaluating the interaction between breed and microbial composition (Kim et al., 2015; Marmion et al., 2021). Additionally, one study used highly established genetic lines to determine that the abundance of 29 fecal microbiome species was different between two egg-laying chicken breeds after 54 generations of selection for high or low market body weight (Zhao et al., 2013). One review evaluating the ileal microbiota of Ross and Cobb broilers emphasized that these breeds have different microbiota compositions, yet taxonomical enrichment was not consistently present in either breed over time (Kers et al., 2018). The results presented in this study and others suggest that the gut microbiome can be distinct between two different broiler breeds and the level of this distinction may be influenced by the selected trait and the length of selection as well as age.

The clearest sign that breed affected broiler intestinal microbiome in the current study was at the youngest age. On day 7, slow-growing broilers had lower diversity and richness in the ileum compared to fast-growing, conventional broilers. Not only was a change in the ileal microbiome observed, but on the same day, conventional broilers were found to have greater villus height and crypt depth in the jejunum based on a concurrent study, suggesting better intestinal health (Snyder et al., 2022). Thus, the microbial communities between a slow-growing broiler and a conventional broiler may vary because of the difference in the morphology of the small intestines. The impact of breed on microbiota diversity measures became less clear in the ileum as the birds grew older and their intestinal morphology developed. Indeed, current literature shows age commonly has a greater impact on the alteration of the microbiome compared to breed (van der Wielen et al., 2002; Hume et al., 2003; Gong et al., 2008). This, together with our data, suggests that observing the direct impact of breed on the microbiome becomes more difficult as the host grows older and the microbiome becomes altered potentially due to intestinal tissue development and exposure to new environmental conditions (Díaz-Sánchez et al., 2019). This may be of importance in regard to identifying beneficial feed additives that have the purpose of affecting the microbiome. A more apparent response might be expected between different broiler breeds when the additive is administered at a younger age.

Differences seen in beta diversity between slow-growing and conventional broilers are further understood when examining differences in taxonomic profile. On day 7, several taxa from Firmicutes that have been shown to be advantageous were differentially abundant in slow-growing and conventional broilers. For example, only *Sporosarcina* was significantly enriched in slow-growing broilers and previous research has indicated that *Sporosarcina* may have potential probiotic characteristics (Priyodip and Balaji, 2019). On the other hand, conventional broilers in the current study were found to have a single amplicon sequence variant of *Oscillibacter*, *Lachnospiracea* NK4A136, and *Ruminococcus gauvreauii* that were differentially abundant in the ileum on day 7. Both *Oscillibacter* and *Lachnospiracea* NK4A136 are short chain fatty acid producers while *Ruminococcus gauvreauii* may be involved with feed efficiency (Madigan-Stretton et al., 2020).

Along with genetics and age, it was found that challenge with *Salmonella* Typhimurium caused community shifts in alpha and beta diversity in both the ileum and cecum. Beneficial microbes such as *Mollicutes* RF39, *Shuttleworthia*, *Ruminiclostridium* 9 and *Flavonifractor* were found to be decreased in the intestinal tract of challenged broilers in the cecum on day 17, the ileum on day 21, and in both intestinal locations on day 24. *Flavonifractor* is positively correlated with body weight and average daily gain in broilers given *Bacillus subtilis* as a probiotic (Zhang et al., 2021). *Shuttleworthia* may also contribute to nutrient absorption because it contributes to carbohydrate and lipid metabolic pathways (Chen et al., 2020). *Ruminiclostridium* 9 plays a role complex carbohydrate metabolism (Joat et al., 2021) and members from the Mollicutes class are involved with energy harvesting in the gastrointestinal

tract (Turnbaugh et al., 2008). From our results, the introduction of *Salmonella* affected the relative abundance of certain commensals in the microbiome, resulting in a change in taxonomic profile and overall composition of the microbial community. This could be of significance because *Salmonella* challenge may be a contributing factor to dysbiosis or further bacterial invasion in the intestinal microbiota. These possible side-effects would need to be tested further to be confirmed.

The present study evaluated the interaction between genetic line and *Salmonella* challenge to find the intestinal microbiome was impacted differently by *Salmonella* between broilers of each breed at specific time points. These varying results have been found in several other studies including one that analyzed the outcome of *Campylobacter* infection between slow-growing and fast-growing broilers, finding fast-growing broilers infected with *Campylobacter jejuni* had greater incidences of pododermatitis compared to slow-growing breeds (Williams et al., 2013). Organic flocks composed of slow-growing broilers had a significantly higher prevalence of *Campylobacter* compared to broilers in conventional flocks (Heuer et al., 2001). Despite being colonized with *Salmonella* Typhimurium to the same extent, there were significant differences in how slow-growing and conventional broilers were impacted by *Salmonella* challenge. Alpha diversity measures decreased in the cecum of challenged conventional broilers on day 17 whereas alpha diversity measures deceased in the ileum of challenged slow-growing broilers on day 21. These results suggest that the microbiome of these two genetic lines are susceptible to *Salmonella*-induced dysbiosis at different times and intestinal locations. Further research is warranted to understand the specific immune response to *Salmonella* challenge in these broiler lines; it may be associated with the upregulation of genes related to T-cell activation in response to *Salmonella* challenge (van Hemert et al., 2000) or that conventional broilers have significantly higher concentrations of IgA and IgG on day 21 (Snyder et al., 2022).

The results from this experiment provide insight regarding the role of broiler genetic selection on the microbiome and how it may impact enteric colonization resistance. A breed effect on the ileal and cecal microbiome occurred between slow-growing and conventional broilers and the effect was dependent on the age and specific intestinal location, as a greater difference between the breeds was observed in the ileum in younger broilers, and in the cecum in older broilers. *Salmonella* Typhimurium challenge caused a shift in the microbial communities, and differences in bacterial relative abundances were observed in certain ages and intestinal regions. Some potentially beneficial microbes were depleted in broilers challenged with *Salmonella* Typhimurium. An interaction between broiler genetic line and *Salmonella* Typhimurium challenge was found and showed the microbiomes of the two different breeds were each negatively affected by *Salmonella* challenge, but at different ages. Results from the present study demonstrate the dynamic nature of the broiler microbiome and how pathogen exposure can result in temporary (or inconsistent across all ages) and localized changes to the intestinal microbiota.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, Bioproject PRJNA803251 and Biosamples SAMN25640069-SAMN25640548.

Ethics statement

The animal study was reviewed and approved by the University of Maryland Animal Care and Use Committee (IACUC#: R-NOV-19-55).

Author contributions

SW and TJ contributed to the conception and design of the study. CW assisted AS with the experimental design and *Salmonella* enumeration. TS led laboratory procedures and TJ assisted TS with statistical analyses. TS and TJ led in writing the manuscript. All authors approve the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Interaction between the hypothalamo-pituitary-adrenal and thyroid axes during immobilization stress

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The location of corticotropin-releasing hormone receptor 2 (CRH-R2) on thyrotropes within the avian anterior pituitary (APit) and its activation by different stressors indicate a possible communication between hypothalamo-pituitary-adrenal (HPA) and thyroid (HPT) axes. Therefore, an experiment was designed to 1) compare the timing of major components of the HPT axis to those of the HPA axis; 2) address whether stressors activating the HPA axis may simultaneously upregulate components of the HPT axis. Blood, brain, and APit were sampled from chicks prior to stress (control) and 15, 30, 60, 90, and 120 min following immobilization (IM) stress. The nucleus of the hippocampal commissure (NHpC) and paraventricular nucleus (PVN) were cryo-dissected from brains for RT-qPCR. Gene expression of thyrotropin-releasing hormone (TRH) and its receptors (TRH-R1 and TRH-R3), urocortin3 (UCN3), deiodinase 2 (D2), and the second type of corticotropin-releasing hormone (CRH2) within the NHpC and PVN was measured. Additionally, gene expression of TRH receptors, thyroid stimulating hormone subunit beta (TSHβ), and D2 was determined in the APit and corticosterone assayed in blood. In brains, a significant upregulation in examined genes occurred at different times of IM. Specifically, UCN3 and CRH2 which have a high affinity to CRH-R2 showed a rapid increase in their mRNA levels that were accompanied by an early upregulation of TRHR1 in the NHpC. In the APit, a significant increase in gene expression of TSHβ and TRH receptors was observed. Therefore, results supported concurrent activation of major brain and APit genes associated with the HPA and HPT axes following IM. The initial neural gene expression originating within the NHpC resulted in the increase of TSHβ mRNA in the APit. Specifically, the rapid upregulation of UCN3 in the NHpC appeared responsible for the early activation of TSHβ in the APit. While sustaining TSHβ activation appeared to be due to both CRH2 and TRH. Therefore, data indicate that CRH-producing neurons and corticotropes as well as CRH- and TRH-producing neurons and thyrotropes are activated to produce the necessary energy required to maintain homeostasis in birds undergoing stress. Overall, data support the inclusion of the NHpC in the classical avian HPA axis and for the first time show the concurrent activation of the HPA axis and components of the HPT axis following a psychogenic stressor.

KEYWORDS

immobilization stress, NHpC, trh, TRHR1, CRH2, TSH, CRHR2

1 Introduction

In birds as in mammals, the HPA axis is essential and important for regulating stress. The hypothalamic PVN contains two peptides, CRH and arginine vasopressin/vasotocin (AVP/AVT), that drive the HPA axis. The two neuropeptides are transported to the APit and bind to G protein-coupled receptors located on the cell membrane of corticotropes to activate proopiomelanocortin (POMC) genes within the cells that ultimately produce an adrenocorticotropic hormone (ACTH) (Blas, 2015). Secretions of ACTH into the cardiovascular system are carried to the adrenal glands for synthesis and release of glucocorticoids [cortisol in humans and corticosterone (CORT) in birds and other vertebrates] to redirect energy resources to meet real or anticipated demands (Romero, 2004; Carsia, 2015; Herman and Tasker 2016).

The corticotropin-releasing hormone binds primarily to its receptor 1 (CRH-R1) in mammals and other vertebrates located on corticotropes (Aguilera et al., 2004). Less attention has been paid to the discovery of a second CRH receptor (CRH-R2) in birds located in APit thyrotropes (De Groef et al., 2004). Within the CRH family, the gene UCN3 was found to be expressed predominantly, but not exclusively in the hypothalamus, pons, and medulla of chick brains (Grommen et al., 2017). UCN3 modulates the behavioral and neuroendocrine system that helps maintain homeostasis in response to stress (Jamieson et al., 2006) and has a much higher affinity for CRH-R2 than CRHR-R1 (Lewis et al., 2001). Recently, a second CRH peptide was discovered, termed CRH2, and found across vertebrate classes (Grone and Maruska, 2015). The gene was identified in chickens (CRH2) and shown to be 15-fold more potent in activating CRH-R2 than CRH-R1 and potently stimulates TSHβ in pituitary cell culture studies (Bu et al., 2019). All brain regions showed high relative brain levels of CRH2 mRNA including the hypothalamus. Additionally, the pituitary gland showed the highest mRNA levels compared to the whole brain and 13 other tissues and organs sampled (Bu et al., 2019).

The presence of CRH-R2 on the thyrotropes indicates a direct positive or negative effect of CRH and/or CRH-like peptides (UCN3 and CRH2) on thyrotropes *via* CRH-R2. Ultimately, this could influence gene expression of TSHβ in addition to the effect of central secretion of TRH on the TSH secretion from APit *via* its receptors (TRH-R1 and TRH-R3). Hence, the net effects of CRH and CRH like peptides (CRH2 and UCN3) are to increase or decrease thyroid hormone (T4 and T3), indicating a possible interaction between the HPA and HPT axes, where the latter classically played an important role in growth, differentiation, and metabolism (De Groef et al., 2005; Brent, 2012; Mullur et al., 2014). However, the role of the HPT axis in regulating stress has received much less attention. Nonetheless, high concentrations of TRH have been shown to occur in the chicken hypothalamus and anterior pituitary (Geris et al., 1999) and TRH-R1 is expressed in the anterior pituitary (De Groef et al., 2003) and TRH-R3 is highly expressed in the hypothalamus and anterior pituitary (Li et al., 2020).

The distribution of CRH neurons is well characterized in the chicken brain (Richard et al., 2004). We found CRH neurons in a septal brain structure called the NHpC (Nagarajan et al., 2017a). Following stress, CRH neurons in the NHpC responded rapidly after initiation by two different stressors, feed deprivation (Nagarajan et al., 2017b; Kadhim et al., 2019) and immobilization (IM; Kadhim et al., 2021). The two stressors were used to determine the sequence of gene activation within the avian HPA axis. Since each of two different stressors activated CRH-R2 located on thyrotropes suggest strongly that stressors not only activate the HPA axis but also the HPT axis. We hypothesized that stress stimulates the HPA and components of the HPT axis, and both axes are activated concurrently. The following aims were addressed: 1) timing of neural secretion from the NHpC and PVN in TSHβ gene expression during stress; 2) gene expression of TRH, TRH-R1, and TRH-R3; 3) role of CRH2 and UCN3 in TSHβ gene activation; 4) possible role of the HPT axis in the neuroendocrine regulation of stress.

2 Materials and methods

2.1 Animals

Broiler chicks (one-day old male Cobb 500) were obtained from a commercial hatchery and raised on our poultry farm directly north of the University of Arkansas. Chicks were provided feed (a standard, broiler starter diet) and water *ad libitum* and exposed to continuous light for the first 3 days so that they could locate both the feed and water. Thereafter, birds were maintained under a daily photoperiod of 16 h: 8 h light/dark cycle (LD 16:8)T, lights on at 6:00 a.m. A heating program was started initially at 32°C and reduced by 2.5°C weekly until reaching 24°C. At 5 weeks of age, experiments were initiated, and sampling occurred between 8:00 a.m. and 4:00 p.m. Each experimental group of chicks had an equal representation of chicks sampled throughout the 8 h of daily sampling times by establishing four, consecutive 2 h time blocks. The same pattern of sampling was followed and repeated until 4:00 p.m. in order to minimize any potential diurnal variation on plasma samples analyzed for CORT levels by RIA (see Section 2.3). All procedures utilized (i.e., immobilization, housing conditions, handling, and sampling) were approved by the University of Arkansas Institutional Animal Care and Use Committee.

TABLE 1 List of primers.

Gene	Accession #	Sequences (5′-3′) (forward/reverse)	Amplicon size (bp)	References
CRH2	KU887752	CGGAGCAGCGGCAGCGGTAT	139	Bu et al. (2019)
		CTGCAGCGGGGAGCAGCTCT		
TRH	XM_025154454	CTGGATGACATCCTGCAGAG	110	Li et al. (2020)
		CATTGTGGCAGAGGCATG		
TSHβ	XM_025143670	CCACCATCTGCGCTGGAT	128	Designed in our lab
		GCCCGGAATCAGTGCTGTT		
TRH-R1	NM_204930	TGAATCCCATCCCTTCGGAC	202	Li et al. (2020)
		ACCACCAGTGTTCGATAGGG		
TRH-R3	XM_004947049	GCAGGGGTTTGGGTGGATAA	163	Li et al. (2020)
		GCTTCAGCCAGTTTCCAAGC		
UCN3	XM_001231710	CCACCAACATCATGAACATCCTGCCTTCGCCCTCAAGTTCTT	61	Ogino et al. (2014))
D2	NM_001324555	TGCGCGCGGTCAAACTT	117	Fallahshahroudi et al. (2019)
		ACTTGGTTCCACACTTGCCA		

2.2 Stress procedure and sample collection for gene expression

Immobilization (IM) stress was initiated on week 5, with feed and water provided *ad libitum*. Chicks were randomly divided into six treatment groups (10–12 birds/group): one group was control, and the rest included five stressed groups: 15, 30, 60, 90, and 120 m following IM, where m = minutes of stress. In stressed groups, birds were secured in a harness where they could not move their wings nor stand, however, did have access to water and feed during the period of restraint. Stress treatments were designed so that all birds would be sampled individually directly after their stress period ended. First, blood was withdrawn from the brachial vein, transferred to a heparinized tube, and refrigerated. Each bird was then cervically dislocated and the brain and APit were rapidly dissected. Brain samples were immersed in two-methyl butane at −30°C for 15 s to maintain their structural morphology for cryo-sectioning and placed in dry ice before being stored at −80°C. Anterior pituitaries were likewise frozen in dry ice and stored at −80°C. Later, thick coronal sections of brain samples were cut at 100 µm using a cryostat and the targeted structures were punched with a stainless steel cannula (brain punch; Palkovits, 1973) using a glass pipette. The NHpC and PVN were micro-dissected, transferred to TRIzol, and stored at −20°C until processed for RNA extraction. Some samples were excluded due to insufficient RNA concentration. Final sample size per group was 8–10 birds/treatment. The procedural details were described in our recent publications (Nagarajan et al., 2017a; Nagarajan et al., 2017b; Kadhim et al., 2019; Kadhim et al., 2020; Kadhim et al., 2021).

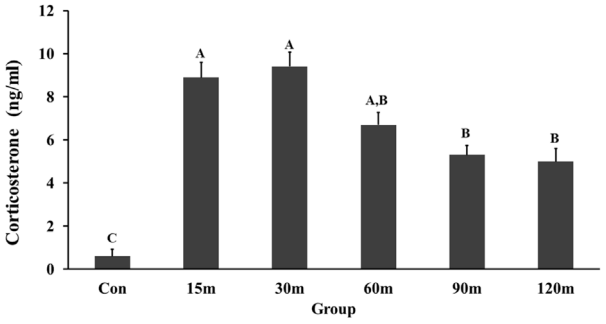

FIGURE 1
Changes in plasma corticosterone concentration in response to different times of immobilization stress. Histograms show the mean ± SEM. Significant differences ($p < 0.05$) among treatment groups are indicated by different letters above each time point. Abbreviations: Con, controls; m, minutes.

2.3 Radioimmunoassay utilized for blood samples

Plasma was obtained from heparinized blood (n = 10–12/treatment) *via* centrifugation at 3,000 rpm for 20 m at 4°C and stored at −20°C until analysis of CORT concentrations by RIA (Proudman and Opel, 1989; Madison et al., 2008; Kadhim et al., 2021). Hemolyzed samples (1-2 samples/group) were excluded. Briefly, primary antibody (100 µl, polyclonal rabbit anti-CORT # 377, kindly provided by Dr. Proudman) and ^{125}I corticosterone tracer (100 µl, MP Biomedicals Inc., Orangeburg, NY, US477A) were incubated with each sample for at least 24 h at 4°C. Sheep anti-rabbit antibody (200 µl) was used as the secondary antibody (MP Biomedicals Inc., Orangeburg, NY, United States). Counts/

tubes were determined using a Perkin Elmer Wizard gamma counter. Samples were assayed in duplicate. After statistical analysis, data were expressed as the mean ± SEM for each group. In all studies, $p < 0.05$ was considered statistically significant. The intra-experimental coefficient of variance was less than 11%.

2.4 RNA extraction and purification, reverse transcription and qPCR procedure

Total RNA was extracted from frozen micro-dissected brain tissue and APit using Trizol-chloroform (Life Technologies) and described previously (Kadhim et al., 2020, 2019, 2021). Briefly, total RNA was extracted and treated with DNase I (Ambion, Austin, TX, United States) to eliminate genomic DNA followed by purification using an RNeasy mini kit (Qiagen). Total RNA concentration was estimated using Synergy HT multi-mode microplate reader (BioTek). Samples with an insufficient amount of RNA (1-3 samples) were excluded. First-strand cDNA was synthesized in 40 µl from total RNA (300 ng of NHpC, 500 ng of PVN, and 700 ng of APit) using Superscript® III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. In the NHpC and PVN, TRH, TRHR1, TRHR3, UCN3, CRH2 and D2 gene expression were measured. In the APit, TSHβ, TRHR1, TRHR3, and D2 mRNA levels were determined. The primer pair for the examined genes was chosen depending upon past studies. Electrophoresis on a 3% agarose gel as well as the melting curve was utilized to identify the specificity of primers (Table 1). The PCR assay was run in triplicate for each sample and performed in 30 µl using the following conditions: one cycle at 95°C for 10 m and amplified for 40 cycles at 95°C for 30 s, 60°C for 1 m, and 72°C for 30 s. The chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (βA) were used as internal controls to normalize the mRNA levels. Relative gene expression levels of each specific gene were determined by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Relative expression of control groups was set to 1.

2.5 Statistical analysis

Gene expression statistical analyses were conducted using JMPR pro 14.0 (SAS Institute Inc., NC). Normal distribution was first tested and thereafter differences among six independent groups for the NHpC, PVN, and APit were analyzed separately using one-way analysis of variance (ANOVA). Comparison for all pairs using Tukey's Kramer HSD test was used to evaluate relative changes in gene expression between control and each immobilized group. Data are presented as the mean ± SEM. A probability level of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Immobilization induced corticosterone increases in blood plasma

During immobilization, there was a robust and rapid increase in CORT concentration in the blood plasma (Figure 1). Specifically, the first significant increase in CORT concentration was observed at 15 m ($p < 0.001$) and peaked at 30 m ($p < 0.001$). Thereafter, CORT level declined at 60 m compared with 15 and 30 m, although it remained higher than controls until the end of all treatments. The overall significance of the change in CORT concentration between immobilized groups compared to the control group was ($p < 0.001$).

3.2 Gene expression data in the nucleus of the hippocampal commissure and paraventricular nucleus of the brain

3.2.1 Thyrotropin-releasing hormone and its receptors

Messenger RNA of TRH and its receptors was detected in both structures (NHpC and PVN) and showed a significant difference among treatment groups ($p < 0.01$; Figure 2A). In the NHpC, the TRH mRNA expression showed no significant differences between treatment groups compared with controls until 120 m of immobilization stress (Figure 2A). The only significant upregulation was observed at 120 m of restraint. Similarly, gene expression of TRH in the PVN did not respond until 90 m when the first significant increase was reported and peaked at 120 m of treatment (Figure 2A). In terms of receptors, a rapid, significant upregulation of TRH-R1 gene expression in the NHpC occurred at 15 m followed by a return to baseline levels throughout the treatments. While in the PVN, no changes in the mRNA expression between control and TRH-R1 treatment groups occurred (Figure 2B). Furthermore, mRNA expression of TRH-R3 in the NHpC showed a significant increase at only 60 and 90 m of immobilization stress, while TRH-R3 mRNA expression in the PVN decreased gradually, and a significant downregulation was observed at only 90 m of stress treatment before returning to the unstressed, control level at 120 m (Figure 2C).

3.2.2 Type II iodothyronine deiodinase 2

Gene expression of D2 in the NHpC was upregulated gradually, and a first significant increase was observed at only 60 m before returning to control levels at the end of treatments,

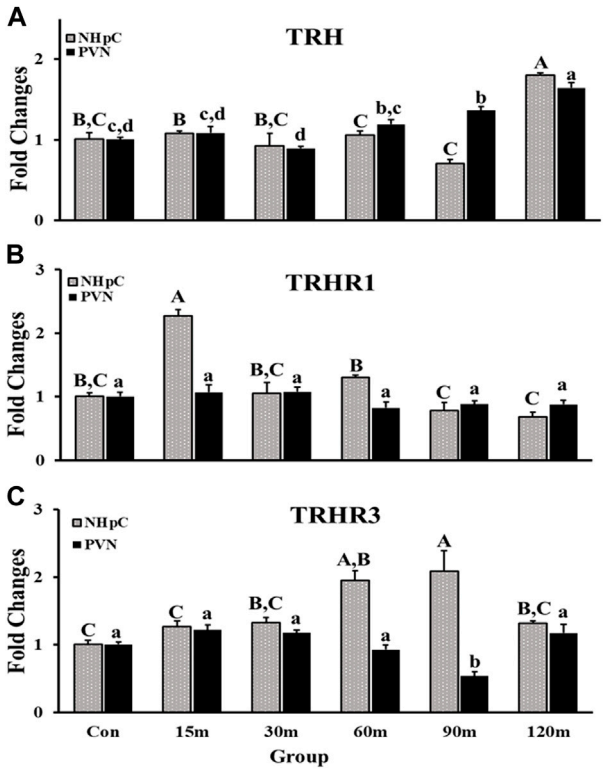

FIGURE 2
Effects of immobilization stress on relative mRNA expression levels of TRH **(A)**, TRH-R1 **(B)**, and TRH-R3 **(C)**, in the NHpC and PVN. Fold changes in relative expression levels were found using the $2^{-\Delta\Delta Ct}$ method after normalization with internal controls (GAPDH or β-Actin). Means ± SEM were determined for each gene. Significant differences ($p < 0.05$) among groups were specified by different letters above each bar or histogram. The gray box and uppercase letters refer to treatment differences among NHpC samples while the black box and lowercase letters refer to treatment differences among PVN samples.

120 m ($p < 0.01$; Figure 3). In contrast to the NHpC, the PVN showed no changes throughout treatments compared with controls ($p > 0.05$).

3.2.3 Corticotropin-releasing hormone 2 and urocortin 3

Corticotropin-releasing hormone 2 (CRH2) and urocortin 3 (UCN3) mRNA expression were detected in the NHpC as well as PVN and showed significant differences among treatment groups compared with controls ($p < 0.001$; Figure 4). Specifically, gene expression of CRH2 in the PVN was downregulated initially at 15 m followed by the lowest decrease at 30 m. After that, CRH2 gene expression showed a significant upregulation at 120 m, the last treatment time. In contrast, in the NHpC, a marked 3-fold increase in CRH2 mRNA occurred at 60, 90, and 120 m after IM (Figure 4A). In the NHpC, UCN3 gene expression was 3-fold higher solely at 15 m, then returned to the normal level at 30 m that lasted to 60 m. At the last sampling time, 120 m, a significant upregulation occurred (Figure 4B), however it was significantly less than the peak response of the NHpC at 15 m. In contrast, UCN3 gene expression in the PVN declined gradually and was downregulated at 90 and 120 m compared to controls (Figure 4B).

3.3 Gene expression data in the APit

3.3.1 Thyroid stimulating hormone subunit beta, TRH-R1, and TRH-R3

Gene expression of TSHβ in the APit was measured as an indicator of thyrotrope activation during stress. A significant increase of TSHβ mRNA was documented as early as 15 m, with a peak mRNA expression at 30 m before decreasing from the peak, however continued higher than control levels to the end ($p < 0.001$; Figure 5A). Furthermore, mRNA levels of TRH receptors (TRH-R1 and TRH-R3) displayed a significant upregulation in the APit throughout treatments compared with controls. In detail, a significant increase of TRH-R1

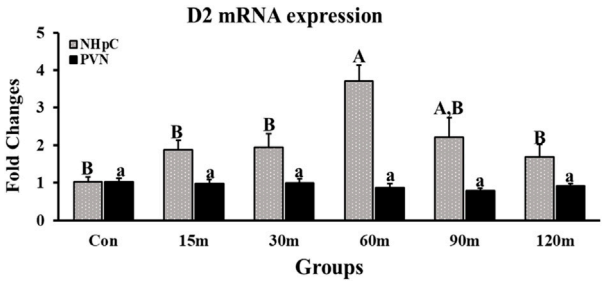

FIGURE 3
Fold changes of relative expression levels of D2 in the NHpC and PVN during immobilization stress. Data were found using the $2^{-\Delta\Delta Ct}$ method after normalization with internal controls (GAPDH or β-Actin). Means ± SEM were determined for each gene. Significant differences ($p < 0.05$) among groups were specified by different letters above each bar or histogram. The gray box and uppercase letters refer to treatment differences among NHpC samples while the black box and lowercase letters refer to treatment differences among PVN samples.

mRNA at 15 m occurred by more than 300% that remained at the same level until 90 m. At 120 m, the peak of gene expression was documented. Additionally, TRH-R3 gene expression showed the same pattern exhibited by TRH-R1 throughout the five treatments ($p < 0.01$; Figure 5B).

3.3.2 Type II iodothyronine deiodinase 2

Deiodinase 2 (D2) mRNA expression showed no changes at 15 and 30 m of IM stress compared with controls. Thereafter, the first mRNA upregulation was demonstrated at 60 m and remained at the same level at 90 m. A significant peak in D2 gene expression occurred at 120 m ($p < 0.01$; Figure 5C).

4 Discussion

The current study utilized a stressor, immobilization, to examine changes in gene expression of a panel of genes related to the thyroid axis occurring in the brain and APit. A goal was to determine their possible roles in the avian neuroendocrine regulation of stress. What is unknown is the timing of neural and endocrine components of the HPT axis compared with the HPA axis utilizing the same stressor. Therefore, we compared the timing of significant changes in gene expression within the same brain structures (NHpC and PVN), TSHβ in the APit, and the production of the stress hormone, CORT. If the timing of neural and APit components of the HPT axis occurs within the HPA axis time frame, then there would be evidence to consider components of the HPT axis as complementary to those of the HPA axis regulating stress.

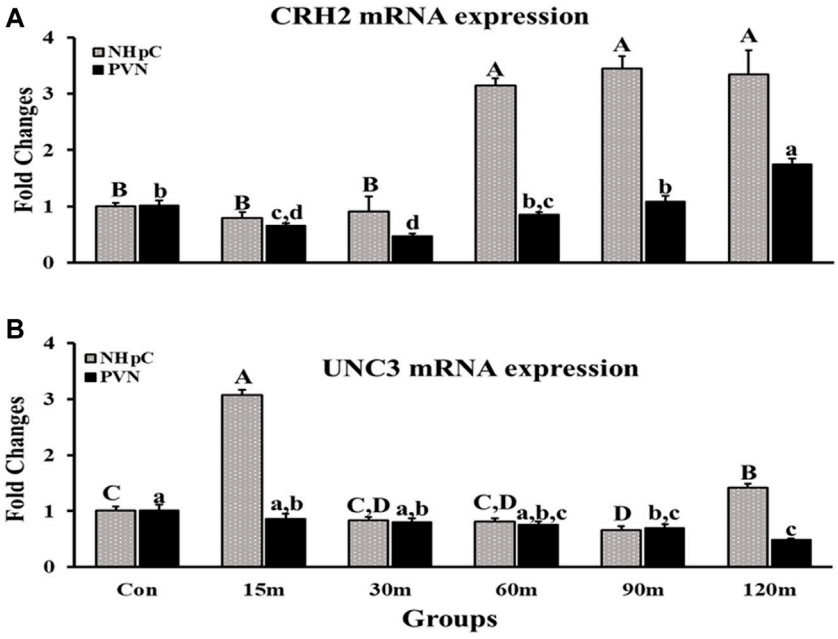

FIGURE 4
Fold changes of relative expression levels of CRH2 **(A)** and UCN3 **(B)** in the NHpC and PVN during immobilization stress. Data were found using the $2^{-\Delta\Delta Ct}$ method after normalization with internal controls (GAPDH or β-Actin). Means ± SEM were determined for each gene. Significant differences ($p < 0.05$) among groups were specified by different letters above each bar or histogram. The gray box and uppercase letters refer to treatment differences among NHpC samples while the black box and lowercase letters refer to treatment differences among PVN samples.

4.1 Role of neural secretion from nucleus of the hippocampal commissure and paraventricular nucleus in the increase of corticosterone concentration in blood and thyroid stimulating hormone subunit beta mRNA levels in the anterior pituitary

The sequence of gene activation within the traditional HPA axis of birds has been completed with two different stressors in our lab. Utilizing feed deprivation (Nagarajan et al., 2017b; Kadhim et al., 2019) or immobilization (IM, Kadhim et al., 2021) suggested that the avian HPA axis may include an additional structure, the NHpC, located in the septal region just dorsal to the hypothalamus. Regardless of the stressor, the sequence of gene activation for maintaining the CORT response was: 1) NHpC (CRH), 2) PVN (CRH), and 3) PVN (arginine vasotocin, AVT). An intriguing result of our recent stress study using IM stress was the robust, increased response of CRH-R2 in the APit (Kadhim et al., 2021). It alerted us that in birds, CRH-R2 was located in thyrotropes, not corticotropes (De Groef et al., 2003). Hence, there may be components of the HPT axis that may play a role in the avian stress response. If so, the APit would need to synthesize both POMC, the pre-pro hormone that produces adrenocorticotropic hormone (ACTH) and TSH following the same stressor. If both the HPA and HPT axes are activated concurrently following a stressor and the NHpC likewise displays components of the HPT axis activated during sampling times, would it provide further evidence to include the NHpC in the avian stress pathway?

In the current study, IM stress induced a significant increase in CORT concentration in the blood plasma that started at 15 m of IM stress, peaked at 30 m, and remained higher than control levels to the end of treatments. Similarly, the major component of the HPT axis, TSHβ, showed a significant increase in mRNA at 15 m that continued to the end of treatments. The upregulation of TSHβ gene in the APit could be mediated *via* the action of CRH-like peptides secreted from the NHpC and PVN activating CRH-R2 located on thyrotropes. Our past HPA data showed rapid upregulation in CRH mRNA in the NHpC and PVN followed by a significant increase of hn/mRNA POMC in the APit. Interestingly, CRH-R2 displayed a significant increase in gene expression in the APit in all immobilized groups (Kadhim et al., 2021). In summary, the significant increase of TSHβ, a component of the HPT axis matched a similar timing of a significant increase in POMC, a component of the HPA axis (Kadhim et al., 2021). The possible contribution of a neural structure contributing to the activation of the HPT axis is covered next.

4.2 Role of thyrotropin-releasing hormone, TRH-R1, and TRH-R3 in the neuroendocrine regulation of stress

A curious result in the study was the significant increase in gene expression of TRH-R1 within the NHpC at 15 m. Additionally, a significant upregulation of TRH-R3 mRNA expression in the NHpC occurred at 60 m near the end of treatment groups. A main issue with TRH-R1 is that one would expect a possible change in expression of TRH within the NHpC, however, no change was observed from 15 to 90 m in that structure. A possible explanation is that the NHpC is

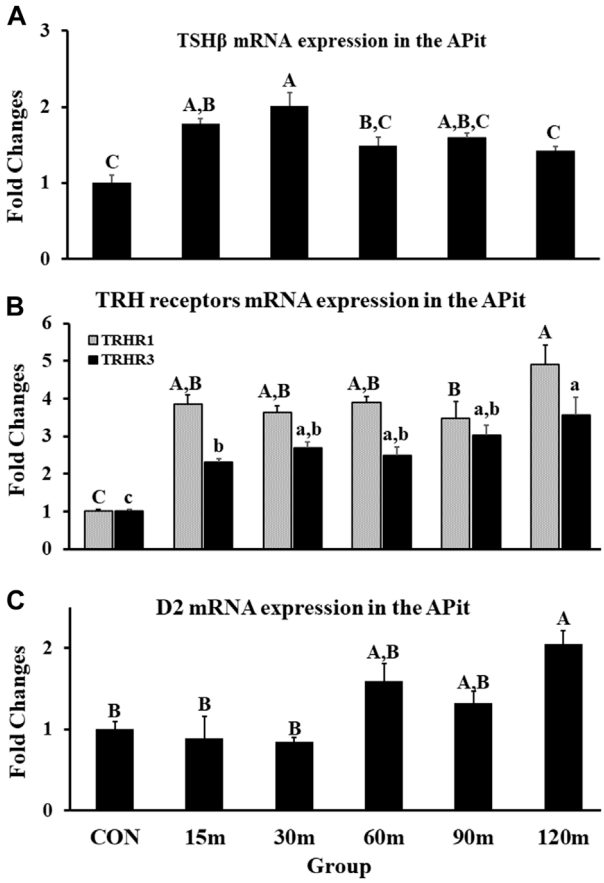

FIGURE 5
Fold changes of relative expression levels of TSHβ **(A)**, TRH receptors **(B)**, and D2 **(C)** in the APit during immobilization stress. Data were found using the $2^{-\Delta\Delta Ct}$ method after normalization with internal controls (GAPDH or β-Actin). Means ± SEM were determined for each gene. Significant differences ($p < 0.05$) among treatment groups were specified by different letters above each bar or histogram for **(A–C)**. In **(B)**, the gray box and uppercase letters show TRHR1 and the black box and lowercase letters show TRHR3 and different letters indicate significant differences among treatment groups for each gene.

surrounded by three circumventricular organs, the organum vasculosum of the lamina terminalis (OVLT), sub-septal organ (SSO), and choroid plexus (CP). The OVLT and SSO reside in the anterior and dorsal region of the third ventricle at its midline while the CP occupies the base of the two lateral ventricles. The NHpC is located at the midline, just dorsal to the third ventricle. A study has shown that the chicken choroid plexus contains type II iodothyronine deiodinase (D2) and produces T_3 (Verhoelst et al., 2004). The epithelial cells lining the walls of the avian lateral ventricles and epithelial cells of the CP contain the D2 protein. Hence, within the avian brain, T_3 can originate in non-neuronal cells in the CP (Verhoelst et al., 2004). Therefore, it is possible that a local source of T_3 was provided by the CP during that stressful 15 m period as evidenced by the significant upregulation of the TRH-R1 in the NHpC (Figure 2B). This suggests that a change in gene expression for TRH is not required to provide the needed source of energy *via* the classical HPT axis.

Our current study shows that both the NHpC and PVN dissected structures likewise contain D2 mRNA. Note that the NHpC produced some increases in D2 mRNA at 15 and 30 m and showed nearly a 4-fold increase in gene expression from controls at 60 m following IM stress. Currently, we do not know whether the functional gene, D2, occurs in glia or neurons in the NHpC and PVN. Birds appear to have at least three sources within the brain for the production of T_3: 1) the traditional HPT axis, 2) the choroid plexus of the brain (Verhoelst et al., 2004) and 3) the presence of the D2 gene in individual brain structures, such as the NHpC and PVN (current study).

4.3 Role of corticotropin-releasing hormone and urocortin3 in upregulation of thyroid stimulating hormone gene *via* CRH-R2 during immobilization stress

Our data, for the first time, documented changes in CRH2 mRNA following IM stress occurring in the NHpC and PVN. Specifically, gene expression significantly increased 3-fold at 60, 90, and 120 m in the NHpC and significantly increased in the PVN at the last sampling point, 120 m. Note that gene expression for CRH2 displayed an extended delay of 1 h following IM stress before a significant increase was first detected in the NHpC. Gene expression in the PVN showed a significant decline at 15 and 30 m and returned to baseline at the 60 and 90 m sampling times. A significant increase in CRH2 mRNA was demonstrated solely during the last sampling point of 120 m. Overall, the response of the avian CRH2 gene to IM stress appears to be dependent upon the neural structure, was totally different between the hypothalamic PVN and the extra-hypothalamic NHpC and in both structures displayed a late, positive response to IM stress. Since CRH2 has a high affinity for CRH-R2 located on thyrotropes (Bu et al., 2019), its upregulation appears to sustain thyrotrope activation as measured by TSHβ mRNA levels in the APit.

Besides CRH and CRH2, another peptide urocortin 3 (UCN3) displayed a highly significant 3-fold increase in gene expression within the NHpC at 15 m. Past studies in mammals showed the peptide UCN (Vaughan et al., 1995) and specifically UCN3, within the family of CRH-like peptides, has a high affinity for CRH-R2 (Lewis et al., 2001). Thereby UCN3 may contribute to the HPT axis. Past studies, using human UCN3 (hUCN3, stresscopin), showed that hUCN3 was effective in releasing chicken TSH; however, was less effective than CRH (De Groef et al., 2003). The chicken UCN3 has been cloned and its possible function/s have been suggested (Grommen et al., 2017). Is there any evidence that the significant increase in UCN3 mRNA in the NHpC at 15 m following immobilization could rapidly affect APit production of TSH? This would require a direct or efficient neural connection between the NHpC and median eminence resulting in a fast release of the peptide into the portal capillary system for transport to the APit. A past behavioral study using mature broiler male-male and male-female interactions provides evidence. In the experiment, four groups were designed. In all experimental groups, the fixed brains were sectioned and used for visualization of FOSir cells. Results showed that all treatments revealed intense immunoreactive (ir) cells in the dorsal region of the NHpC where CRH neurons are located. The greatest ir occurred with the male-male interactions (Xie et al., 2010). Hence that male-male interaction as well as the other three male-female interactions resulted in activating the HPA axis *via* increased activity of neurons known to help initiate the ultimate production of the stress hormone, CORT.

In the current study, the peak response of UCN3 mRNA occurred at 15 m in the NHpC and likewise a significant increase in TSHβ was documented in the APit at 15 m. We, therefore, suggest that UCN3 neurons in the NHpC have contributed to that significant increase in APit TSHβ at 15 m and thereafter. We have no immunocytochemical data showing that UCN3 neurons exist in the NHpC. However, the dramatic gene expression changes shown in the NHpC of UCN3 provide strong evidence of their existence, particularly contributing to the early significant increase in TSHβ within the APit. Similarly, we have no direct evidence that UCN3 neurons in the NHpC project to the ME. However, there exists an alternative possibility. The NHpC resides at the midline just dorsal to the hypothalamus and sits directly above the third ventricle (VIII). Cells within the NHpC could have axons projecting to the ME or directly beneath the NHpC into the VIII containing cerebrospinal fluid. Therefore, NHpC secretions of UNC3 into the VIII would eventually be transported to the ME.

4.4 Role of the HPT axis in the neuroendocrine regulation of stress

Data collected in the current experiments suggest that genes associated with the major components of the HPT axis should be seriously examined in future studies involving the avian HPA axis and the neuroendocrine stress response. There appear to be at least three neuropeptide genes in the HPT axis impacting the neuroendocrine regulation of stress: TRH, CRH2, and UCN3. Additionally, the APit hormone gene TSHβ, two receptors, TRH-R1, TRH-R3 and enzyme gene D2 likewise play a role. Importantly, all the previously named genes were activated within the time span when genes associated with the avian HPA axis displayed clear changes in gene expression following activation of the same stressor. The following summarizes the timing of some of the seven HPT genes and their possible role in interacting with the HPA axis. Specifically, a significant, delayed increase in gene expression of TRH occurred in the PVN at 90 and 120 m following IM stress, while a highly significant increase in TRH mRNA occurred in the NHpC at 120 m. A second distinct gene, CRH2, showed a highly significant increase of its mRNA at 60, 90, and 120 m in the NHpC while the PVN showed a delayed activation at 120 m.

The third gene listed above as part of the avian HPT axis was UCN3. This gene displayed activation early and late in the NHpC, specifically at only the first (15 m) and last sampling points (120 m) following IM stress. Gene expression of UCN3 was six times greater at 15 m compared to its significant increase at 120 m. UCN3 was shown to bind to CRH-R2 and failed to activate CRH-R1 even at high concentrations. Similarly, Hsu and Hsueh (2001) reported that human UCN3 or stresscopin did not stimulate ACTH in cell culture studies nor *in vivo* (Hsu and Hsueh 2001). In chickens, human UCN3 was shown to stimulate TSH release in APit glands (De Groef et al., 2003). The large increase at 15 m suggests another possible function of UCN3, perhaps related to a stress behavioral response at the level of the brain. In an open field behavioral test in rodents or a dark-light emergence test, mice injected with 20 ng UCN3 intracerebroventricularly (icv) showed significantly less anxiety-like behavior than controls that was associated with no changes in plasma concentrations of ACTH and CORT (Venihaki et al., 2004). However, mice lacking the CRH-R2 were shown hypersensitive to stress and displayed anxiety-like behavior (Bale et al., 2000). Hence, in mammals, UCN3 is associated with anxiety-like behavior. Our UCN3 data suggest a possible behavioral role of UCN3 in birds related to anxiety. Our stress protocol involved our picking up each bird, wrapping it in a harness so that it could not move its wings nor stand up, then quickly returning the bird to its cage. Thus, it is likely that the protocol we utilized for immobilizing each bird was initially quite stressful and UCN3 may have enabled coping with that restraint. Additionally, the delayed, significantly increased UCN3 mRNA at 120 m appears to be a natural response of the NHpC to activate the HPT axis to generate the needed energy to cope with the continued stressor IM.

Activation of the three genes (TRH, CRH2, and UNC3) is reflected in the sustained, continued significant upregulation of TSHβ gene expression and increase of TRH-R1 and -R3 mRNA in the APit, while the significant increase of D2 at 120 m in the APit appears to generate T_3 from T_4 (Figure 5). Hence, three genes acting within two neural structures associated with the HPA axis appear to likewise activate TSHβ, the critical APit hormone of the HPT axis, to produce the necessary energy for providing a complementary means to address IM stress. Overall, concurrent activation of both the HPA and HPT axes appears to be an essential, physiological response in an attempt to maintain homeostasis in birds undergoing a physiological and/or mental challenge.

5 Summary

The study addressed a group of genes associated with the HPT axis to ascertain their change in gene expression following IM stress. Genes in two distinct brain structures and regions (NHpC in the septum and PVN in the hypothalamus) and the APit were examined to compare them with activation of genes regarded as components of the avian HPA axis. The objective was to determine whether genes of the HPT axis may clarify our understanding of the neuroendocrine regulation of stress in birds. Results showed that the timing of the pattern of expression of the major gene, TSHβ of the HPT axis, at the level of the APit, matched well with the increase in CORT which is the final product of HPA axis activation. At the neural level, two peptides in the NHpC responded rapidly to IM stress. Both CRH and UNC3 showed significantly increased mRNA at 15 m, contributing to activation of the HPT axis, as each is known to bind to CRH-R2. In both the NHpC and PVN, CRH2 and TRH responded late to IM with significant increases in mRNA, thereby sustaining activation of the HPT axis. Overall, the timing of significant changes in APit and neural components of the HPT axis following IM stress occurred within the time frame of previous data addressing the HPA axis. Additionally, current data show that the NHpC is a major player in both avian HPT and HPA axes, supporting the proposal that the structure should be included in the neuroendocrine regulation of stress in birds.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by The University of Arkansas Institutional Animal Care and Use Committee protocol #19,054.

Author contributions

HK and WK designed the experiments and sampled tissue at all time points described in materials and methods section of the paper. HK performed statistical analyses of data obtained and wrote the first draft and intermediate draft of the manuscript. WK wrote the second and final drafts of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Chronic heat stress part 1: Decrease in egg quality, increase in cortisol levels in egg albumen, and reduction in fertility of breeder pekin ducks

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Global warming poses detrimental effects on poultry production leading to substantial economic losses. The goal of our experiment was to test the hypothesis that heat stress (HS) would alter welfare and egg quality (EQ) of breeder ducks. Furthermore, we wanted to test if HS would increase cortisol levels in egg albumen. Adult Pekin ducks were randomly assigned to two different rooms at 85% lay with 60 hens and 20 drakes per room. Baseline data including body weight, body condition scores (BCS), and egg production/quality were collected the week preceding heat treatment. Ducks were subjected to cyclic HS of 35°C for 10h/day and 29.5°C for the remaining 14h/day for 3 weeks while the control room was maintained at 22°C. Eggs were collected daily and analyzed weekly for quality assessment, and for albumen glucocorticoid (GCs) levels using mass spectrometry. One week before the exposure to HS, 10 hens and 5 drakes were euthanized and the same number again after 3 weeks and birds necropsied. Data analyses were done by 1- or 2-way ANOVA as appropriate with a Tukey-Kramer *post hoc* test. BCS were analyzed using a chi-squared test. A $p \leq 0.05$ was considered significant. Circulating levels of corticosterone were significantly ($p < 0.01$) elevated at week 1 only in the HS hens. The circulating levels of cortisol increased significantly at week 1 and 2 ($p < 0.05$), and week 3 ($p < 0.01$) in the hens and at weeks 2 and 3 only ($p < 0.05$) in the drakes. Feather quality scores ($p < 0.01$), feather cleanliness scores ($p < 0.001$) and footpad quality scores ($p < 0.05$) increased significantly in the HS group. HS elicited a significant ($p < 0.001$) decrease in egg production at weeks 1 and 3. Hens in the HS group showed significantly decreased BW ($p < 0.001$) and number of follicles ($p < 0.05$). Shell weight decreased significantly at week 1 only ($p < 0.05$) compared to controls. Yolk weight decreased significantly at week 3 ($p < 0.01$) compared to controls. HS elicited a significant increase in albumen cortisol levels at week 1 ($p < 0.05$) and week 3 ($p < 0.05$). Thus, cortisol may provide critical information to further understand and to improve welfare.

KEYWORDS

climate change, heat stress, welfare, cortisol, corticosterone, egg quality, pekin duck

Introduction

Environmental stressors are major concerns facing poultry production. HS is one of the most prominent, having detrimental effects on the production, performance, welfare, meat quality, immunity, and egg quality of poultry (Ebeid et al., 2012; Honda et al., 2015; Barrett et al., 2019; He et al., 2019). According to St-Pierre et al. (2003), the United States incurred an annual loss of $1.69–2.36 billion in the whole livestock industry of which $128–165 million occurs in poultry industry because of HS. Poultry is known as an excellent source of protein in the form of meat and egg (Nawab et al., 2018) and poultry meat accounts for 34.3% of global meat production in 2012 (Pawar et al., 2016). Generally, poultry are more susceptible to HS because of their high body temperature and lack of sweat glands for heat dissipation (Wolfenson et al., 2001). A growing number of studies have exposed meat type and laying ducks to high temperature to assess the effect on performance and reproductive parameters. The effects of heat stress on ducks include decreased daily feed intake and decreased egg quality in shelducks. Studies have suggested that heat stress reduces fertility due to decreased oviduct length and number of ovarian follicles in shelducks (Ma et al., 2014), however actual fertility data is lacking. Heat stress also elicits production and biochemical effects in ducks such as decreased growth performance and breast meat quality in pekin ducks (Sun et al., 2019), increased mRNA expression of heat shock protein and inflammatory factors in muscovy and pekin ducks (Zeng et al., 2014), and decrease in hypothalamic expression of antioxidant and pro-oxidant enzymes genes in laying shanma ducks (Luo et al., 2018). Heat stress has also been suggested to increase the stress response as evidenced by an increase in adrenal gland weight in pekin ducks (Hester et al., 1981), HS activates the sympathetic adrenomedullary (SAM) and hypothalamic-pituitary-adrenal (HPA) axes that lead to the secretion of catecholamines and glucocorticoids (GC) respectively (Nagarajan et al., 2017). Several studies have shown that acute, chronic, and cyclic HS all affect circulating levels of GC (reviewed by Scanes, 2016). Increased circulating levels of GC induces gluconeogenesis, cell trafficking and proliferation, cytokine secretion, and antibody production that can affect immunity, promote inflammation and lead to hypersensitive HPA axis (Shini et al., 2008; Lara and Rostagno, 2013). In addition, HS can dysregulate the hypothalamic-pituitary-gonadal (HPG) axis thereby altering the secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus, and luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland (Lu et al., 2021). HS also depresses the gonads thereby reducing the secretion of testosterone from testes and estradiol from the ovaries, causing a decline in ova maturation, sperm quality, morphology, mobility, and egg penetration (reviewed by Ayo et al., 2011). Thereby the effect of HS on the HPG axis can lead to a decrease in fertility.

The purpose of our study was to evaluate the effects of HS on egg quality, the stress response, fertility, and welfare of pekin ducks. To achieve this, we treated 80 adult Pekin ducks at 85% lay to HS and an equal number as controls. Our results suggest that HS elicited a selective deposition of cortisol, not corticosterone, into the egg albumen. Further, HS decreased shell and yolk weights, caused a decline in duck welfare, and affected ovarian but not testes morphometrics.

Materials and methods

Animals

160 developer pekin ducks of approximately 20 weeks of age were obtained from Maple Leaf Farms Inc. (Leesburg, IN) and randomly allocated into 2 rooms in equal numbers at industry standard density. They were allowed *ad lib* access to water and feed for 8 h per day as per standards (Chen et al., 2021). We utilized 60 hens and 20 drakes per treatment. None of the ducks had any prior exposure to HS. The ducks were placed in the single rooms with an 18:6 light cycle, temperature of 20–22°C for both treatment groups until 85% lay (~35 weeks of age). Water nipple lines (5 ducks per nipple) were placed over a pit covered with raised plastic flooring, and the remaining area of the rooms were covered with pine shavings and added to or replaced as necessary at the same time for both rooms. Nest boxes were placed along one wall of the room with 4 hens per nest box as per industry standards (Pfaus et al., 2001; Chen et al., 2021). All procedures were approved by the Purdue Animal Care and Use Committee (PACUC # 2109002195).

Experimental design

At 85% lay, the 2 rooms were randomly allocated as control or HS group such that there is one room per treatment group. The HS group was subjected to cyclic temperature of 35°C for 10h/day and returned to 29.5°C for the remaining 14h/day for 3 weeks while the control room was modified to an industry-standard temperature of 22°C. Both rooms had data loggers (Hobo, Onset Inc.) for temperature and humidity that were placed to monitor both variables at the level of the ducks' heads. Ammonia readings were taken twice per week using a NH_3 meter (Forensic Detectors, NH3000).

Sample collection and preparation

Blood smears were collected for HLR on weeks 0, 1, 2, and 3 relatives to the onset of heat treatment from different ducks at

TABLE 1 Body condition scoring rubric for welfare assessment[a].

Structure	Score level	Description
Eyes	0	**Best:** Eyes clear, clean, and bright.
	1	**Moderate:** Dirt and/or staining around the eye area. Any evidence of wet eye ring or inflamed eye lid.
	2	**Worst:** Eyes sealed shut with or without conjunctivitis.
Nostrils	0	**Best:** Nostrils with clean and clear air passageways.
	1	**Dirty:** Nostril air passageways block with dust or mucus.
Feather Cleanliness	0	**Best:** Clean and unstained breast, back feathers or down depending on age.
	1	**Dirty:** Adhering manure or staining on down or feathers.
Feather Quality	0	**Best:** good feather coverage for age—down in younger and feathers for developing and older birds
	1	**Moderate:** Some evidence of feather picking, down and/or feather damage less than 2 cm^2.
	2	**Worst:** Feathers/down damaged, short, and stubbly. Large patchy feathers/down over back greater than 2 cm^2 and/or evidence of severe feather picking (presence of blood on back, tail, neck, or wings).
Foot pad	0	**Best:** Heel and toe pads free of any lesions or ingrained dirt.
	1	**Moderate:** Dirt pervades the heel or toe pads and skin papillae raised typically dark brown on heel or toe pads. Lesions covering less than 50% of heel or toe pad. Free of any bloody lesions.
	2	**Worst:** lesions or callouses cover 50% or more of heel or toe pad, any bleeding lesions.

[a]Adapted from Fraley et al. (2013) and Karcher et al. (2013).

each collection (n = 6/sex/treatment/week), with the week preceding heat treatment designated as 0 and analyzed by a certified pathologist in a diagnostics laboratory unaware of the treatment groups. Body condition scores (BCS) (Fraley et al., 2013; Karcher et al., 2013; Colton et al., 2014) such as foot pad quality scores, eyes scores, nostrils scores, feather cleanliness and quality scores were also assessed on weeks 0, 1, 2 and 3 for all ducks at each assessment (n ~ 65/treatment) using the scoring rubric shown in Table 1.

Blood samples were collected from the ducks' tibial veins at the same time as blood smears on weeks 0, 1, 2, and 3 relative to the onset of HS from randomly selected ducks at each time point (n = 6/sex/treatment/week) and placed into a serum separator tubes, centrifuged, and the serum was stored at −20°C until assayed by ELISA for GC (Tetel et al., 2022a; Tetel et al., 2022b). Daily eggs laid (n = 35–60) were counted and 3 consecutive day eggs (thus n = 3 per time group) were averaged to reduce daily lay variability and compared between treatment groups beginning 1 week prior to HS treatment (n = 3/group/treatment): groups -1 and 0 (week preceding heat stress), groups 1 and 2 (week 1), groups 3 and 4 (week 2), and groups 5, 6 and 7 (week 3). All eggs from each treatment group were also collected daily over the 4-week period and assessed for egg quality weekly (N = 33–44 per daily collection per week) as described below. Yolk and albumen (N = 10 each per treatment group) samples were collected into tubes during egg quality assessment and stored at -20°C until assayed for GC using mass spectrometry. Ducks were weighed on days 0 and 21 relative to the onset of HS. One week before the exposure to HS, 10 hens and 5 drakes were euthanized and the same number again after 3 weeks of HS or control exposures using pentobarbital (Fatal Plus, 396 mg/ml/kg) and birds necropsied. Spleen, testes, and liver were collected and weighed, the number of maturing follicles recorded, and a final blood sample was obtained and treated as described above.

Egg incubation

Eggs from the last 3 days of the treatment were collected from both groups (n = 127 for control and n = 113 for HS group). They were stored at 4°C for 5 days. After storage, the eggs were sorted and incubated per industry standard (Chen et al., 2021). Candling was done on day 10 and the number of fertile eggs was counted and calculated as percentage for fertility. Eggs hatched on days 28 and 29 and number of dead embryos were counted and expressed as a percentage of embryo mortality. Number of hatched chicks were counted and calculated for hatchability.

ELISA for glucocorticoids

The kits utilized for this project were from Cayman Chemicals (corticosterone: kit #16063; cortisol kit #560360) and the assays were run according to the manufacturer's recommendations. Details of the kit verification have been reported previously (Tetel et al., 2022a; Tetel et al., 2022b). Plates were incubated with samples overnight at 4°C. Plates were developed for 90 min and were read at 405 nm (SynergyLx, Biotek).

Egg quality assessments

Eggs were identified by room and collected on weekly basis for the duration of the trial for quality assessment, refrigerated at 4°C overnight, and weighed before analyses (n = 33–44/treatment/

week). Shell compression strength was analyzed using the TA.XT Plus Texture Analyzer (Texture Technologies, Hamilton, MA) with a 10 kg load cell. The egg was placed on its side using the egg holder (TA-650) and compressed with the compression disc (TA-30). The albumen height was measured with a micrometer and the Haugh unit was calculated. The yolk was separated and weighed, then the vitelline membrane compression strength was analyzed using TA.XT Plus Texture Analyzer with a 500 g load cell. The yolk was placed in a petri dish and compressed with the compression disc. Albumen and yolk samples were collected into tubes and stored at—20°C for GC assays. The eggshells with intact membranes were washed, placed on trays, and dried at room temperature for 48 h. When the eggshells were fully dried, the weights were obtained, and the shell thickness was measured at three different points around its equator and the values averaged. Data from 1 week before treatment were classified as 0 and from weeks 1, 2 and 3 after the exposure to HS.

Mass spectrometry for yolk and albumen glucocorticoids

Yolk (n = 10/treatment/week) and albumen (n = 10/treatment/week) samples from the same egg were stored at −20°C before extraction and analysis. At the time of analysis, each albumen sample was thawed, 500 mg of albumen was transferred to an extraction tube while 5 g of each thawed yolk sample was transferred to an extraction tube. The samples were extracted according to a previous report with minor modifications (Caulfield and Padula, 2020). To each sample, 10 µL of an internal standard mixture containing 5 ng of deuterated corticosterone (d8-corticosterone solution in methanol) and 1 ng of deuterated cortisol (d4-cortisol solution in methanol) was added to the albumen and yolk samples and vortexed for 1 minute. The internal standards d8-corticosterone (# C695702) and d4-cortisol (# C696302) were purchased from Toronto Research Chemicals (Ontario, Canada). Next, 2.5 ml and 5 ml of acetonitrile +1% formic acid was added to albumen and yolk respectively to extract corticosterone and cortisol. The samples were vortexed for 10 min and centrifuged at 3,220 g for 5 min at room temperature. The supernatants were collected and transferred to new tubes. The extract was then washed with equal volumes of hexane. The hexane was discarded, and the bottom phase was collected and dried using nitrogen gas. The pellet was then reconstituted in 0.1 ml of methanol, followed by 0.9 ml of water. The samples were mixed well by vortexing before loading onto a Water's HLB PRIME solid-phase extraction cartridge, 1cc, 30 mg (part number 186008055; Waters Corp., Milford MA). The cartridges were washed with 1 ml of water followed by 1 ml of hexane. The samples were eluted with 2 volumes of 1 ml of ethyl acetate. This fraction was dried using a nitrogen stream or speed vacuum. The samples were then stored at -80°C until ready for analysis.

For analysis, each sample was derivatized with 50 µL of Amplifex keto reagent (# 4465962, AB Sciex, Framingham, MA) according to the kit directions just before instrument analysis. After 1 hour 10 µl of water was added and the sample mixed well before transferring to an autosampler vial for analysis by LC/MS/MS. An Agilent 1260 Rapid Resolution liquid chromatography (LC) system coupled to an Agilent 6470 series QQQ mass spectrometer (MS/MS) was used to analyze corticosterone and cortisol in each sample (Agilent Technologies, Santa Clara, CA). An Agilent Eclipse plus C18 2.1 mm × 50 mm, 1.8 µm column was used for LC separation. The buffers were (A) water +0.1% formic acid and (B) acetonitrile +0.1% formic acid. The linear LC gradient was as follows: time 0 min, 10% B; time 1.0 min, 10% B; time 1.5 min, 25% B; time 21.5 min, 35% B; time 22 min, 100% B; time 23 min, 100% B; time 24 min, 10% B; time 30 min, 10% B. The flow rate was 0.3 ml/min. Corticosterone eluted at 6.6 min and cortisol at 5.8 min. Multiple reaction monitoring was used for MS analysis. The data were acquired in positive electrospray ionization (ESI) mode (Tetel et al., 2022a; Tetel et al., 2022b). The jet stream ESI interface had a gas temperature of 325°C, gas flow rate of 8 L/min, nebulizer pressure of 45 psi, sheath gas temperature of 250°C, sheath gas flow rate of 7 L/min, a capillary voltage of 4000 V in positive mode, and nozzle voltage of 1000 V. The ΔEMV voltage was 500 V. Agilent Masshunter Quantitative analysis software was used for data analysis (version 10.1). For quantitation of corticosterone/d8-corticosterone, the transition 461.3 → 402.2/469.3 → 410.2 was used. For cortisol/d4-cortisol, the transition 477.3 → 418.3/481.3 → 422.3 was used.

Statistical analyses

The duck was considered the statistical unit. Given the limitations of our model design, we ran power analyses following a simulation approach (i.e., simulating data with parameters taken from previous studies) to establish the sample size per each treatment variable. All our power analyses were run in MacJMP SAS (JMP Pro 15). We ran four power analyses considering four indicators of our response variables: BCS, blood cell counts, hormone levels, and egg quality. We found that the sample sizes described above would provide us 85% power to detect significant effects ($p < 0.05$) taking into account inter-duck variability. All data were analyzed by 1-, or 2 -way ANOVA as appropriate. Body condition scores were analyzed using a chi-squared test. *Post hoc* analyses were done by Tukey Kramer pairwise comparison test. A $p < 0.05$ was considered significant.

Results

Environmental conditions

Data loggers confirmed cyclic heat temperatures in the HS room and control conditions. Bedding was replaced as

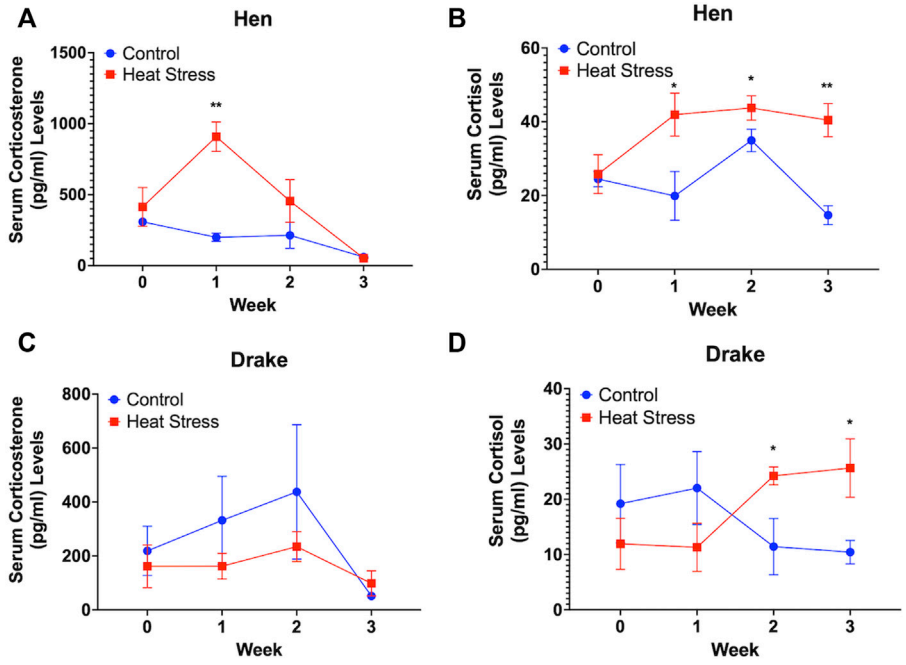

FIGURE 1
Serum corticosterone **(A)** and cortisol levels **(B)** in Hens and serum corticosterone **(C)** and cortisol **(D)** in Drakes exposed to cyclic heat stress (35°C for 10h/day and 29.5°C for the remaining 14h/day) or control. Data shown are means ± SEM, n = 6/sex/treatment/week. Ducks exposed to heat stress showed a significant increase in serum cortisol levels compared to controls at weeks 1, 2, and 3 for hens and weeks 2 and 3 for drakes after the onset of treatment. Only hens exposed to heat stress showed a significant increase in serum corticosterone levels compared to controls at week 1 only after onset of treatment. * = $p < 0.05$, ** = $p < 0.01$.

needed in the HS room, and in control room at the same time. Regardless, analyses of NH_3 showed that control room had below measurable levels, while the HS room averaged 12.5+/− 2.36 ppm during the 3 weeks of HS. The necessary use of electric heating units on our farm facility led to low relative humidity levels with the control room averaging 37.4 +/− 2.43% and the HS room averaging 64.7 +/− 4.66%. The differences in NH_3 and humidity levels in between groups are possible confounding factors.

Serum glucocorticoids

Circulating levels of corticosterone in the hens were significantly ($p < 0.01$) elevated at week 1 only in the HS group (Figure 1A) while the circulating levels of cortisol increased significantly at week 1 ($p < 0.05$), week 2 ($p < 0.05$), and week 3 ($p < 0.01$) in the hens compared to the controls for each hormone (Figure 1B). In contrast, there was no significant increase in the circulating levels of corticosterone in drakes in either group (Figure 1C), while HS significantly increased the serum levels of cortisol at week 2 and 3 only ($p < 0.05$) in the drakes compared to control drakes (Figure 1D).

Welfare assessment

No significant differences were observed in the HLR for either hens (Figure 2A) or drakes (Figure 2B). We observed a significant increase in feather quality scores ($p < 0.01$, Figure 3A), and feather cleanliness scores ($p < 0.001$, Figure 3B), although significant, ducks with feather cleanliness scores below 0.1 are not considered dirty (Fraley et al., 2013; Karcher et al., 2013; Colton et al., 2014). Footpad quality scores ($p < 0.05$, Figure 3C) increased significantly in the HS group compared to controls. Higher BCS indicate a decline in welfare (Fraley et al., 2013; Karcher et al., 2013). All other welfare parameters (Figure 3D) showed no significant differences. Eye score data are not illustrated due to the fact that throughout the experiment, nearly all ducks had "0" scores regardless of treatment, suggesting that the slightly elevated NH_3 levels in the HS had minimal, or even negligible, confounding effects.

Egg production, hatchability, and fertility rate

HS elicited a significant ($p < 0.001$) decrease in egg production at weeks 1 and 3 compared to controls as shown in Figure 4. HS elicited a descriptive decrease in the number of

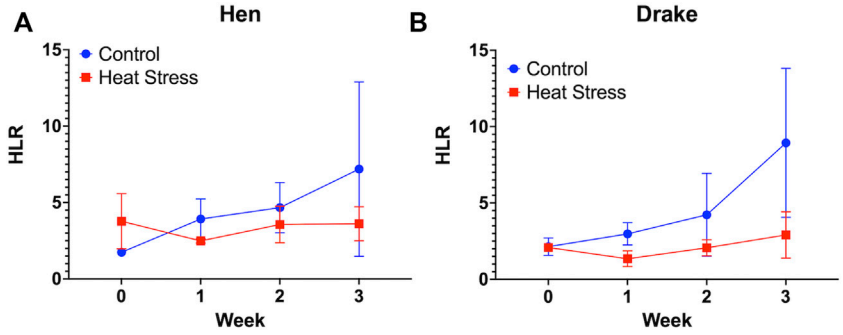

FIGURE 2
Heterophil and Lymphocyte ratio in Hens **(A)** and Drakes **(B)** exposed to cyclic heat stress (35°C for 10h/day and 29.5°C for the remaining 14h/day) or control. Data shown are means ± SEM, *n* = 6/sex/treatment/week. No significant difference were observed in the HLR of both drake and hen, however elevated levels in both groups suggest a hypersensitivity to the handling procedures.

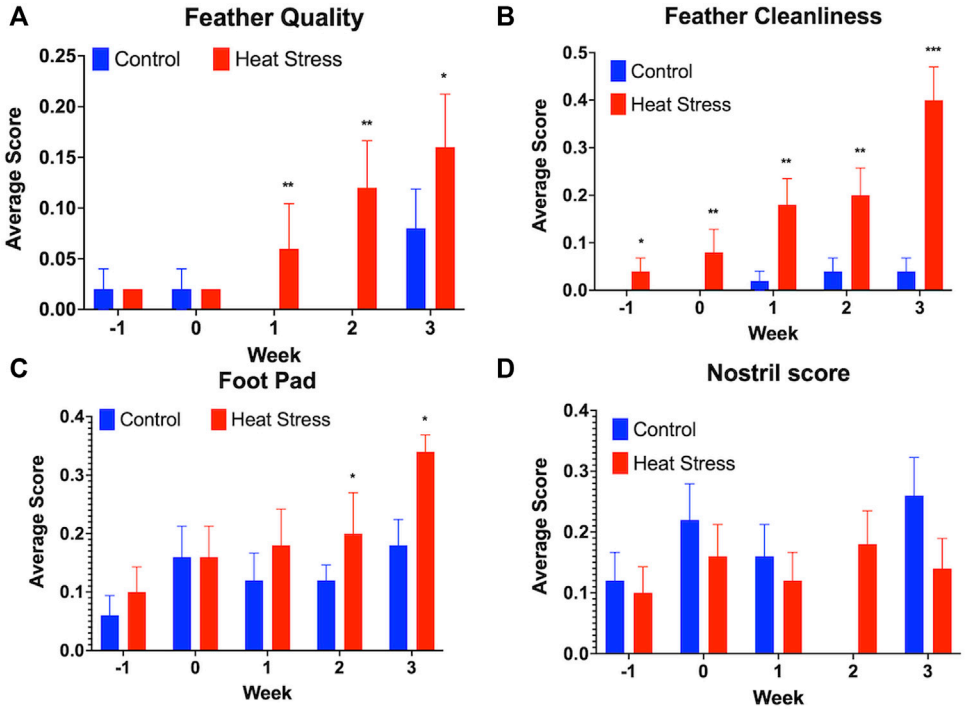

FIGURE 3
Welfare assessment score for Feather quality **(A)**, Feather cleanliness **(B)**, Foot pad quality **(C)**, and Nostril quality scores **(D)** in ducks exposed to cyclic heat stress (35°C for 10h/day and 29.5°C for the remaining 14h/day) or control. Data shown are means ± SEM, *n* = 65–80/treatment/week. Ducks exposed to heat stress showed higher feather quality scores at all time-point, higher feather cleanliness scores compared to the controls at weeks 1, 2, and 3, and higher foot pad scores compared to the controls at weeks 2, and 3 after onset of treatment and higher scores indicate a decline in welfare. No significant differences in the nostril quality scores were observed between treatment groups. * = $p < 0.05$, ** = $p < 0.01$.

fertile eggs upon candling at 10 days of incubation with values of 98.4% in the control and 92.9% in the HS group, decrease in the number of hatched chicks after the incubation period with values of 76% in the control and 69% in the HS group, and an increase in the number of dead embryo upon hatching with values of 23.2% in the control and 25.7% in the HS group.

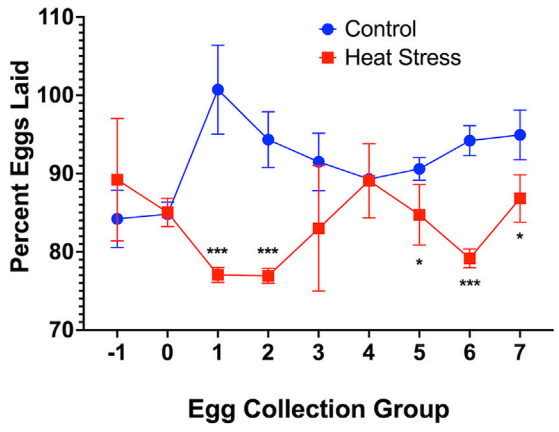

FIGURE 4
Percent Eggs Laid of Ducks Exposed to Cyclic HS (35°C for 10h/day and 29.5°C for the remaining 14h/day) or control. Data shown are means ± SEM, n = 3/time group and all eggs laid over 3 consecutive days averaged per time group. Ducks exposed to heat stress showed a significant lower percent of eggs laid compared to the controls at weeks 1 and 3 after onset of treatment. Egg groups relative to week; groups −1 and 0 (week preceding heat stress), groups 1 and 2 (week 1), groups 3 and 4 (week 2), and groups 5, 6 and 7 (week 3). * = $p < 0.05$, *** = $p < 0.001$.

Egg quality

We observed a significant ($p < 0.001$) decrease in shell weight at week 1 only compared to the control. Yolk weight decreased in the HS group but only significant ($p < 0.05$) at week 3 compared to controls. There were no significant differences in other egg quality parameters such as egg weight, shell thickness, shell and vitelline membrane compression strengths. Table 2 illustrates these results.

Morphometrics

Hens in the HS group showed a significant decrease in body weight ($p < 0.001$) and number of maturing ovarian follicles ($p < 0.05$) compared to controls. There were no significant differences in the relative weights of spleen or testes in drakes between treatment groups. There were also no significant differences in the relative liver weights of either hens or drakes between treatment groups. Table 3 illustrates these results.

Mass spectrometry for yolk and albumen GCs

No measurable levels of corticosterone were observed in the albumen from either control or HS groups. We found a significant increase in the albumen cortisol levels at week 1 ($p < 0.05$) and week 3 ($p < 0.05$, Figure 5A) in the HS group compared to the control. There were no measurable levels of corticosterone (Figure 5B) or cortisol (Figure 5C) in the egg yolk from either treatment group.

Discussion

The purpose of our study was to determine the effects of HS on reproduction, welfare, and glucocorticoid secretion in the Pekin duck. To achieve this, we treated 160 adult drakes and hens at peak lay to HS or control conditions. We observed a decrease in reproductive parameters in hens as seen by reduced egg production, egg quality variables, and follicle numbers. The decrease in fertilized eggs suggests a decrease in male fertility, however the lack of significant differences in testes size suggest a physiological mechanism not evident in this study. Further a sex difference in GC responses, and cortisol, not corticosterone, is deposited into the egg albumen but not yolk. Our data suggest that the increase in albumen cortisol could elicit epigenetic effects on future generations. Further, we observed sex differences in how HS can affect ducks and that these sex differences may be related to sex differences in the glucocorticoid responses as shown by previous studies from our lab (Tetel et al., 2022a; Tetel et al., 2022b).

Numerous studies have reported that differences in the level of GC in mammalian females are higher than in males (Turner, 1990; Ferrini et al., 1997; Bourke et al., 2012). Madison et al. (2018) demonstrated sex differences in hippocampal responses to stressors in zebra finches. These authors further stated that females showed upregulated hippocampal mineralocorticoid receptors while males downregulated both mineralocorticoid and glucocorticoid receptors in response to social stressors. These findings confirms our results that further suggest a sex difference in the HS response, although, we could have taken earlier blood samples to see the acute phase of GC secretion after the onset of HS. Our lab has consistently observed a sex difference in the time of peak of GC secretion in ducks and we have shown that the peak secretion is very dependent upon when that individual first perceives the onset of stressor, so knowing exactly when to collect early blood samples in this current study to observe peak secretion of GC would have been problematic (Tetel et al., 2022a; Tetel et al., 2022b). The changes in circulating glucocorticoid levels associated with HS is likely related to changes in body morphometrics associated with this stressor.

HS has been shown to cause decrease in the body and organ weights of poultry, as further demonstrated in our study. The decline in body weight can partly be attributed to the reduction in feed intake and impairment of the intestinal integrity (Quinteiro-

TABLE 2 Effect[a] of a 3-week chronic cyclic heat stress[b] on egg quality parameters[c] of breeder ducks.

	Weeks relative to treatment			
Parameter and treatment group	**0**	**1**	**2**	**3**
Egg Weight (g)				
Control	83.8 ± 0.78	85.8 ± 0.78	86.1 ± 1.00	85.9 ± 1.75
Heat stress	83.8 ± 0.73	83.9 ± 0.96	83.7 ± 0.85	84.4 ± 0.91
Shell Weight (g)				
Control	7.96 ± 0.088	8.08 ± 0.072[a]	8.06 ± 0.087	8.26 ± 0.083
Heat stress	8.06 ± 0.101	7.65 ± 0.101[b]	7.80 ± 0.101	8.01 ± 0.095
Shell Thickness (mm)				
Control	0.45 ± 0.003	0.45 ± 0.003	0.44 ± 0.003	0.43 ± 0.004
Heat stress	0.45 ± 0.004	0.43 ± 0.004	0.43 ± 0.004	0.44 ± 0.004
Yolk Weight (g)				
Control	24.0 ± 0.336	24.2 ± 0.32	24.6 ± 0.30	25.4 ± 0.42[a]
Heat stress	24.0 ± 0.29	24.1 ± 0.39	24.0 ± 0.31	23.6 ± 0.34[b]
Haugh Unit				
Control	98.7 ± 0.50	97.1 ± 0.65	95.8 ± 0.95	94.1 ± 0.87
Heat stress	97.8 ± 0.80	99.5 ± 0.69	94.4 ± 0.95	94.1 ± 0.80
Shell Strength (N)				
Control	51.3 ± 1.25	50.9 ± 0.99	49.9 ± 1.38	51.9 ± 1.05
Heat stress	47.3 ± 1.81	46.2 ± 1.54	49.0 ± 1.69	48.3 ± 2.11
Vitelline membrane strength (N)				
Control	2.04 ± 0.100	2.14 ± 0.136	2.09 ± 0.097	1.89 ± 0.109
Heat stress	1.84 ± 0.115	1.89 ± 0.125	2.23 ± 0.130	1.84 ± 0.106

[a]Data shown are means ± SEM, n = 33–44/treatment/week.
[b]Breeder ducks exposed to cyclic heat stress (35°C for 10h/day and 29.5°C for the remaining 14h/day) or control measured weekly including week preceding HS.
[c]The table shows Tukey-kramer pairwise comparisons of HS and control groups. Different letter coding within parameter is significantly different ($p \leq 0.05$).

TABLE 3 Effect[a] of a 3-week chronic cyclic heat stress[b] on immune organ parameters in breeder ducks.

	Week 0		Week 3	
Parameters[c, d]	**Control**	**Heat stress**	**Control**	**Heat stress**
Hen				
Body weight (kg)	3.55 ± 0.078	3.38 ± 0.038	3.58 ± 0.057[a]	3.16 ± 0.038[b]
Spleen (g/kg)	0.41 ± 0.028	0.40 ± 0.025	0.48 ± 0.034	0.44 ± 0.018
Follicles (#)	6.1 ± 0.31	5.7 ± 0.45	5.2 ± 0.20	4.4 ± 0.31
Liver (g/kg)	24.3 ± 0.93	24.4 ± 2.03	22.2 ± 0.67	22.6 ± 0.10
Drake				
Body weight (kg)	3.71 ± 0.100	3.78 ± 0.160	3.97 ± 0.150	3.56 ± 0.066
Spleen (g/kg)	0.57 ± 0.051	0.54 ± 0.040	0.52 ± 0.017	0.55 ± 0.021
Testes (g/kg)	30.0 ± 3.29	24.2 ± 2.72	25.8 ± 2.58	25.4 ± 1.58
Liver (g/kg)	18.4 ± 0.73	16.5 ± 1.16	15.4 ± 0.77	14.3 ± 0.85

[a]Data shown are means ± SEM, n = 10/treatment for hens and 5/treatment for drakes.
[b]Breeder ducks exposed to cyclic heat stress (350C for 10h/day and 29.50C for the remaining 14h/day) or control measured 1 week before HS and 3 weeks after.
[c]The table shows Tukey-kramer pairwise comparisons of HS and control groups. Different letter coding within parameter is significantly different ($p \leq 0.05$).
[d]Values of spleen, liver and testes are relative to the body weight (g/kg).

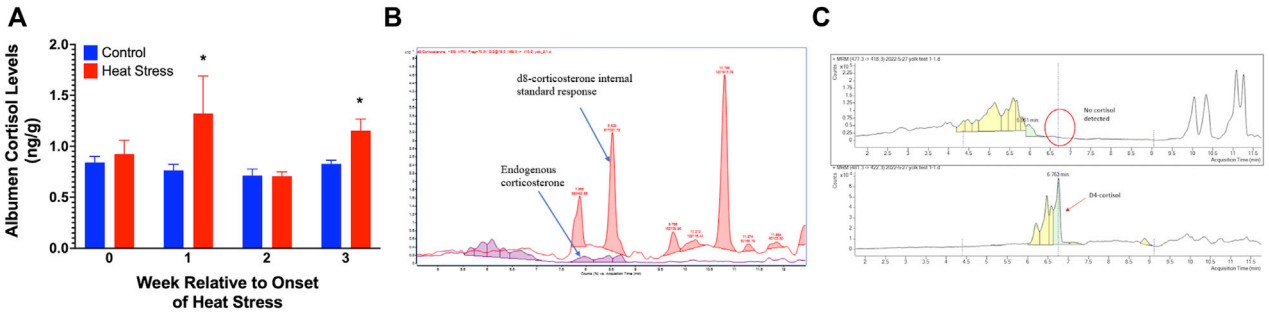

FIGURE 5
Egg albumen concentration of cortisol **(A)**, Chromatograph overlay for yolk corticosterone comparison **(B)**, and yolk cortisol comparison **(C)** of ducks exposed to cyclic heat stress (35°C for 10h/day and 29.5°C for the remaining 14h/day) or control. Data shown for Figure 5A are means ± SEM, n = 6/treatment/week. Albumen cortisol levels were significantly increased at weeks 1 and 3 after onset of treatment compared to controls. The endogenous corticosterone in the samples were below detectable limits. Cortisol and corticosterone were not detected in yolk samples. * = $p < 0.05$.

Filho et al., 2010; Quinteiro-Filho et al., 2012; Sohail et al., 2012; Khatlab et al., 2018). It has been reported that HS negatively affects intestinal mucosa and microbiota composition (Liu et al., 2009; Kers et al., 2018). Further, the damage to mucosal epithelium can directly affect intestinal barrier function, nutrient absorption and impair production performance (Moeser et al., 2007). This finding confirms our result that showed a decrease in the body weight of hens in the HS group. Feed intake data would have added strength to this observation but was, unfortunately, not possible during this study. We also observed a decrease in the number of maturing ovarian follicles, similar to that described by others. Rozenboim et al. (2007) reported the reduction in ovary weight and number of maturing follicles after 6 days of exposure to HS and decline in plasma progesterone and estradiol levels. Other studies also showed a decrease in ovary weight, oviduct weight, hierarchical follicle number and weight following exposure of Japanese quail to HS (Pu et al., 2019). The decline in the number of maturing follicles might be attributed to reduction in blood flow to the ovary during heat stress (Wolfenson et al., 1981). The reduction of maturating follicles in heat stressed hens may also, in part, be caused by the increase in the level of prolactin that decreases the secretion of gonadotropins by suppressing pituitary gonadotrophs leading to ovarian regression (Rozenboim et al., 1993; You et al., 1995).

Several studies have reported the adverse effects of heat stress on egg production and the egg quality of laying hens including a reduction in egg weight, shell strength and weight, albumen deposition, yolk weight, and Haugh unit (Mashaly et al., 2004; Ebeid et al., 2012; Ma et al., 2014; El-Tarabany, 2016; Barrett et al., 2019). These effects are mainly observed in heat-stressed ducks where the decrease in egg quality was attributed to the reduction in feed intake, respiratory alkalosis, and reduction in blood flow to the oviduct (Ma et al., 2014). The decrease in yolk weight was attributed to impairment in the ovary that is caused by the reduction of blood flow to this organ (Mashaly et al., 2004; El-Tarabany, 2016). Ebeid et al. (2012) demonstrated that heat stress leads to respiratory alkalosis due to hyperventilation and the increased blood pH reduces the amount of Ca^{+} that is essential for shell formation. These authors further suggested that the defect in transport of intestinal calbindin leads to a further decrease in calcium absorption. However, others have shown no effects of GC on egg or albumen weight (Wolfenson et al., 1979; Deng et al., 2012). Similar to these previous studies, we found that heat stress decreased shell weight and yolk weight. We conclude that HS affected shell weight and yolk weight but that to achieve decrease in egg weight probably required an extended or greater heat stress exposure in laying ducks, or possibly ducks are less sensitive to HS compared to other poultry species.

Historically, it was suggested that corticosterone is the main plasma GC in birds produced by adrenal cortical cells (Deroos, 1961). A quest to develop a non-invasive measure of stress in chickens lead to the measure of GC in egg albumen and it was discovered that subcutaneous injection of corticosterone leads to its direct deposition in egg (Downing and Bryden, 2008). Campbell et al. (2018) reported an increase in corticosterone deposition in the egg of birds in non-enriched cages compared to birds housed in an enriched environment. However, recently Caulfield and Padula. (2020) showed that it is cortisol that is deposited in albumen, not corticosterone, as suggested by previous studies. Lechner et al. (2001) investigated the presence of steroidogenic enzymes in the Bursa of Fabricius and thymus and reported that the steroidogenic pathways lead to the synthesis of cortisol, and not corticosterone, within these organs. These authors further stated that cortisol has a higher affinity to the GC receptor than does corticosterone in the bursa

and thymus of chickens. Our results support the idea that cortisol is a physiologically important part of the stress response in ducks and that cortisol is selectively deposited in egg albumen and could be used as a non-invasive marker of welfare.

To determine if corticosterone is present in egg yolk, we analyzed yolk extract using mass spectrometry and detected extremely low to non-measurable levels of corticosterone and non-detectable levels of cortisol. We conclude that cortisol is not deposited in yolk but might be synthesized and deposited into the albumen during egg formation in the magnum of the oviduct. Therefore, there is need for further research on extra-adrenal synthesis of steroid hormones in the reproductive tract and other tissues. Several studies have reported increase in yolk immunoreactive corticosterone following the administration of corticosterone or exposure to heat, restraint, or rearing system stress (Okuliarová et al., 2010; Hayward et al., 2015; Della Costa et al., 2019; Pu et al., 2019; Miltiadous and Buchanan, 2021). Corticosterone in avian blood is predominantly bound to proteins, such as corticosterone binding globulin (CBG) and albumin which might also be the case for yolk corticosterone (reviewed by Scanes, 2016). Rettenbacher et al. (2005) were unable to demonstrate the presence of corticosterone in the yolk following the administration of radiolabeled corticosterone or injection of ACTH but suggested the presence of gestagens, a class of steroid hormones also known as progestogens, cross-react with corticosterone antibodies and thus responsible for confounding the corticosterone concentration levels. To investigate this possibility, Rettenbacher et al. (2009) analyzed yolk extracts and reported the absence of immunoreactive compound for cortisol, but rather detected concentrations of progesterone, pregnenolone, gestagen metabolites and a very small peak for corticosterone, all of which support the findings in our study. Interestingly, Rettenbacher et al. (2009) further stated that progesterone antibody revealed 3 peaks representing at least 3 immunoreactive steroids while corticosterone antibody detected immunoreactive compounds at the same elution position as progesterone; confirming that gestagens cross-react with corticosterone antibodies. Quillfeldt et al. (2011) also reported low corticosterone and high gestagen concentrations in the yolk of rockhopper penguins and imperial shag egg yolks. Given the high concentration of gestagens compared to other steroid hormones in the yolk (Von Engelhardt and Groothuis, 2005; Quillfeldt et al., 2011) even low cross-reactivity is capable of confounding results, so it is likely that GC activity measured in the yolk by other studies using antibody-based detection systems are due to cross-reactivity with gestagens or their metabolites. There is need for further studies to investigate the cross-reactivity of GC with other steroid hormones and their metabolites in the yolk, and the validation of these antibody-based, steroid hormone detection assays. However, the presence of cortisol in albumen has been confirmed in our lab and by others (Caulfied and Padula, 2020) using mass spectrometry.

The epigenetic effect of maternal glucocorticoids is under current investigation by our lab, as maternal exposure to stressors can alter the phenotype of offspring thereby affecting both pre-hatch and post hatch welfare and development. Several studies have reported the effect of *in ovo* administration of corticosterone on embryonic and post-hatch development of the offspring (Saino et al., 2005; Janczak et al., 2007; Peixoto et al., 2020). In addition, embryonic exposure to corticosterone led to reduced hatchability, body weight, competitive ability, growth rate, increase embryo mortality and fearfulness (Janczak et al., 2006; Eriksen et al., 2010). Hayward and Wingfield. (2004), reported decrease in hatching weight, growth rate, and increase in HPA axis response to restraint stress in the offspring of Japanese quail implanted with corticosterone. Further studies are underway in our lab to explore the effect of maternal GC on F_1 development, welfare, and adaptation to stress.

In summary, we showed that there are sex differences in GC response of ducks exposed to chronic HS with hens been more responsive to the stressor as evidenced by higher circulating levels of GCs. In addition, we demonstrated the selective deposition of cortisol in egg albumen but not yolk following HS. Therefore, we emphasize the importance of studying the effect of cortisol on embryonic and post-hatch development. Further, HS causes significant decrease in reproductive parameters in hens as seen by reduced egg production, egg yolk, shell weight, and follicle numbers and the decrease in fertility as evident by the reduction in number of fertile eggs and hatchability. Our results suggest that GC elicit differential effects and although corticosterone has been stated to be the predominant GC in avian species, cortisol may provide critical information to further understand and to improve welfare. Finally, the measure of cortisol in egg albumen can be used as a non-invasive marker of stress.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Purdue Animal Care and Use Committee.

Author contributions

GF is the PI and was responsible for funding, design, confirmation of analyses, and editing proof reading manuscript. EO, VT, and JS were all equally responsible for helping to design experiment, collect and assay samples, analyze data, interpret analyses. EO was primary writer of manuscript, but VT and JS were equally responsible for proof reading and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Catecholamine concentrations in duck eggs are impacted by hen exposure to heat stress

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Rapid "fight-or-flight" responses to stress are largely orchestrated by the catecholamines. Moreover, catecholamines and catecholamine precursors are widely recognized to act as interkingdom signaling molecules among host and microbiota, as well as to serve as chemotactic signals for bacterial foodborne pathogens. While albumen and yolk concentrations of glucocorticoids have received extensive attention as non-invasive indicators of hen response to stress, little is known regarding the impact of the hen's stress response on *in ovo* catecholamine and catecholamine precursor concentrations. The aim of the present study was to determine norepinephrine and L-dopa concentrations in albumen and yolk of eggs laid by hens maintained under normal or heat stress conditions. Norepinephrine and L-dopa concentrations were also measured in oviductal tissue. Breeder ducks (~35 weeks/age) were kept under normal (22°C) conditions or subjected to cyclical heat stress (35°C day/29.5°C night) for 3 weeks. Eggs (n = 12 per timepoint/group) were collected on a weekly basis. Hens were sacrificed at baseline or after 3 weeks of heat stress for oviductal tissue collection. Albumen, yolk, and oviduct concentrations of norepinephrine and L-dopa were determined using ultra high-performance liquid chromatography with electrochemical detection. Norepinephrine and L-dopa were detected in oviductal tissue as well as egg albumen and yolk. Norepinephrine concentrations were elevated ($p < 0.05$) in the yolk of eggs laid by the heat stress group compared to those of the control group. Norepinephrine concentrations in albumen were elevated ($p < 0.05$) in the heat stress group compared to control group at week 2. L-dopa concentrations were not significantly affected ($p > 0.05$) by heat stress in albumen, yolk, or oviductal tissue. Together, the present study provides the first evidence of the stress neurohormone, norepinephrine, in duck eggs and identifies that hen exposure to heat stress can affect *in ovo* norepinephrine concentrations. These data highlight the potential utility of *in ovo* catecholamine concentrations as non-invasive measures of the hen's response to stress, as well as warrants future research into whether hen deposition of stress-related neurochemicals into the egg could serve as a chemotactic signal in the vertical transmission of foodborne pathogens.

KEYWORDS

norepinephrine, microbial endocrinology, avian, heat stress, duck, egg, catecholamine

Introduction

Stress is a critical consideration in all aspects of poultry production (Chen et al., 2021) and is also an important factor in *Salmonella* spp. control in poultry flocks (Humphrey, 2006). As such it should not be surprising that animal welfare is a major determinant in shaping disease susceptibility and influencing foodborne pathogen carriage in birds. Avian, as well as mammalian, neuroendocrine responses to stress involve the production of stress-related neurochemicals that are released into circulation, the gut lumen and other peripheral sites (Villageliu and Lyte, 2017), as well as deposited into yolk and albumen contents during egg formation (Moudgal et al., 1990b). While having impact on host physiology, stress-related neurochemicals, such as norepinephrine, have been recognized for decades to act as interkingdom signaling molecules, including to elicit an increase in bacterial growth and colonization as well as to upregulate virulence in the host both *in vitro* and *in vivo* (Lyte et al., 2022).

A thorough understanding how heat stress can affect norepinephrine concentrations in egg albumen and yolk can serve two complementary outcomes for poultry producers. First, measuring catecholamine concentrations, including norepinephrine and l-3,4-dihydroxyphenylalanine (L-dopa), in egg contents can serve as a novel non-invasive measure of hen's response to stress (Moudgal et al., 1990a). Second, norepinephrine concentrations can inform the design of a potential microbial endocrinology-based strategy for the control of vertical transmission of *Salmonella* spp. to prevent bacterial migration from an infected hen oviduct into the albumen and yolk (Methner et al., 2008). Indeed, stress-related neurochemicals, including the catecholamine norepinephrine, can exert a chemotactic effect on bacteria (Lopes and Sourjik, 2018). Hence it can be hypothesized that increased norepinephrine concentrations in egg contents due to hen's stress response could provide a chemotactic signal for the migration of *Salmonella* spp. into the egg contents. Nevertheless, very limited attention has been directed towards the impact of stressors in modern poultry production, including the climate change challenge of heat stress (Lara and Rostagno, 2013), on hen deposition of catecholamines in egg contents or in the oviduct tract. Therefore, we sought to determine whether heat stress can increase norepinephrine and L-dopa concentrations in egg albumen and yolk, as well as oviductal tissue of healthy breeder ducks. The results of this study provide poultry researchers with the foundation to explore whether egg neurochemical concentrations could be used as a non-invasive measure of hen's stress response and are detectable at relevant concentrations to plausibly serve as a chemotactic attractant for vertical transmission of *Salmonella* spp.

Materials and methods

Ducks and egg collection

None of the breeder ducks in this experiment had any prior exposure to heat stress (HS). The ducks were placed in single rooms with an 18:6 light cycle, temperature of 20–22°C for both treatment groups until 85% lay (~35 weeks of age). Water nipple lines (5 ducks per nipple) were placed over a pit covered with raised plastic flooring, and the remaining area of the rooms were covered with pine shavings and added to or replaced as necessary at the same time for both rooms. Nest boxes were placed along one wall of the room with four hens per nest box as per industry standards (Chen et al., 2021), and all eggs collected daily. Details of the study design were published elsewhere (Oluwagbenga et al., 2022). Briefly, heat stress or control conditions began when the hens reached 85% lay. The HS group was subjected to cyclic temperature of 35°C for 10 h/day and returned to 29.5°C for the remaining 14 h/day for 3 weeks while the control ducks were housed at an industry-standard temperature of 22°C. These temperature ranges were selected as being typical temperatures during periods of heat stress in the midwestern United States and to be coincident to decreases in fertility observed in commercial duck barns. All procedures and management practices were approved by Purdue University Institutional Animal Care and Use Committee (Protocol #2109002195) prior to the start of the study.

Eggs from control and heat stress groups (n = 12 eggs/group/timepoint) were collected on the same day prior to the onset of HS (baseline), and then at the end of each week over the 3 weeks heat stress period. Details of the egg collection procedure were previously published (Oluwagbenga et al., 2022). Due to the number of hens compared to number of nest boxes typical for industry and research, we are not capable at this time to associate an egg with the hen whom laid that specific egg. To collect oviductal tissue, breeder ducks (n = 9–10/group/timepoint) were euthanized (intravenous pentobarbital; FatalPlus, 396/mg/mL/kg) at baseline (i.e. before the start of heat stress) or at the end of the 3 weeks heat stress period for oviductal tissue collection. An n = 12 eggs per group/timepoint and an n = 9–10 ducks/group/timepoint for oviduct samples were used in the present study as these n numbers provided sufficient Power to detect a significant effect ($p < 0.05$) of stress on egg yolk stress-related neurochemical concentrations (Moudgal et al., 1990a; Moudgal et al., 1990b). After breaking the eggshell, the yolk and albumen were carefully separated. 900 µL of yolk or albumen were separately pipetted into individual 2 mL reinforced tubes each containing 100 µL of 2N perchloric acid, vortexed, then snap frozen on dry ice. Oviductal tissue was collected from the magnum region of the reproductive tract, weighed, and then acidified in individual 2 mL reinforced tubes containing 1 mL of 0.2N perchloric acid and six ceramic beads, then snap frozen on dry ice. All yolk, albumen, and oviduct samples were stored at −80°C until analysis.

Ultra-high performance liquid chromatography with electrochemical detection

All samples were processed and analyzed as previously described (Lyte et al., 2022). Briefly, thawed samples were homogenized in a BeadRuptor and then centrifuged at 3,000 × g and 4°C for 15 min. Yolk samples required heating to 37°C and diluting 1:10 with mobile phase in order to pass through the spin filters. Sample supernatant was passed through 2–3 kDa spin filters, and flow-through was stored at −80°C until ultra-high performance liquid chromatography with electrochemical detection (UHPLC-ECD). The UHPLC-ECD consisted of a Dionex Ultimate 3,000 autosampler, a Dionex Ultimate 3,000 pump, and Dionex Ultimate 3,000 RS electrochemical detector (ThermoFisher-Scientific, Sunnyvale, CA). Mobile phase was buffered 10% acetonitrile (Catalog #: NC9777698, ThermoFisher-Scientific) and the flow rate was 0.6 mL/min on a 150 mm (length) 3 mm (internal diameter) 3 µm (particle size) Hypersil BDS C18 column (Catalog #: 28,103-153030,

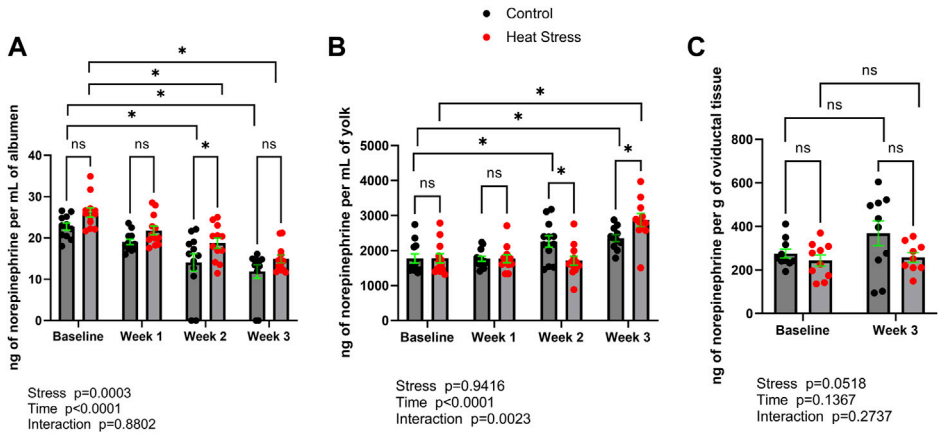

FIGURE 1
Norepinephrine concentrations in albumen **(A)**, yolk **(B)**, or oviductal tissue **(C)** in control or heat stress groups at baseline or after 1, 2, or 3 weeks of heat stress. Significant differences ($p < 0.05$) were indicated with *. Values are ng per mL of albumen or yolk, or ng per g of oviductal tissue. All values are expressed as mean ± SEM (n = 12 eggs/timepoint/group or for oviductal tissue n = 9–10 ducks/timepoint/group).

ThermoFisher-Scientific). A 6041RS glassy carbon electrode set at 400 mV was used for electrochemical detection. Data were analyzed using the Chromeleon software package (version 7.2, ThermoFisher-Scientific), and neurochemical identification was confirmed using the retention time of the corresponding analytical standard (Millipore-Sigma, St. Louis, MO) (for norepinephrine, Catalog #: 636-88-4; for L-dopa, Catalog #: 59-92-7).

Statistical analysis

Grubbs' test (alpha = 0.05) was used to identify outliers in each dataset (GraphPad Prism v9.4.1, San Diego, CA). Data were analyzed with outliers removed followed by two-way ANOVA with Šídák's *post hoc* test (GraphPad Prism v9.4.1). Stress and time were considered as main effects. Differences were considered significant at the threshold of $p < 0.05$.

Results

Norepinephrine (Figure 1) was detected in albumen, yolk, as well as oviductal tissue in both control and heat stress groups. Norepinephrine concentrations were not different ($p > 0.05$) between control and heat stress groups at baseline in albumen, yolk, or oviduct. No differences between control and heat stress groups were observed in norepinephrine concentrations in either albumen or yolk at week one. In albumen, norepinephrine was significantly elevated ($p < 0.05$) at week two in the HS group compared to the control. In yolk, norepinephrine was lower at week two relative to control, and significantly elevated ($p < 0.05$) compared to control at week three. There was a significant interaction for stress x time in yolk norepinephrine ($p = 0.0023$). Oviduct norepinephrine concentrations did not differ ($p > 0.05$) between control and HS groups at week three. Time-related changes were observed in both control and HS groups. In albumen, at weeks two and three norepinephrine concentrations were lower in both control and HS groups compared to baseline levels. In yolk, norepinephrine levels were higher at week two and three in control compared to baseline, and greater at week three in HS group compared to respective baseline samples. Differences between week three and baseline in oviduct norepinephrine concentrations were not observed ($p > 0.05$) for either control or HS groups.

L-dopa concentrations are reported in Figure 2. L-dopa concentrations were not significantly different ($p > 0.05$) between control and HS groups at any timepoint in albumen, yolk, or oviduct. Time-related changes in L-dopa concentrations were found in both control and HS groups. In albumen, lower concentrations relative to baseline were found in the HS group at weeks two and three, while in control group only at week three compared to respective baseline group. In yolk, a decrease in L-dopa concentrations was observed ($p < 0.05$) in the HS group at weeks two and three compared to baseline. There was a significant interaction for stress x time in yolk L-dopa ($p = 0.0203$). Decreased oviduct L-dopa concentrations in week three relative to baseline groups were not statistically significant ($p > 0.05$).

Discussion

While glucocorticoids are detectable in egg contents (Caulfield and Padula, 2020; Oluwagbenga et al., 2022), limited attention has been directed towards how stressors encountered in modern poultry production, such as heat stress, may affect catecholamine and catecholamine precursors in albumen and yolk. Previously it was reported that catecholamines are detectable in chicken egg yolk and are elevated in response to stress (Moudgal et al., 1990a; Moudgal et al., 1990). The present study is the first to demonstrate that catecholamines and catecholamine precursors, specifically norepinephrine and L-dopa, respectively, are also present in duck hen eggs and their levels are impacted by stress similarly to that observed in chickens (Moudgal et al., 1990b; Moudgal et al., 1990b).

The concentrations of norepinephrine reported here (Figure 1) are similar to those previously identified in chicken egg yolk (Moudgal et al., 1990a), indicating translational, or phylogenetic, utility of findings that describe neurochemical concentrations between ducks and chickens exposed to stress. Interestingly, when compared to the

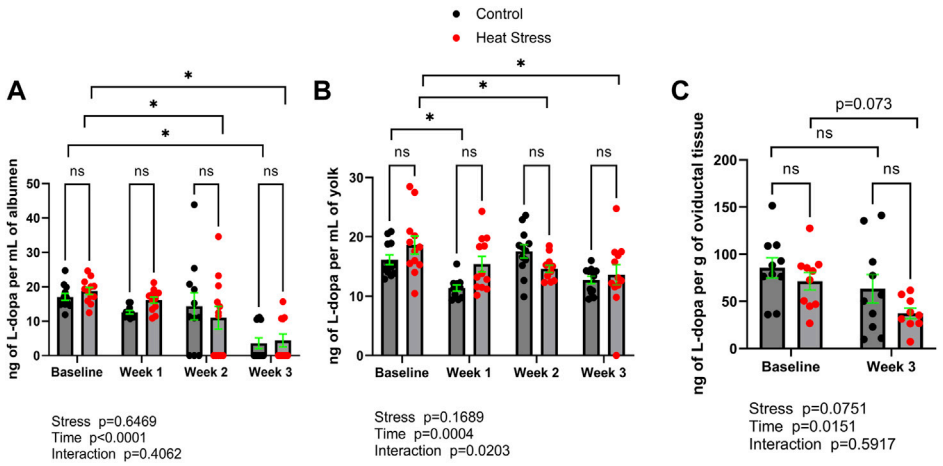

FIGURE 2
L-3,4-dihydroxyphenylalanine (L-dopa) concentrations in albumen **(A)**, yolk **(B)**, or oviductal tissue **(C)** in control or heat stress groups at baseline or after 1, 2, or 3 weeks of heat stress. Significant differences ($p < 0.05$) were indicated with *. Values are ng per mL of albumen or yolk, or ng per g of oviductal tissue. All values are expressed as mean ± SEM (n = 12 eggs/timepoint/group or for oviductal tissue n = 9–10 ducks/timepoint/group).

matching control group, we observed that norepinephrine concentrations in egg yolk of the heat stress group were lower and elevated at week two and week three, respectively. This result may suggest temporary adaptation of the hen's stress response to heat stress. Indeed, previous reports in mammals have shown that chronic repeated forms of stress, including temperature based stressors such as cold exposure, cause a prolonged dampening of norepinephrine concentrations and other neuroendocrine components of the sympathetic stress response (Stone and McCarty, 1983; Konarska et al., 1990). As eggs were collected in the present study at the end of each week of heat stress, future studies should investigate whether at the beginning of the heat stress period (i.e. sampling eggs at the beginning of week one of heat stress) there may be an initial immediate stress response.

We also sought to quantify a precursor to norepinephrine synthesis, L-dopa, concentrations (Figure 2) in egg contents and oviduct. L-dopa is a precursor to norepinephrine and is able to be added to feed and has been reported to directly modulate catecholamine synthesis in stress-susceptible pigs *in vivo* (Erlander et al., 1985). While L-dopa was not found to be increased ($p > 0.05$) in albumen, yolk, or oviduct in response to stress, the fact that L-dopa was detectable in egg contents and the oviduct provides a possible platform for the design of in-feed manipulation of L-dopa, and therefore *in ovo* norepinephrine concentrations. This could be especially useful as a potential microbial endocrinology-based strategy to control the impact of stress on vertical transmission of *Salmonella* spp. via hen norepinephrine biosynthesis. It was previously shown that norepinephrine not only promotes *Salmonella* spp. colonization in chicken layers *in vivo*, but that in egg whites *in vitro* can enable *Salmonella* spp. growth (Methner et al., 2008). Moreover, as norepinephrine can exert chemotactic effects on bacteria (Lopes and Sourjik, 2018), *Salmonella* spp. can migrate from albumen to the yolk (Braun and Fehlhaber, 1995), it is reasonable to hypothesize that environmental stress that causes the hen to deposit norepinephrine in the egg contents could serve to encourage vertical transmission of *Salmonella* spp. from infected oviduct to the egg. Alternatively, stress hormones could be deposited into egg albumen as a way of transferring information about the environment to the embryo.

Avian stress hormones have been demonstrated to be transferred to egg contents, including the yolk (Hayward and Wingfield, 2004). Maternal hormones in the avian egg are means of transferring information from one generation to the other showing how the mother can contribute to the coping mechanism of the offspring to environmental variability (von Engelhardt and Groothuis, 2011). Glucocorticoids, androgens, and nutrients can be transferred from mother to egg via the yolk and albumen and can have significant effects on the phenotype of the developing offspring (Eising and Groothuis, 2003; Hayward and Wingfield, 2004; Caulfield and Padula, 2020). In the same eggs analyzed in this study, we separately assessed glucocorticoid levels and found increased cortisol in the albumen (Oluwagbenga et al., 2022). The combination of both catecholamine and glucocorticoid may lead to specific patterns of DNA methylation during embryogenesis, although this has yet to be determined and requires further studies. However, several studies have elevated yolk androgen concentrations experimentally to assess the effects on the developing offspring. These effects include earlier hatching time in black-headed gull (Eising and Groothuis, 2003), increased muscular development in red-winged blackbird (Lipar and Ketterson, 2000), enhanced post-hatching growth in canaries (Schwabl, 1996), more intense food solicitation behavior in black-headed gulls (Eising and Groothuis, 2003), improved nestling survival in zebra finches (von Engelhardt et al., 2006), and decreased early immune function in Chinese painted quail (Andersson et al., 2004). Thus, there is ample evidence for elevated hormone levels in egg to alter offspring phenotypes.

Together, the present study establishes the presence of norepinephrine and L-dopa in duck egg contents, and that hen exposure to heat stress can alter catecholamine concentrations in both albumen and yolk.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Purdue University Institutional Animal Care and Use Committee.

Author contributions

JL conceived and designed the project. GF and EO designed the heat stress paradigm, conducted the bird study, and collected all eggs and samples. ML and KD designed, performed all UHPLC analyses. JL analyzed the UHPLC data and wrote the first draft of the manuscript. All authors reviewed, edited, and approved the final version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of a variable light intensity lighting program on the welfare and performance of commercial broiler chickens

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Our previous variable-light intensity lighting program studies indicate the light intensity preference behavior of broilers for their daily activity including eating and resting. To evaluate the effects of variable-light intensity lighting program on performance and welfare of broilers, four commercial trials were conducted for looking at behaviors, mortality, leg-health, performance, and brain welfare indicator genes including tryptophan hydroxylase 2 and tyrosine hydroxylase (TH), glucocorticoid receptor (GR), brain-derived neurotropic factor (BDNF), and melanopsin (Opn4) gene expression. One-day-old broilers were housed in four commercial broiler houses. Each quadrant (section) of the house was placed with 4,800 chicks. A total of four lighting programs began on day 7 with 5 lux (lx), 20 lx, natural light (NL, 480 lx), and variable light (2–5/40 lx) using LED lights on a 16L:8D photoperiod. In the variable-light house, the number of dustbathing holes was significantly higher than that in natural-light houses and 5-lx and 20-lx houses. Daily physical activities, footpad condition, fear response to novel objects, body weight, feed conversion ratio, and the number of leg-problem induced culled birds were affected by the variable-light intensity lighting program. Expression of tryptophan hydroxylase 2 in the DRN and VTA of variable-light treated birds was lower than that of 5-lx- and 20-lx-treated birds on day 42 ($p < 0.05$). Higher expression of VTA-TH in 5-lx-treated birds than that in 20-lx-, NL-, and variable-light-treated birds suggests the high stress-susceptibility of 5-lx treated birds. Lower VTA-GR expression in 20-lx- and variable-light-treated birds indicates lower stress than that in NL- and 5-lx-treated birds ($p < 0.05$). The VTA-BDNF expression of NL-treated birds was 2.5 fold higher than that of 5-lx-, 20-lx-, and variable-light-treated birds ($p < 0.05$), and variable-light-treated birds showed the lowest level of BDNF expression ($p < 0.05$), suggesting the chronic social defeat stress in NL-treated birds. The result of VTA-Opn4 expression on day 42 suggests the possible role of VTA-Opn4 in broiler welfare through central light perception. Taken together, the variable-light intensity lighting program increased volunteer natural behaviors and physical activity, which may improve footpad condition and leg health of birds, consequently. Performance data including the increased daily weight gain and the lowered feed conversion ratio and results of brain welfare indicator gene expression showed the beneficial effect of the variable-light intensity lighting program on the performance and welfare of commercial broilers.

KEYWORDS
variable light intensity, broilers, welfare, serotonin, dopamine, BDNF, melanopsin

1 Introduction

Light is a critical environmental factor that can affect behaviors, welfare, and production for intensively housed commercial broilers (Alvino et al., 2009; Deep et al., 2010; Olanrewaju et al., 2016; Wu et al., 2022). One of the most noticeable effects of light on birds is the effect of light intensity on birds' health and behavior (Deep et al., 2010; Blatchford et al., 2012; Olanrewaju et al., 2018). Light intensity has been shown to affect the activity of birds, but most studies have focused on constant light intensities to determine their effect on welfare and performance. Intriguingly, research by Blatchford et al. (2012) showed a strong effect of light intensity contrast on the behavior and health of broilers and suggested that high contrast in light intensity was associated with strong daily rhythms of behavior. In the previous light preference studies, broiler chickens showed preference for different light intensities (Berk, 1995; Prayitno et al., 1997; Raccoursier et al., 2019; Kang et al., 2020). Broiler chickens showed preference for the higher intensity light when they are performing active behaviors but prefer dimmer areas when resting (Newberry et al., 1985; Berk, 1995; Raccoursier et al., 2019; Kang et al., 2020).

Leg health is one of the most prevalent causes of culling and late mortality during grow-out of commercial broilers. It has been suggested that increasing locomotor activity in broilers may improve their welfare (Bizeray et al., 2002; Kristensen et al., 2004; Reiter and Bessei, 2009; Kang et al., 2020). Our previous variable light intensity studies indicate that when birds have a dual light choice, they consumed more feed in the lighter intensity area (20 lux (lx)) than in the lower intensity area (2 lx) (Raccoursier et al., 2019; Kang et al., 2020). There was no significant difference in production parameters (body weight and feed conversion ratio). However, the results of central welfare indicator studies suggest better central welfare in VL intensity-treated birds (Kang et al., 2020).

Most physiological evaluations used in the broilers' welfare assessment tend to determine negative rather than positive welfare indicators under the assumption that a lack of a negative effect is indicative of well-being (Marcet Rius et al., 2018). Serotonin (5-HT), dopamine (DA), and brain-derived neurotropic factor (BDNF) were suggested as positive indicators in the assessment of animal welfare (Boissy et al., 2007; Polter and Kauer, 2014; Rault et al., 2018; Kang et al., 2020). 5-HT is a major neurotransmitter in the central nervous system (CNS) involved in emotional states caused by stress, pain, or the availability of food (Chamas et al., 1999; Mosienko et al., 2012). In mammals, chronic stress stimulated tryptophan hydroxylase 2 (TPH2: rate-limiting enzyme of 5-HT biosynthesis) gene expression levels in the raphe nuclei of the brainstem (Chamas et al., 1999), suggesting the activation of 5-HT metabolism. A recent animal study showed the increasing of brain 5-HT levels during a novel object test (Ursinus et al., 2013), suggesting an important role of 5-HT in the behavioral responses of animals when confronted with a challenging situation. The amygdalar complex and nucleus accumbens are associated with positive emotional states, and the nucleus accumbens is the terminal site of the DAergic mesolimbic axis from the midbrain VTA (Ikemoto, 2007; Holly and Miczek, 2016). DA-releasing neurons of the VTA are located near the base of the midbrain and play central roles in reward-related and goal-directed behaviors (Morales and Margolis, 2017). The VTA-DAergic neurons are involved in integrating complex inputs to convert multiple signals that influence motivated behaviors (Beier et al., 2015; Bouarab et al., 2019). The avian VTA contains cell bodies that label positively for tyrosine hydroxylase (TH; the rate-limiting enzyme in catecholamine biosynthesis) and have been investigated in broilers as a welfare indicator in the brain (Kang et al., 2020). BDNF is a stress- and activity-dependent neurotrophic factor involved in many activities and modulated by the hypothalamic–pituitary–adrenal (HPA) axis (Jerzemowska et al., 2012; Phillips, 2017; Rault et al., 2018). Animal studies have shown that physical exercise is associated with increased expression of BDNF in the brain and may improve memory performance and reduce depressive symptoms by promoting neurogenesis and neuronal differentiation (Hötting and Röder, 2013; Arosio et al., 2021). In addition, BDNF concentrations in the brain and blood are correlated, offering the opportunity to use peripheral BDNF as a minimally invasive measure of effective enrichment reflecting neural changes. Another important regulator of VTA activity is glucocorticoid (GC), which is synthesized in response to a range of stimuli including stress and is regularly measured in the assessment of animal welfare (Ralph and Tilbrook, 2016). Its action relies on the GC receptor (GR) which translocates into the nucleus upon ligand binding and regulates the transcription of a battery of genes. VTA-GR signaling was suggested to be involved in stress and reward system, regulating the feeding behavior (Daftary et al., 2009; Hensleigh and Pritchard, 2013; Ferrario et al., 2016; Mizoguchi et al., 2021).

Therefore, addressing the central 5-HT, and DA, VTA-GR, and VTA-BDNF in broilers may provide data to aid in understanding possible adaptive behavioral or physiological responses of commercial broilers to the environment. In the present study, we hypothesized that when broilers in commercial houses are provided with the VL intensity lighting environment, it will stimulate birds' innate natural behavior, causing volunteer movement for consumption of feed and water and consequently improving physical activity and helping leg health and welfare of birds. To test this hypothesis, we have focused on the effects of the VL intensity lighting program on the gene expression of the indicators of natural behaviors, footpad condition, performance, and central welfare in the brain compared to other constant light intensities and natural light.

2 Materials and methods

2.1 Experimental design, animal housing, and sample preparation

One-day-old broilers (Cobb 700, mixed sex, and 19,200 birds/house) were housed in four commercial broiler houses (Tyson Foods Broiler Welfare Research Farm (BWRF)). Four replicate trials were performed, and each house was composed of four quadrant sections (compartment). Each quadrant of the house was placed with 4,800 chicks with all source flocks equally represented in each quadrant. Birds were raised for 56, 51, 49, and 55 days in trails 1, 2, 3, and 4, respectively. Each house was equipped with standard feeders, waterers, and brooders (12.8 m × 122 m, wood shavings). Two of the houses have a 60-cm-wide strip of clear plastic that runs

the length of the houses from 120 to 180 cm high on the sidewall and allows for natural light to enter. The natural light window can be sealed to convert that house to internal illumination. In each trial, four different light intensity lighting programs were installed, and the light intensity (LED) was measured at nine different areas of the house. Averages of light intensity in 5-lx, 20-lx, natural-light (NL), and VL houses were 6.16 ± 0.16 lx, 26.16 ± 0.70 lx, 483.76 ± 42.02 lx, and 2.07/40.4 ± 0.04 lx, respectively. A diet was formulated to meet minimum industry standards (Council, 1994). Light was switched on at 6 a.m. on day 1–3 (23L1D_40 lx), and then on day 4–7, the photoperiod schedule was changed to 20L4D_20 lx. The NL house received supplemented light for maintaining the same photoperiod. On day 7, lighting programs were started for 5-lx, 20-lx, NL, and VL houses (16L8D; light switched on at 6 a.m.). The VL house received about 40 lx light intensity over the feed lines and dimmer light intensity at the sidewalls (2–5 lx). Data on dustbathing holes and other natural behaviors were obtained weekly without interruption of the time schedules. In trials 1 and 3, the brains of the birds in each section were sampled on days 14, 28, and 42. Birds in each section (n = 12/treatment; male) were randomly selected, weighed, and transported to the brain sampling room. Daily body weight gain and feed conversion ratio were obtained from the processing plant at the end of the trial for each house. The guidelines for care and experimental use of animals were followed, and all birds were maintained in accordance with the protocol of Tyson Foods BWRF.

2.2 Observation of welfare-related behaviors

2.2.1 Dustbathing holes and daily physical activity

In trial 3, the number of dustbathing holes was counted within six identified areas of the section (4 sections/house) to evaluate the effects of the four different light intensity lighting programs (5 lx, 20 lx, NL, and VL) on the dustbathing behavior. In each section, dustbathing holes as the evidence of dustbathing behavior were counted at 9, 16, and 23 days of age. The number of holes per 10 m^2 was determined. Data were compared among treatments. In trial 4, daily broiler activity was monitored using a 22-g activity tracker, Animo (www.surepetcare.com, activity and behavior monitor), which monitors animals' activity and behavior including sleep quality, energy burnt, and shaking *via* tri-axial accelerometer technology. A similar animal activity tracker, FitBark (www.fitbark.com), was used for monitoring animal's movement in the behavior study (Delgado et al., 2022). At 38 days of age, birds were randomly selected and body weight was measured (n = 16/house; 20 lx and VL house). An Amino tracker was installed for each bird using a commercially available chicken harness and uninstalled at 43 days of age. Average daily activity (joules/day) of each bird was obtained from the installed software. Animo energy calculation is based on an industry standard calculation that takes into account the bird's weight. The energy burnt is tracked against each movement type for birds.

2.2.2 Footpad condition test

In trial 3, in each section of houses, randomly selected broiler feet (n = 10/section and four sections/house) were evaluated with a scoring range of 0–2. Footpad data were analyzed in a pass and fail manner. Lesion scorings on the feet of birds were given as follows: 0—no evidence of footpad dermatitis (pass), 1—minimal evidence of footpad dermatitis (fail), and 2—evidence of footpad dermatitis (fail). Pass birds have normal color and minimal swelling, and no lesion was found in more than half the area of the central pad. Fail birds have discoloration and swelling, and lesions were found in more than half the area of the central pad. Footpad condition was checked at 28 and 42 days of age, and the percentage of failed birds to the inspected birds was obtained and compared among treatment groups.

2.2.3 Novel object test

In trial 3, the novel object test was conducted for each quadrant (section) of the four houses. The test was carried out every 7 days in the morning, starting at 31 days of age. The test was conducted by placing a novel object in the center of the section by an observer. Observation points were set the same every week. The novel object was a cone-shaped container (30 cm H x 24 cm D) and had identical proportions of green, yellow, and red colors. The observer moved away from the object, and the bird activity was evaluated by counting the number of birds that approached toward the novel object in different timepoints (30 s, 1 min, 5 min, 10 min, and 15 min).

2.3 Leg-problem-induced culled birds and mortality

In each section of trials 1 and 3, the accumulated number of birds that were culled because of leg problems and total dead birds were obtained from day 7 to 14, day 7 to 28, and day 7 to 49. To evaluate the effects of different light intensity lighting programs on leg health and total mortality, the number of total dead birds and leg-problem-induced culled birds was obtained on day 7. Leg problems of culled birds included any leg issue that prevented the bird to access feed or water and/or marked a gait score of 2 based on the modified Tyson Foods' gait scoring system. Mortality was recorded daily, and birds were removed when they died, were unable to move to access feed and water, appeared to be sick or injured, and exhibited signs of lameness (one or both legs splayed and severe hock/paw). Accumulated mortalities were obtained from day 7 to 14, day 7 to 28, and day 7 to 42. The same counting process was used for counting the number of birds which were culled due to leg problems (one leg or two legs). Data were compared among treatments.

2.4 Dissection of the dorsal raphe nucleus (DRN) and caudal raphe nucleus (CRN) of the brainstem and ventral tegmental area (VTA) of the midbrain

According to the previous studies on avian species and a chick brain atlas (Kuenzel and Masson, 1988; Kang et al., 2009; Kang et al., 2020), two major 5-HTergic regions in the brainstem, DRN and CRN, and VTA regions were dissected in a cryostat microtome. The dimensions of the dissected section are as follows: 2.5–3 mm (W) x 1–1.5 mm (H) x 2.5–3.0 mm (L) for DRN; 2–2.5 mm (W) x

TABLE 1 Primers used for RT-qPCR.

Gene	GenBank #	Primer sequence (5′–3′)	Size (bp)	Annealing Tm (°C)
TPH2	NM_001001301.1	F: AGGACCTCCGCAGTGATCTA	111	58
		R: CAGCATAAGCAGCTGACAACA		
TH	NM_204805	F: CTTTGATCCTGATGCTGCTG	103	56
		R: CCTCAGCTTGTTTTTGGCAT		
GR	NM_001037826	F: GCCATCGTGAAAAGAGAAGG	95	54
		R: TTTCAACCACATCGTGCAT		
BDNF	NM_001031616	F: GACATGGCAGCTTGGCTTAC	167	60
		R: GTTTTCCTCACTGGGCTGGA		
Opn4	AY036061	F: AAGGTTTCGCTGTCATCCAGC	128	58
		R: CTGCTGCTGTTCAAACCAAC		
GAPDH	NM 204305	F: CTTTGGCATTGTGGAGGGTC	128	58–60
		R: ACGCTGGGATGATGTTCGG		
β-actin	L08165	F: CACAATGTACCCTGGCATTG	158	54–56
		R: ACATCTGCTGGAAGGTGGAC		

1–1.2 mm (H) x 2.5–3.0 mm (L) for CRN; and 3–3.5 mm (W) x 2–3 mm (H) x 1–1.2 mm (L) for VTA. The thickness (W, H, and L) of the dissected brain tissue block was proportionally increased from young birds to older birds based on the brain size and structure. Inside the cryostat, the brain areas shown as rectangles were dissected from each flattened brain section using a scalpel handle and blade (#11) and were quickly transferred to TRIzol reagent and then stored at -80°C until total RNA extraction.

2.5 RNA isolation and two-step real-time quantitative RT-PCR

Total RNA was extracted from dissected frozen tissues using TRIzol® reagent (Invitrogen Life Technologies, Palo Alto, CA, United States) followed by DNase I treatment and purification of total RNA using the RNeasy Mini Kit (Qiagen, Valencia, CA, United States). The RNA quality and quantity were determined using agarose gel electrophoresis and NanoDrop 1000 (Thermo Scientific, Wilmington, DE, United States). A volume of 2 μg of the total RNA from each sectioned tissue was converted into cDNA with oligo $(dT)_{16}$ primer and SuperScript III reverse transcriptase (Invitrogen, Grand Island, NY, United States), as previously described (Kang and Kuenzel, 2014; Nagarajan et al., 2017; Kang et al., 2017, 2020). The specific oligonucleotide primers were designed using the Primer3 program (http://frodo.wi.mit.edu). A total of five primer sets for chicken TPH2, TH, GR, BDNF, and Opn4 were designed, and conventional RT-PCR was performed for optimizing annealing temperature for each primer set (Table 1). The PCR products were analyzed by agarose gel electrophoresis (3%). Melting curve analysis and PCR efficiency for each selected primer set were validated with the default settings on the ABI 7500 system (Applied Biosystems LLC, Foster, CA, United States). The efficiency of PCR was evaluated by performing a dilution series experiment, and the slope of the standard curve was translated into an efficiency value. The efficiency of the PCR within 95%–100% was accepted for this study. A portion of the cDNA was subjected to real-time quantitative RT-PCR (qRT-PCR) using an ABI 7500 system with Power SYBR Green PCR Master Mix (Invitrogen, Grand Island, NY, United States). Chicken glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were used as internal controls. Dissociation curves were constructed at the end of amplification for validating the quality of the data. All qRT-PCR experiments were performed in triplicate, and the values of the average cycle threshold (Ct) were determined, and the delta-Ct scores for gene transcripts in each sample were normalized using the delta-Ct scores for GAPDH/β-actin and expressed as the relative fold change in gene expression using the $2^{-\Delta\Delta Ct}$ method. The gene name, NCBI accession numbers, primer sequences, PCR product size, and annealing temperatures used in the present study are shown in Table 1.

2.6 Statistical analyses

Statistical analyses were performed using JMP® 14.0 (SAS Institute Inc., NC). Normal distribution was first tested, and differences among the groups were analyzed using one-way analysis of variance (ANOVA) followed by mean comparison using Tukey's HSD test at a significance level of $p < 0.05$. Multiple comparisons of group means by Tukey's HSD test were used to evaluate behavior data, including dustbathing holes, daily physical activity, footpad lesion, and novel object test, accumulated mortality, the number of culled birds, and relative changes in TPH2, TH, GR, BDNF, and Opn4 mRNA expression among treatment groups for each gene. Data are presented as the mean ± SEM. A probability level of $p < 0.05$ was considered statistically significant.

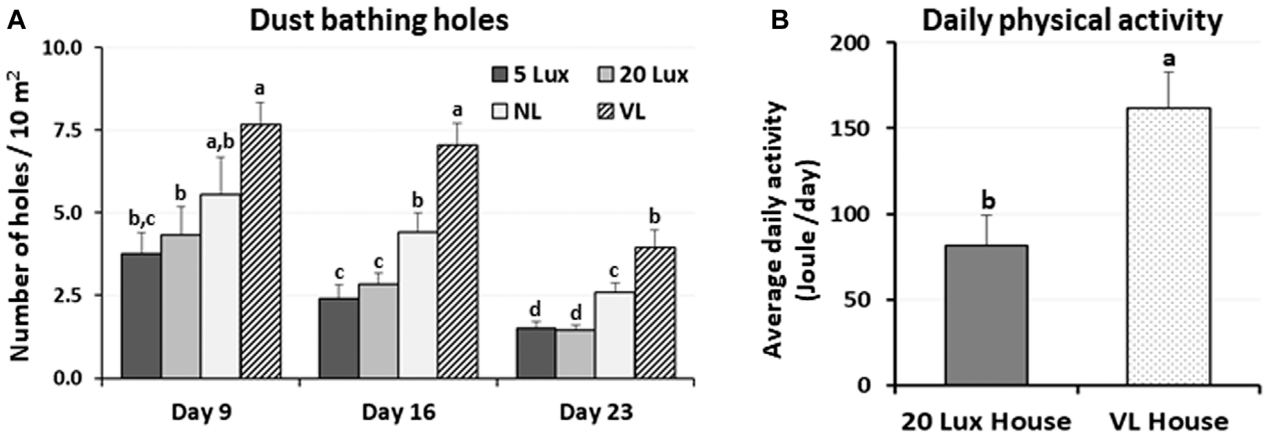

FIGURE 1
Effects of the four different lighting programs (5 lx, 20 lx, NL, and VL) on the number of dustbathing holes and daily physical activity at different ages. **(A)** In the each section of trial 3, dustbathing holes as the evidence of dustbathing behavior were counted at 9, 16, and 23 days of age. Dustbathing holes were observed in six parts of each section and number of holes per 10 m^2 was determined. Data (mean ± SEM) were compared among treatments. Different lower-case letters above the bars denote significant differences ($p < 0.05$) among groups, where a>b and a,b is not different from a or b. **(B)** In trial 4, an activity tracker, Animo, was installed on bird using harness at 38 days of age, and uninstalled at 43 days of age (total $n = 16$, 4/sections, 20 lx and VL house). Average daily activities (calorie consumption) for each bird from day 39 to day 42 obtained. Data (mean ± SEM) were compared between 20 lx and VL houses. Different lower-case letters above the bars denote significant differences ($p < 0.05$) between groups.

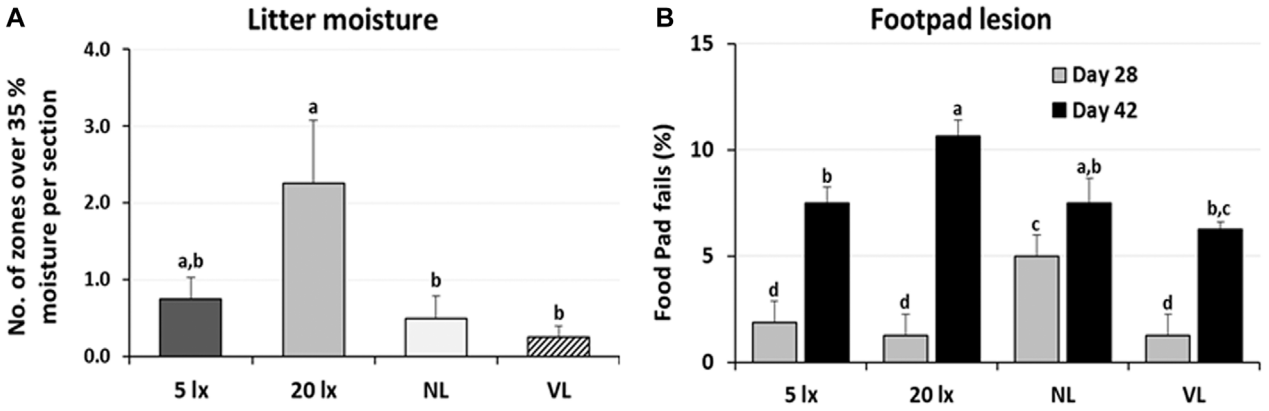

FIGURE 2
Effects of the four different lighting programs (5 lx, 20 lx, NL, and VL) on the litter moisture and footpad health. **(A)** In trial 1, condition of litter moisture was accessed by the hand-clumping method. When litter begins to retain moisture and moisture content is over 35%, it clumps together. Seven different areas (zones) of each section were tested if the litter clumps by hand. Average of number of zones per section with positive hand-clumping was obtained in each treatment of light programs and compared among treatment groups. **(B)** In trial 3, footpad condition was checked at 28 and 42 days of age in each section of the treatment houses ($n = 10$/section, 4 sections/house). Percentage of footpad failed birds from the inspected birds was obtained and compared among treatment groups. Data (mean ± SEM) were compared among treatments. Different lower-case letters above the bars denote significant differences ($p < 0.05$) among groups, where a>b>c. b,c is not different from b or c.

3 Results

3.1 Effects of different light intensity programs in a commercial broiler house on welfare-related behaviors

The dustbathing behavior made holes on the floor of commercial broiler houses. Weekly counting of the numbers of holes in each section of the house was performed and compared (Figure 1A). The numbers of dustbathing holes in the NL and VL houses were higher than those in the 5-lx house on day 9 (2 days light treatment) ($p < 0.05$). The numbers of dustbathing holes in both NL and VL houses were higher than those in 5-lx and 20-lx houses ($p < 0.05$), and in the VL house, the number of dustbathing holes was higher than that in the NL house ($p < 0.05$). To evaluate the effect of light intensity programs in 20-lx and VL houses on the daily activity of broilers, Animo, an activity tracker, was used in the 20-lx and VL houses of trial 4 (n = 16). Average body weights

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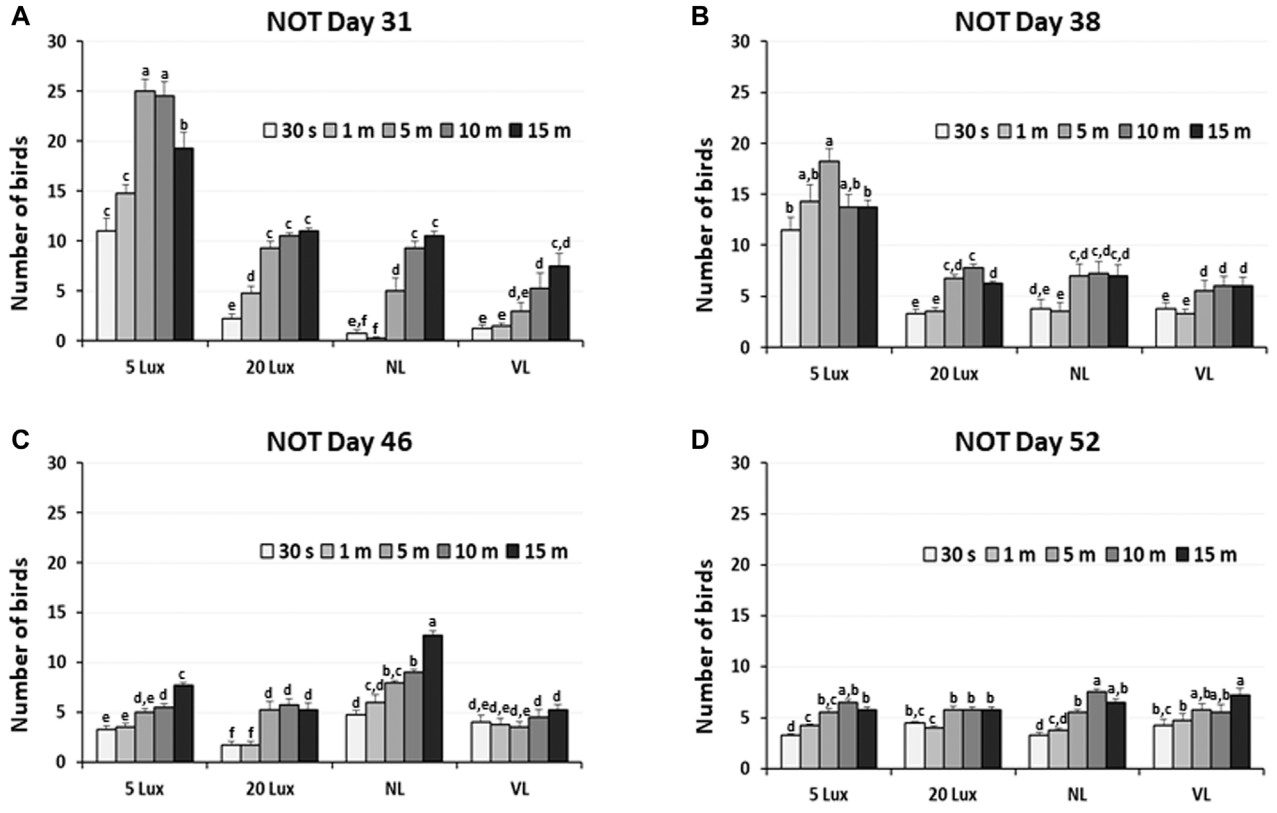

FIGURE 3
Novel object test in four different lighting program houses (5 lx, 20 lx, NL, and VL) at different ages **(A)** age of day 31, **(B)** age of day 38, **(C)** age of day 46, and **(D)** age of day 52. Numbers of birds approaching the novel object were obtained in the sequential time points (30 sec, 1, 5, 10, and 15 min) in each section of the houses, and data (mean ± SEM) were compared among treatments. Different lower-case letters above the bars denote significant differences ($p < 0.05$) among groups, where a>b>c>d>e>f and a,b is not different from a or b.

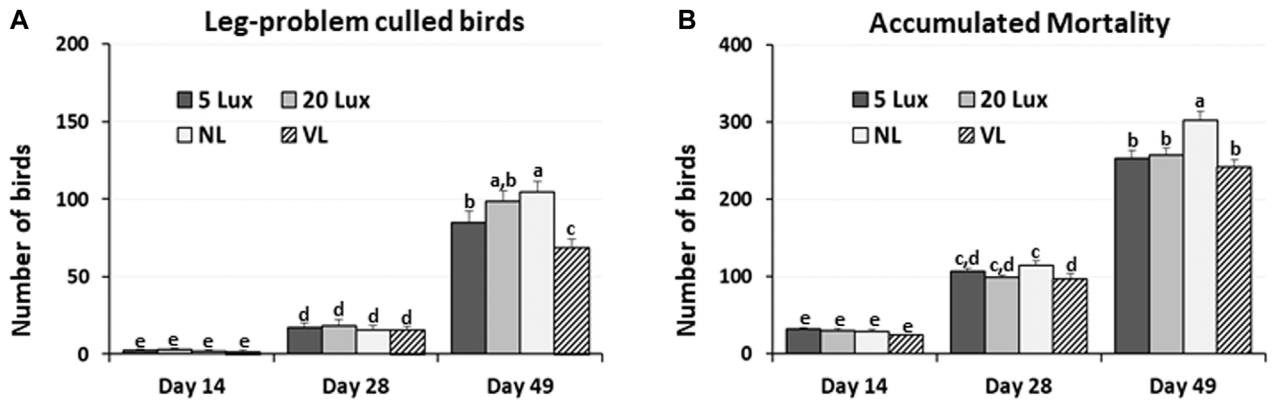

FIGURE 4
Accumulated number of birds that were culled by leg-problems and mortality in four different lighting programs (5 lx, 20 lx, NL, and VL) at different ages. **(A)** In each section of trial 1 and 3, the accumulated number of birds that were culled because of leg-problems and total dead birds were obtained from day 7 to day 14 (Day 14), day 7–28 (Day 28) and day 7–49 (Day 49). **(B)** Accumulated mortality from trial 1 and was obtained from day 7 to day 14 (Day 14), day 7–28 (Day 28) and day 7–49 (Day 49). Data (mean ± SEM) were compared among treatments. Different lower-case letters above the bars denote significant differences ($p < 0.05$) among groups, where a>b>c>d>e>f and a,b is not different from a or b.

TABLE 2 Effects of different light programs on the production performance of commercial broilers.

Trial/treatment	Daily weight gain (g)	Feed conversion ratio (FCR)
Trial 1 (56 days)		
5-lux house	67.13	1.958
20-lux house	66.27	1.977
NL house	65.68	1.996
VL house	67.31	1.932
Trial 2 (51 days)		
5-lux house	66.36	1.858
20-lux house	64.23	1.842
NL house	63.96	1.871
VL house	66.54	1.821
Trial 3 (49 days)		
5-lux house	63.87	1.922
20-lux house	66.18	1.905
NL house	61.64	1.993
VL house	66.27	1.890
Trial 4 (55 days)		
20-lux house	68.95	1.970
20-lux house	67.63	2.070
NL house	68.67	1.944
VL house	70.62	1.953

(mean ± SEM) of birds randomly selected for activity tracking in 20-lx and VL houses were 2.622 ± 0.061 and 2.752 ± 0.055, respectively. Average daily consumed energy by moving activity was obtained for 4 days (from day 39 to day 42) (Figure 1B). For their moving activity, VL-treated birds used twice as much energy per day as 20-lx-treated birds ($p < 0.05$). The percentage of footpad-failed birds to the inspected birds (see Section 2.2.2 for grading scale) was obtained and compared among treatment groups. On day 28, the NL house had a significantly higher footpad-failed percentage than the other groups (Figure 2). On day 42, the 20-lx house had the higher number of footpad-failed birds than 5-lx and VL houses ($p < 0.05$). The novel object test was performed on four different light intensity lighting program houses (5 lx, 20 lx, NL, and VL) at different ages. The numbers of birds approaching toward the novel object were obtained in the sequential timepoints (30 s, 1 min, 5 min, 10 min, and 15 min) in each section of the houses. On day 31, the first testing day of the novel object, there was a significant difference in the number of birds approaching toward the novel object in the 5-lx house compared to other houses ($p < 0.05$). The numbers of approached birds in the 5-lx house in different timepoints were higher than those in other houses on days 31 and 38 (Figures 3A, B). On days 46 and 52, the difference in the number of approached birds among houses was decreased (Figures 3C, D).

3.2 Effects of different light intensity programs on broiler performance in a commercial broiler house

In each section of trials 1 and 3, the accumulated number of birds that were culled because of leg problems and total dead birds in each section were obtained from day 7 to 14, day 7 to 28, and day 7 to 49. Leg problems of culled birds included any leg issue that prevented the bird to access feed or water and/or had a gait score of 2. The number of culled birds on day 49 decreased by 19%, 30%, and 34% in VL-treated birds compared to 5-lx-, 20-lx-, and NL-treated birds (Figure 4A). Total mortality was 25% lower in VL-treated birds than NL-treated birds (Figure 4B). There were slight decreases in mortality in the VL house compared to 5-lx and 20-lx houses on days 28 and 49 ($p > 0.05$). The average daily weight gain of the birds in the VL house was 4.3, 1.2, and 4.1% higher than that in 5-lx, 20-lx, and NL houses, respectively (Table 2). From the feed conversion ratio (FCR) of four trials (total house numbers of 20-lx and VL house were 5), the average FCR of 20-lx-treated and VL-treated birds was 1.953 and 1.908, respectively. The average FCR of VL-treated birds was 2.2% lower than that of 20-lx- and NL-treated birds.

3.3 Differential effects of different light intensity programs on 5-HTergic activity in dorsal raphe nucleus (DRN) and caudal raphe nucleus (CRN) of the brainstem of commercial male broiler chickens

As an indicator of serotonergic activity, the TPH2 mRNA level was determined in two brainstem areas, DRN and CRN, at 14, 28, and 42 days of age (Figure 5). Changes in TPH2 gene expression were determined in the DRN and CRN of the brainstem and were compared among treatments at 14, 28 and 42 days of age (Figure 6). In the DRN of the broiler brainstem, TPH2 gene expression was significantly higher in NL-treated birds than in other light treatment groups at 14 days

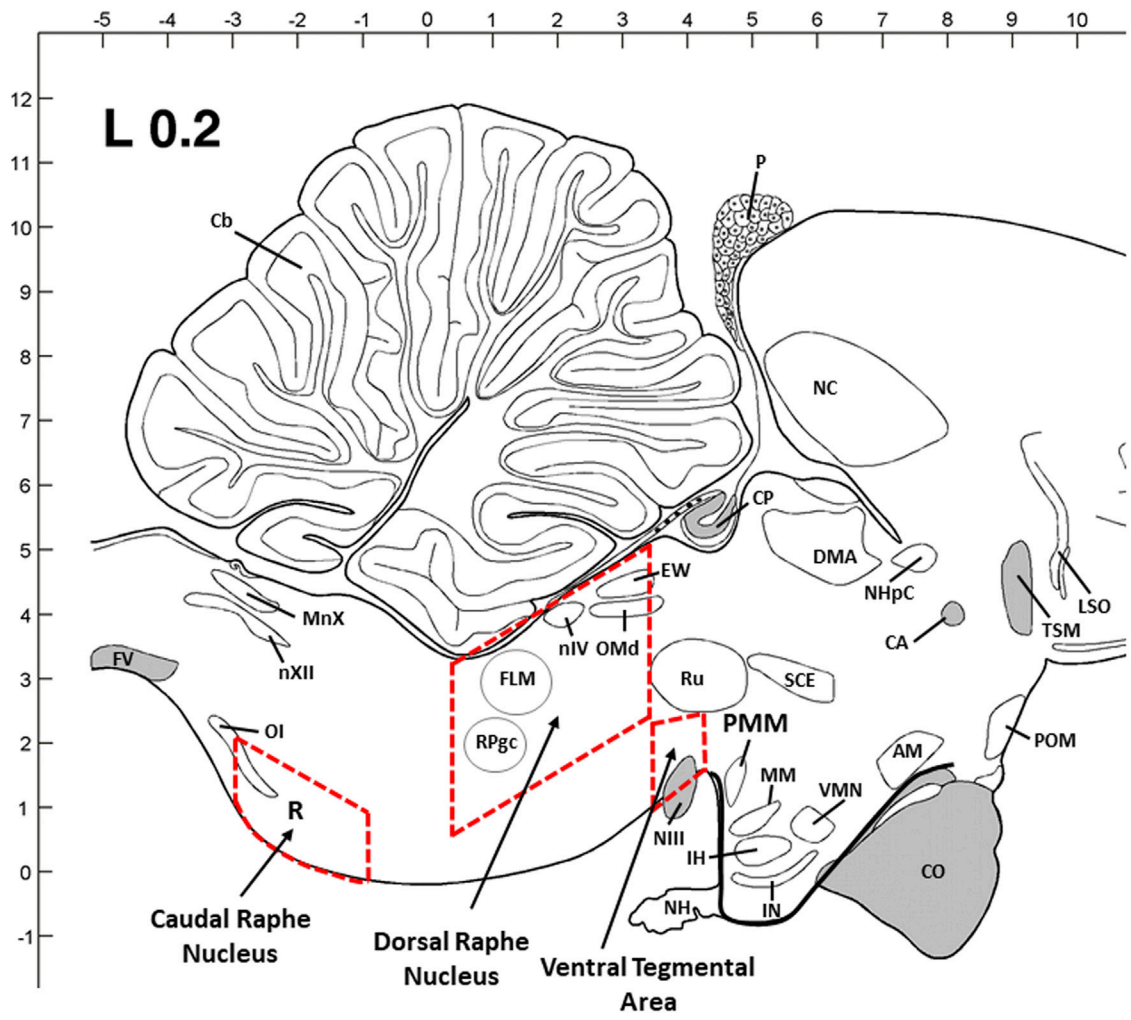

FIGURE 5
Sagittal view of three dissection areas of the chicken brain [dorsal raphe nucleus (DRN) and caudal raphe nucleus (CRN) of the brainstem and ventral tegmental rea (VTA) of the midbrain]. Dimensions of the dissected tissues are coronal with 2.5–3 mm (W) x 1–1.5 mm (H) x 2.5–3.0 mm (L) for DRN, 2–2.5 mm (W) x 1–1.2 mm (H) x 2.5–3.0 mm (L) for CRN, and 3–3.5 mm (W) x 2–3 mm (H) x 1–1.2 mm (L) for VTA. The thickness (W, H, and L) was adjusted proportionally from young birds to older birds based on the brain size and structure. Abbreviations: AM: anterior medial hypothalamic nucleus; CA: anterior commissure; Cb: cerebellum; CO: optic chiasma; CP: posterior commissure; DMA: dorsomedial nucleus; EW: Edinger–Westphal nucleus; FLM: medial longitudinal fasciculus; FV: ventral fasciculus; IH: inferior hypothalamic nucleus; IN: infundibular hypothalamic nucleus; LSO: lateral septal organ; MM: medial mammillary nucleus; MnX: nucleus motorius dorsalis nervi vagi; NC: caudal neostriatum; NH: neurohypophysis; NHpC: nucleus of the hippocampal commissure; NIII: oculomotor nerve; nIV: trochlear nerve nucleus; nXII: hypoglossal nerve nucleus; OI: inferior olivary nucleus; OMd: dorsal oculomotor nucleus; P: pineal gland; POM: medial preoptic nucleus; PVN: paraventricular nucleus; RPgc: nucleus of caudal pontine reticular gigantocellular; Ru: red nucleus; SCE: stratum cellular externum; TSM: septopallio-mesencephalic tract; VMN: ventromedial hypothalamic nucleus.

of age ($p < 0.05$). TPH2 expression in 5-lx-treated birds was significantly lower than that in the 20-lx- and VL-treated birds on day 14 (Figure 6A). However, on day 28, DRN-TPH2 expression of all treatment birds decreased and was insignificant. On day 42, TPH2 expression in the DRN was the lowest in NL- and VL-treated birds ($p < 0.05$) and highest in 20-lx-treated birds compared to other treatment groups. In the CRN of the brainstem, TPH2 expression was significantly lower in 5-lx- and VL-treated birds on day 14 than that in the 20-lx-treated birds ($p < 0.05$) (Figure 6B). There was significant lower expression of TPH2 in the CRN of 5-lx-treated birds than in 20-lx-treated birds on days 28 and 42. On days 28 and 42, TPH2 expression of each treatment group of birds became less significant ($p < 0.05$).

3.4 Regulation of welfare marker and melanopsin genes in the ventral tegmental area (VTA) by different light intensity programs in a commercial broiler house

To evaluate the long-term effects of different light intensity programs on the previously identified welfare marker genes, expression of TPH2, TH, GR, and BDNF genes was determined at 42 days of age (Figure 7). The expression of TPH2 mRNA in the VTA of NL-treated and VL-treated birds was lower than that of 5-lx-treated and 20-lx-treated birds ($p < 0.05$). The TPH2 mRNA level in the VTA of 5-lx-treated birds was the highest compared to other light-treated birds ($p < 0.05$). TH mRNA expression, an indicator of DAergic activity, was the highest in 5-lx-treated birds compared to other light-treated birds

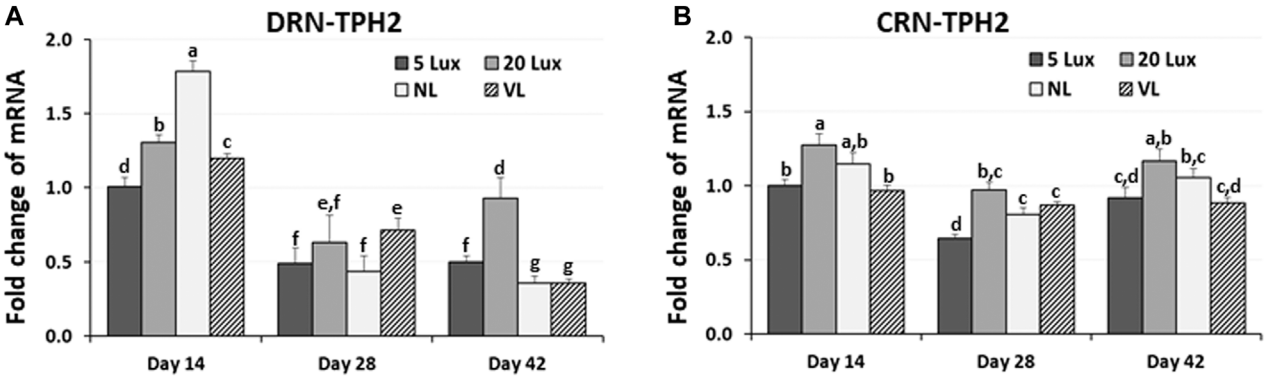

FIGURE 6
Expression changes of TPH2 in **(A)** the dorsal raphe nucleus (DRN) and **(B)** caudal raphe nucleus (CRN) of brainstem. Light intensity programs were changed at day 7–5, 20, 480 lx (NL house), 2–5 lx/40 lx (VL house). Brains of male bird were sampled on days 14, 28 and 42 (n = 12/section, 4 sections/house). DRN and CRN of the brainstem from each bird were dissected as described in Figure 5. Total RNA was extracted and used for real time RT-qPCR for TPH2 expression. Data were set as the relative fold changes of expression levels using the ΔΔCt method with GAPDH and β-actin as internal controls. Data (mean ± SEM) were expressed from a value set for 1.0 for 5 lx birds at 14 days of age. Different lower-case letters above the bars denote significant differences ($p < 0.05$) among groups, where a>b>c>d>e>f>g and a,b is not different from a or b.

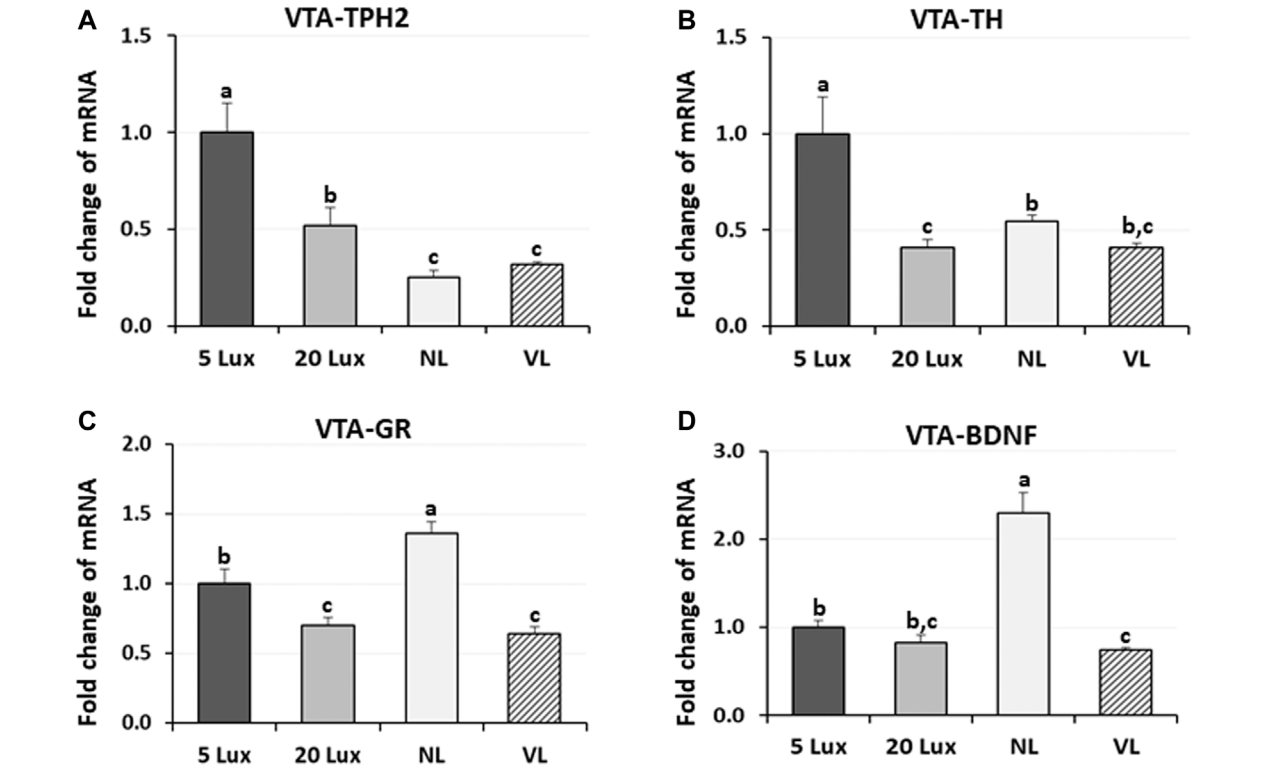

FIGURE 7
Regulation of VTA welfare related marker genes. Expression changes of **(A)** TPH2, **(B)** tyrosine hydroxylase (TH, the rate-limiting enzyme of dopamine biosynthesis), **(C)** glucocorticoid receptor (GR), and **(D)** brain-derived neurotropic factor (BDNF) mRNA in the ventral tegmental area (VTA) of birds at 42 days of age were measured in four different lighting program houses. Light intensity programs were changed at day 7–5, 20, 480 lx (NL house), 2–5 lx/40 lx (VL house). Brains of male bird were sampled on day 42 (n = 12/section, 4 sections/house). VTA of the brainstem from each bird was dissected as described in Figure 5. Total RNA was extracted and used for RT-qPCR. Data were set as the relative fold changes of expression levels using the ΔΔCt method with GAPDH and β-actin as internal controls. Data (mean ± SEM) were expressed from a value set for 1.0 for 5 lx birds for each gene. Different lower-case letters above the bars denote significant differences ($p < 0.05$) among groups, where a>b>c and b,c is not different from b or c.

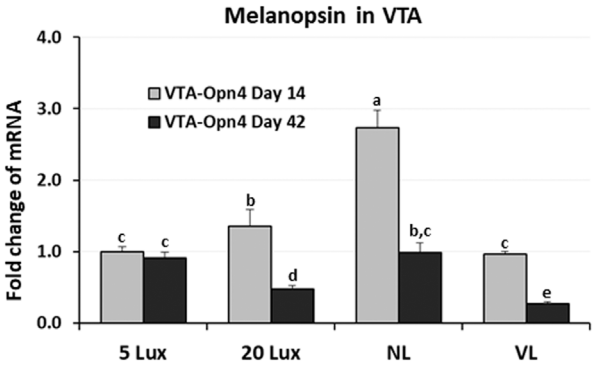

FIGURE 8
Expression changes of melanopsin (Opn4) mRNA in the VTA of birds at 14 and 42 days of age. Light intensity programs were changed at day 7–5, 20, 480 lx (NL house), 2–5 lx/40 lx (VL house). Brains of male bird were sampled on days 14 and 42 (n = 12/section, 4 sections/house). VTA of the midbrain from each bird was dissected as described in Figure 5. Total RNA was extracted and used for RT-qPCR. Data were set as the relative fold changes of expression levels using the ΔΔCt method with GAPDH and β-actin as internal controls. Data (mean ± SEM) were expressed from a value set for 1.0 for 5 lx birds at 14 days of age. Different lower-case letters above the bars denote significant differences ($p < 0.05$) among groups, where a>b>c and b,c is not different from b or c.

($p < 0.05$), and there were no significant differences in TH expression between 20-lx- and VL-treated birds and between NL- and VL-treated birds at 42 days of age. VTA-GR is the stress modulator and involved in the stress-induced plasticity and functioning of VTA (Douma and de Kloet, 2020). Lower VTA-GR expression was observed in 20-lx- and VL-treated birds than that in 5-lx- and NL-treated birds ($p < 0.05$), indicating that the stress is higher in NL-treated birds than in 20-lx- and VL-treated birds ($p < 0.05$). The over-activation of GR in NL-treated birds may stimulate a dysfunctional reward system in VTA and plays an important role in the pathogenesis (Mizoguchi et al., 2021). BDNF is a social stress-related neurotrophic factor in the VTA (Berton et al., 2006). The VTA-BDNF expression of NL-treated birds was 2.5 fold higher than that of 5-lx-, 20-lx-, and VL-treated birds ($p < 0.05$), and VL-treated birds showed the lowest level of BDNF expression ($p < 0.05$), suggesting the chronic social defeat stress in NL-treated birds compared to 5-lx-, 20-lx-, and VL-treated birds (Fanous et al., 2010). These results suggest that social stress can cause long-term neuroadaptations involving both GR and BDNF in the VTA.

For the first time, the avian VTA was suggested as an important area of the midbrain of birds involved in the light perception by melanopsin (Opn4, photoreceptor) and might be involved in the welfare of poultry (Kang, 2021). To evaluate the long-term effects of light intensity programs on the previously identified melanopsin (photoreceptor), the expression of Opn4 gene was determined at 14 and 42 days of age (Figure 8). On day 14, Opn4 expression was the highest in NL-treated birds ($p < 0.05$) and Opn4 expression in 5-lx- and VL-treated birds was significantly lower than that in 20-lx- and NL-treated birds ($p < 0.05$). On day 42, after 5 weeks of light treatment, the expression in VL-treated birds became the lowest among the groups and there was no significant difference between 5-lx- and NL-treated birds, and the expression of Opn4 mRNA in 20-lx-treated birds was intermediate between that in 5-lx/NL- and VL-treated birds ($p < 0.05$).

4 Discussion

4.1 Effects of different light intensity programs on the broiler behaviors in a commercial broiler house

Environments recognized by birds are either threatening their survival or causing homeostatic disruption, resulting in the behavioral responses and physiological impact on birds. Different lighting programs in commercial broiler houses may influence diverse behavioral and physiological impact on birds. The effects of four different light intensity lighting programs on broiler welfare-related behaviors were evaluated in the present study. Dustbathing is performed by many avian species and is considered a comfort behavior (Vestergaard et al., 1997; Louton et al., 2016). Dustbathing holes as the evidence of dustbathing behavior were counted in three timepoints, which indicates that the VL intensity lighting program in the commercial broiler house has a stimulating effect on the dustbathing behavior and dustbathing is the most observed natural activity in VL-treated birds. As the birds got older, evidence of dustbathing decreased, which could be due to age or reduced friable litter.

An activity tracker, Animo, was able to monitor the daily physical activity of birds at an older age. Results showed significant stimulation of birds' daily physical activity in VL-treated birds compared to 20-lx-treated birds. The number of dustbathing holes in the NL house was intermediate between the 5-lx/20-lx and VL houses, suggesting that the bright light in NL also stimulates locomotive activity of NL-treated birds. In fact, several studies have indicated that the stimulatory effect of bright light on broilers increased locomotor activity (Newberry et al., 1988; Blatchford et al., 2009). Footpad condition is critical and associated with birds' leg health and moving activity. The footpad-failed percentage in each section was the highest in the 20-lx house and was the lowest in the VL house on day 42 ($p < 0.05$), indicating that there is a possibility of stimulated natural movement by the VL intensity lighting program, as observed in the dustbathing holes and activity tracking studies, which may be beneficial to footpad health.

Environment lighting has long been recognized as a factor that can change the perceived atmosphere of the environment, and fear is an adaptive emotional response to potentially harmful stimuli and serves to protect animals from injury (Steimer, 2002; 2009; Vasdal et al., 2018).

VTA DAergic neurons are involved in the extinction mechanism of fear responses, and the increase in DA release has been observed in the nucleus accumbens during fear extinction in the fear extinction learning study (Badrinarayan et al., 2012; Salinas-Hernandez et al., 2018).

In the novel object test (Figure 3), we observed a rapid increase in the fear-sensing mechanism in 5-lx-treated birds as these birds have less access to the novel object than other light-treated birds, suggesting that the experienced fear of the novel object was different in the four different light intensity lighting programs we tested. When fear response is not normal or different, birds may have an impaired ability to sense mental alertness. It may be speculative that the fear-sensing mechanism of 5-lx-treated birds was impaired on day 31, at least for an individual's ability to function, but it became adapted in the later test on days 46 and 52. This will be discussed in detail in Section 4.3 with the result of VTA-TH expression in 5-lx-treated birds.

4.2 Effects of different light intensity programs on the leg health and performance of commercial broilers

To increase performance and productivity, commercial broilers are often raised in houses that are dimly illuminated on a near-continuous basis. Several studies on broilers addressing broiler leg health suggest that the stimulatory effect of bright light on locomotor activity can improve their leg condition and thus their welfare (Newberry et al., 1988; Shields et al., 2005; Blatchford et al., 2009). In the present study, fascinating and engrossing results were observed in leg health and performance of the VL house.

Within-treatment house comparison in each trial showed the consistency in the lowest number of leg-problem-induced culled birds and total mortality after light treatments, suggesting that the increased natural movement behaviors, as observed in this study, appear to improve the leg health of VL-treated birds. The improved leg health may contribute to the reduction in mortality in the VL house. In addition, we observed the highest daily weight gain (DWG) and the lowest FCR in the VL house in each trial (Table 2) consistently. These results indicate the economic and welfare benefit of the VL intensity lighting program in broiler production.

4.3 Differential effects of light intensity programs on 5-HTergic activity in dorsal raphe nucleus (DRN) and caudal raphe nucleus (CRN) of the brainstem of commercial male broiler chickens

It was suggested that the acceptable welfare of animals is not simply the absence of negative experiences, but rather it is primarily the presence of positive experiences (Boissy et al., 2007; Marcet Rius et al., 2018). Animals use the sense of vision to examine the surrounding area and locate food sources. Recent studies showed that the 5-HTergic system in the raphe nuclei of the brainstem was involved in this behavioral choice decision which was affected by light (Filosa et al., 2016; Kang et al., 2020). The stress-induced activation of TPH2 expression was observed in the raphe nuclei of mammals, and 5-HT metabolism and turnover were increased in the brain (Inoue et al., 1994; Chamas et al., 1999). The elevation of TPH2 mRNA expression in the raphe nuclei was reported to be involved in the emotional conditions, such as depression in mammals and avian species (Bach-Mizrachi et al., 2008; Kang et al., 2020). To address the welfare of birds under different lighting programs in commercial broiler houses, we measured TPH2 expression in DRN and CRN of the brainstem as an indicator of 5-HTergic activity in the brain. Results showed that there was a significantly higher expression of TPH2 in the DRN of 20-lx- and NL-treated birds than VL-treated birds on day 14 and in the DRN of 20-lx-treated birds than VL-treated birds on day 42, indicating that VL-treated birds experience lower stress than 5-lx- and 20-lx-treated birds on day 42. Results of TPH2 expression in CRN suggest that 5-HTergic activities in the DRN are more associated with the light intensity-related physiological response of birds.

4.4 Regulation of welfare-indicating genes and melanopsin (Opn4) in the ventral tegmental area (VTA) by different light intensity programs in a commercial broiler house

In the VL house, birds rested and slept in the dim light area of the house and actively fed and drank water in the 40-lx light intensity area, which may provide the appropriate environment for the homeostatic control of their autonomic nervous system (ANS). The ANS allows animals to adapt to their environment by equilibrating sympathetic (SNS) and parasympathetic nervous systems (PNS) (Briefer et al., 2015; Daniela et al., 2022). 5-HT and DA are critical neurotransmitters to regulate PNS and SNS, respectively. Compensatory changes in 5-HTergic and DAergic activities were suggested to restore the balance of the brain's homeostatic mechanisms, and these compensatory changes take days to develop (Andrews et al., 2015; Pratelli et al., 2017). Recent studies found that the VTA of the midbrain contains cell bodies of mesolimbic DAergic neurons as well as the 5-HTergic system in mammals and avian species (Kang et al., 2009; Carkaci-Salli et al., 2011; Chaudhury et al., 2013; Grace, 2016). The upregulated 5-HTergic and DAergic activities in VTA of 5-lx-treated birds suggest that the higher synthesis of these two positive welfare indicators in 5-lx-treated birds indicates the high stress-susceptibility and may be regulated by the compensatory mechanism as suggested in the previous reports (Belujon and Grace, 2015; Grace, 2016; Welford et al., 2016; Kang et al., 2020).

There is a growing body of evidence that stress affects learned fear response (Krishnan, 2014; Maren and Holmes, 2016; Steimer, 2002). GCs were suggested for detecting animal welfare (Ralph and Tilbrook, 2016), and our previous study indicated that there was no consistency in corticosterone (CORT) levels in different timepoints, and no long-term effect of lighting programs on CORT was observed in circulating blood (Kang et al., 2020). One of the major actions of GCs is to regulate the transcription of its primary target gene, GR, through genomic GC response elements (GREs) by directly binding to DNA or tethering onto other DNA-binding transcription factors. These GR primary targets trigger physiological and pathological responses of GCs. Therefore, we used the GR gene expression in VTA (VTA-GR) to investigate the effect of different variable light intensity lighting programs on the welfare of birds as described in other animal studies (Daftary et al., 2009; Mizoguchi et al., 2021; Tran et al., 2022). The lower level of GR in 20-lx- and VL-treated birds indicates that the VTA-DAergic system is necessary to initiate fear extinction and reveal a crucial role of DA neurons in this form of safety learning.

Physical exercise is an evidence-based treatment strategy to improve broiler's leg health as discussed previously (Bizeray et al., 2002; Kristensen et al., 2004; Reiter and Bessei, 2009). Several mechanisms may explain the positive impact of physical exercise, including an increase in neurotrophic support. One proposed theory is that the mechanism of action of exercise could involve the neurotrophic pathway, especially the BDNF (Liu and Nusslock, 2018). Animal studies have shown that physical exercise is associated with the increased expression of

BDNF in the hippocampus, which may improve memory performance and reduce depressive symptoms by promoting neurogenesis and neuronal differentiation (Hötting and Röder, 2013; Arosio et al., 2021). However, unlike BDNF in the hippocampus, the activation of VTA-BDNF was suggested to be involved in the long-term social defeat stress, and BDNF gene deletion in the VTA attenuated stress-induced behaviors, such as social avoidance in mice (Berton et al., 2006; Krishnan et al., 2007; Fanous et al., 2010). Taken together, the results of welfare-related gene expression in the brain of commercial broilers against stress indicate the beneficial effects of the VL intensity lighting program on broilers' welfare.

Many studies provide evidence that light can affect the central physiology of animals independent of the retinal function (Underwood et al., 1984; Wade et al., 1988; Fernandes et al., 2013). VTA was suggested as an important area of the midbrain involved in the light perception by melanopsin (Opn4, photoreceptor) and might be involved in the avian welfare (Kang, 2021). The result of long-term effects of light intensity programs on Opn4 expression at 42 days of age in the present study suggests that Opn4 in VTA may be involved in the direct perception of light intensity information for physiological adaptation of birds.

5 Conclusion

The findings of the present study show an extensive understanding of effects of variable light (VL) intensity lighting programs on the welfare and performance of broiler chickens. Here, we evaluated the effects of constant light intensities (5 and 20 lx), natural light (480 lx), and VL intensity lighting programs (2–5 lx/40 lx) on the behavior, performance, and welfare indicators in commercial broiler chickens. We observed an increased broiler dustbathing behavior and better performance in the VL intensity lighting house, suggesting a beneficial effect of the VL intensity lighting program on broiler natural exercise. This lighting program stimulates voluntary walking behavior for consuming feed/water and taking rest as well as improves leg health and performance, providing the valuable information on how to improve broilers' welfare and performance in a commercial broiler farm using the VL intensity lighting program.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials; further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Tyson Foods Broiler Welfare Research Farm.

Author contributions

SK had full access to all the data used in the study and took responsibility for the integrity and accuracy of the data. Study concept and design: SK and KC. Acquisition, analysis, and interpretation of the data: JC, MK, SK, and KC. Drafting and critical revision of the manuscript: SK, KC, and SO. Funding acquisition: SK and KC. All authors have read, edited, and approved the final manuscript.

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Conflict of interest

Authors KC and JC were employed by the company Tyson Foods, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer, CS, declared a shared affiliation with the authors SK, MK, and SO to the handling editor at the time of the review.

Publisher's note

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Chronic treatment with glucocorticoids does not affect egg quality but increases cortisol deposition into egg albumen and elicits changes to the heterophil to lymphocyte ratio in a sex-dependent manner

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During chronic stress, there is an initial increase in glucocorticoid (GC) levels, but they then return to low, albeit not baseline, levels. Recent studies have renewed interest in cortisol in that it may also have important roles in the stress response. The purpose of our study was to test the hypothesis that chronic treatment with low levels of either corticosterone or cortisol would alter HLR and immune organ morphometrics. Further, we wanted to determine if chronic treatment with either GC would elicit an increase in cortisol levels in egg albumen. To test our hypotheses, we implanted silastic capsules that contained corticosterone, cortisol, or empty capsules as controls (N = 5/sex/treatment). Blood serum, smears, body weights, and egg quality data were collected. Ducks were then euthanized and body weight, weights of spleens, livers, and the number of active follicles were recorded. Albumen GC levels were assessed using mass spectrometry. Data were analyzed using a 2- or 3-way ANOVA as appropriate and post-hoc with Fishers PLSD. No treatment elicited differences in egg quality measures or body weight compared to controls. Corticosterone treatment did elicit an increase in serum corticosterone ($p < 0.05$), but not cortisol, levels compared to controls in both sexes. Both cortisol and corticosterone treatments increased ($p < 0.05$) serum levels of cortisol compared to controls. Relative spleen weights were higher ($p < 0.05$) in hens following corticosterone but not cortisol treatment. No other organs showed any differences among the treatment groups. Both GCs elicited an increase ($p < 0.001$) in HLR in hens at all time-points over the 2-week treatment period compared to controls. Cortisol, not corticosterone, only elicited an increase in HLR for drakes ($p < 0.05$) compared to controls but only at day 1 after implants. Chronic treatment with cortisol, but not corticosterone, elicited an increase ($p < 0.01$) in egg albumen cortisol levels compared to other groups. Corticosterone was not detected in any albumen samples. Our results suggest that glucocorticoids elicit differential effects and although corticosterone has been stated to be the predominant GC in avian species, cortisol may provide critical information to further understand bird welfare.

KEYWORDS

chronic stress, cortisol, corticosterone, egg quality, breeder ducks

1 Introduction

Glucocorticoids (GC) are steroid hormones that include cortisol and corticosterone, and their secretion is controlled by the anterior pituitary hormone, adrenocorticotropic hormone (ACTH) (Salem et al., 1970). In birds, ACTH secretion is controlled by both corticotropin-releasing hormone (CRH) and arginine vasotocin (AVT) secreted from the hypothalamus (Kuenzel et al., 2012; Kang and Kuenzel, 2014; Nagarajan et al., 2014; Kang et al., 2018). Poultry experience some stressors that have been reported to elevate circulating serum GC levels (reviewed by Scanes, 2016). The hypothalamus and anterior pituitary control the release of plasma levels of corticosterone and cortisol and this is known as the hypothalamic pituitary axis (HPA). Stress activates the HPA system to cause the release of GC which in turn initiates metabolic processes directed to maintain physiological homeostasis.

For decades, researchers have investigated the effect of the body's response to stressors that includes elevation in the level of plasma GC. These effects include stimulating gluconeogenesis and alteration of immune system functions, including leukopenia and heterophilia (Shini et al., 2008; Vicuña et al., 2015). In particular, the heterophil to lymphocyte ratio (HLR) is an indirect measure of GC function in birds. Prolonged periods of increased circulating levels of GC compromise the immunity, welfare, health, performance, and overall productivity of the bird resulting in economic losses and a decline in product quality (Post et al., 2003; Quinteiro-Filho et al., 2012; Zeng et al., 2014; Honda et al., 2015). The potential for elevated circulating levels of GC to mediate detrimental effects on production and performance parameters can be achieved through the direct introduction of the GC to the birds *via* implantation (Hayward and Wingfield, 2004), injection, oral administration in feed or water (Shini and Kaiser, 2009; Kim et al., 2015), or through the direct introduction of stimuli such as heat (Videla et al., 2020; Oluwagbenga et al., 2022), transportation (Vosmerova et al., 2010; Tetel et al., 2022b), or feed restriction (Najafi et al., 2015) to alter the HPA axis and stimulate the release of GC.

It was previously believed that corticosterone, produced by the adrenal gland, was the main GC found in birds' plasma (deRoos, 1961). However, newer studies suggest that cortisol may also have physiological functions in poultry (Caulfield and Padula, 2020; Tetel et al., 2022b; Tetel et al., 2022a; Oluwagbenga et al., 2022) but there is a lack of research exploring the effects of both GCs, particularly cortisol, on physiological parameters and egg quality and biochemistry in ducks. It is well established that during chronic stress, there is an initial rise in glucocorticoids but after a few hours, their levels return to a low levels that still remain above baseline (reviewed by Scanes, 2016). It is not clear what physiological effects these prolonged periods of increased-baseline glucocorticoids have on the chronic stress response. Therefore, the purpose of our study was to explore the effects of prolonged exposure to increased baseline levels of corticosterone or cortisol on egg quality, HLR, and albumen GC levels. To accomplish this, we treated adult drakes and hens for 2 weeks with either GC or control using subcutaneous implants. Our results suggest that cortisol may be selectively deposited into egg albumen during periods of prolonged stress.

2 Materials and methods

2.1 Animals

Adult breeder Pekin ducks were obtained from Maple Leaf Farms Inc (Leesburg, IN) at approximately 40 weeks of age. Hens and drakes weighed 4.0–4.5 kg, respectively and we utilized five ducks of each sex per treatment, with a total of 30 ducks. The ducks were placed in floor pens in a barn at Purdue University Animal Sciences research farm with an 18:6 light cycle and *ad lib* access to water and 8 h exposure to feed per day, as per industry standards. All procedures were approved by the Purdue University Institutional Animal Care and Use Committee (PACUC #2008002065).

2.2 Experimental design

Pens of hens and drakes were randomly allocated into three treatments (5 ducks/treatment/sex): corticosterone, cortisol, or control. Chronic steroid treatment was delivered using subcutaneously implanted Silastic™ capsules packed with crystalline steroids. Subcutaneous implants were placed behind the neck under propofol anesthesia (2.0—8.0 mL). Each bird received two, 5 mm long 1.57 mm × 3.18 mm (inside vs. outside diameter) capsules packed with pure crystalline steroid. Decades of research using such capsules for steroid delivery have shown that release rate is related to inside and outside diameters and total, additive, length of capsules (Davidson et al., 1978; Fraley and Ulibarri, 2001; Thomas et al., 2006). Although this technique has been utilized in a great many species over the decades, it had not been used previously in ducks. Therefore, the total length of capsules had to be extrapolated based upon body weight of animals to provide the necessary dose to achieve increased basal circulating glucocorticoid levels. Controls were implanted with two empty 5 mm Silastic capsules (the vehicle for crystalline steroid delivery). Incisions were closed using tissue glue. All capsules were pre-charged in 100% ethanol before implantation to ensure immediate delivery of hormone. The use of these capsules has been established for decades as an effective delivery for constant levels of steroid hormones (Smith et al., 1977) and has been routinely used by the PI (Fraley and Ulibarri, 2001; Fraley and Ulibarri, 2002).

2.3 Sample collection and preparation

Blood smears were collected for HLR on days −1, 0.5, 1, 2, 4, 7, 9, 11, 13, and 14 relatives to the day of implant, with the day of implant designated as day 0 (n = 5/time-period/treatment). We attempted to minimize sample collection times in order to minimize handling stress while still providing a complete picture of the 2 week responses. All blood collections were completed within 30–45 s of approaching any given duck. The first post-implant blood smear was done approximately 6 h later and designated as day 0.5. Blood samples were collected from the ducks' tibia veins on days −1, 7 and 14 relatives to subcutaneous implant into a serum separator

tube, centrifuged, and the serum was stored at −20°C until assayed by ELISA for glucocorticoids (n = 5/time/treatment). Eggs were collected daily, combined over 3 days (n = 15/time-period/treatment) to reduce variability and compared among groups for egg quality assessment and albumen assay for GCs. Eggs from the 2 days preceding treatment, including the first day of treatment, were combined and labeled as group 0. Days 1, 2, and 6 were combined and labeled as group 1, days 7, 8, and 11 were combined and labeled as group 2, and days 12, 13, and 14 were combined and labeled as group 3. Albumen samples (n = 15/time-period/treatment) were collected into tubes during egg quality assessment and stored at −20°C until assayed for GCs using mass spectrometry.

Ducks were weighed on days −7, 1, 2, 7, and 14. After 2 weeks of GC or control exposures, all ducks were euthanized using pentobarbital (Fatal Plus, 396 mg/mL/kg) and birds were necropsied. Spleen, testes, and liver were collected, weighed, and expressed relative to the body weight (g organ weight/kg body weight). The final blood sample was obtained and treated as described above. The number of maturing follicles on the ovary were counted. Silastic capsules were removed to confirm placement and diffusion (Fraley and Ulibarri, 2001; Fraley and Ulibarri, 2002).

2.4 ELISA for glucocorticoids

The kits utilized for this project were from Cayman Chemicals (corticosterone: kit #16063; cortisol kit #560360) and the assays were run according to the manufacturer's recommendations. Details of the extensive kit verification have been reported previously (Tetel et al., 2022a; Tetel et al., 2022b). Plates were incubated with samples overnight at 4°C. For the development of the plate, 250 determinations vial of Ellman's Reagent was reconstituted with 50 mL of Ultrapure water. 200 µL of this reagent was added to each well on the plate before being placed on an orbital shaker for 90 min. At end of 90 min, plates were read at 405 nm (SynergyLx, Biotek).

2.5 Egg quality assessments

For the trial, eggs were collected, labelled by pen, and stored in a refrigerator at 4°C overnight. They were then weighed and their shell and vitelline membrane compression strengths were measured using a TA. XT Plus Texture Analyzer (Texture Technologies, Hamilton, MA) with a 10 kg and 500 g load cell, respectively. The procedures for these analyses have been previously described (Oluwagbenga et al., 2022). Additionally, samples of albumen were collected during the egg quality analysis and stored at −20°C for GC assays.

2.6 Mass spectrometry for albumen glucocorticoids

The samples were stored at −20°C before being extracted and analyzed. At the time of analysis, each albumen sample was thawed, and 500 mg was transferred to an extraction tube. The samples were extracted using a method with minor modifications, as previously reported (Caulfield and Padula, 2020). To each sample, an internal standard mixture containing 5 ng of deuterated corticosterone and 1 ng of deuterated cortisol was added and vortexed for 1 min. The samples were then derivatized with Amplifex keto reagent and analyzed by LC/MS/MS, following the kit directions and the previously reported details of extraction and analysis (Oluwagbenga et al., 2022).

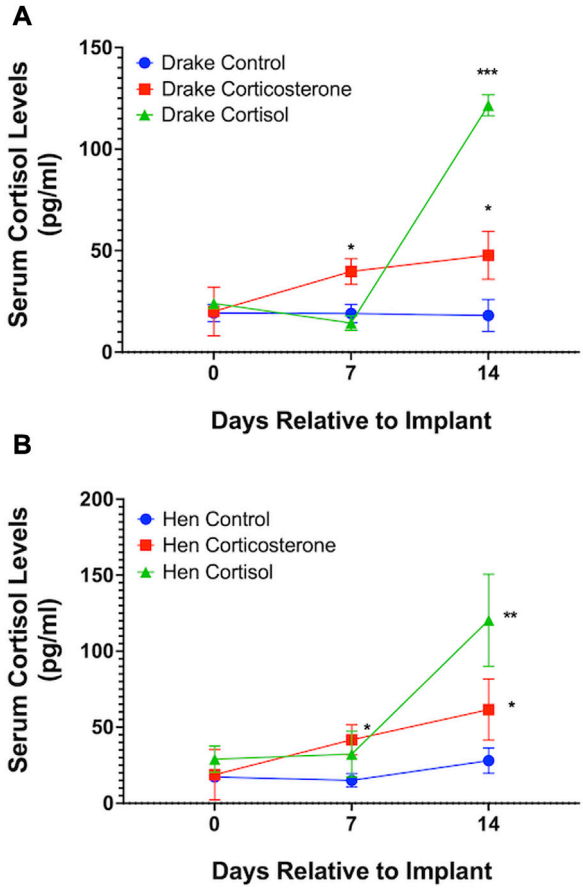

FIGURE 1
Serum cortisol levels in Drakes **(A)** and Hens **(B)**. Ducks treated with either cortisol or corticosterone showed a significant increase in serum cortisol levels compared to controls 7 and 15 days after the onset of treatment. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

2.7 Statistical analyses

Statistics were run using JMP Pro v.15 (SAS Institute, Cary, NC United States). For all duck-related variables, the duck was considered the statistical unit. An *a priori* Power analysis showed that our duck level sample sizes would provide 85% Power at alpha = 0.05 for the three treatment groups and two sexes. For egg quality assessment, eggs were pooled over 3 days interval (approximately N = 15/treatment/time point) and averaged. All data were analyzed by 2 -way ANOVA or repeated measures as appropriate. *Post hoc* analyses were done by Fisher's PLSD test. A $p < 0.05$ was considered significant.

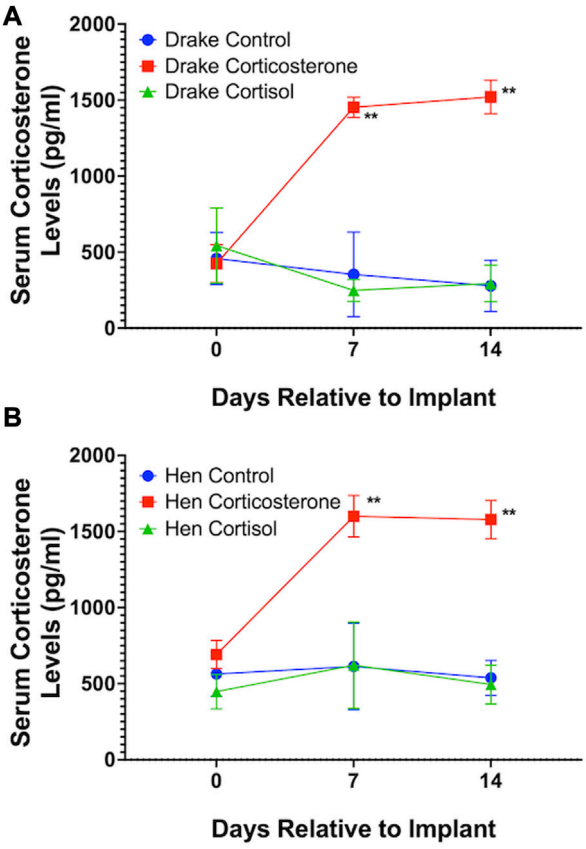

FIGURE 2
Serum corticosterone levels in Drakes **(A)** and Hens **(B)**. Only ducks treated with corticosterone showed a significant increase in serum corticosterone levels compared to controls at 7 and 15 days after onset of treatment. ** = $p < 0.01$.

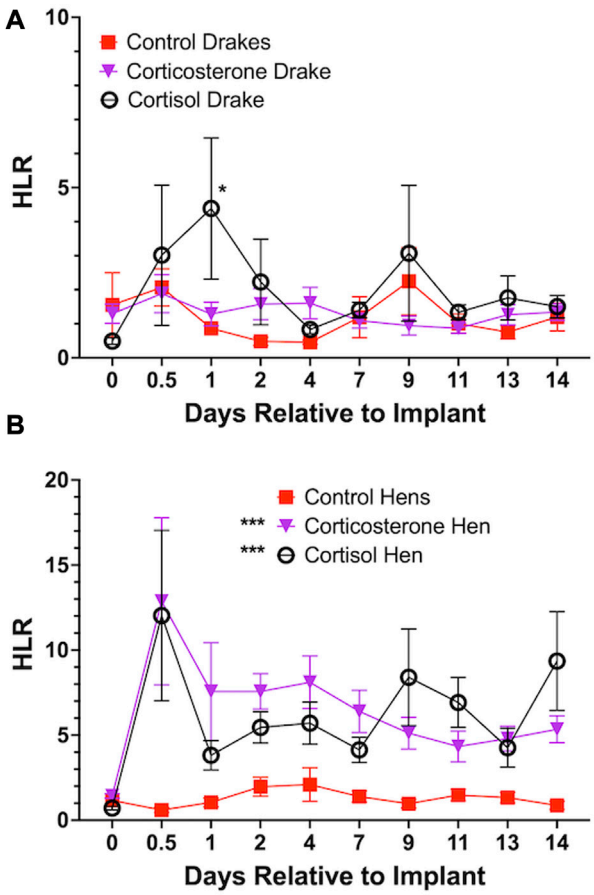

FIGURE 3
Heterophil and Lymphocyte ratio in Drakes **(A)** and Hens **(B)**. Both cortisol and corticosterone showed a significant increase in hen HLR at all time points during the experiment compared to controls. In drakes, only cortisol elicited a significant increase in HLR and only 1 day following onset of treatment compared to controls and corticosterone treatment groups. * = $p < 0.05$, *** = $p < 0.001$.

3 Results

3.1 ELISA for serum GCs

Assays confirmed our goal to achieve increased basal levels of circulating glucocorticoids. Circulating levels of corticosterone, but not cortisol, were elevated in both drakes and hens in the corticosterone treatment group ($p < 0.01$) compared to cortisol treatment and controls. Cortisol treatment increased circulating levels of cortisol in both sexes ($p < 0.01$) compared to corticosterone treatment and controls. Interestingly, both corticosterone and cortisol treatments resulted in elevated cortisol levels ($p < 0.05$) compared to controls in both sexes. Figures 1, 2 illustrate these results.

3.2 Heterophil: Lymphocyte ratio

Both cortisol and corticosterone elicited an increase in HLR for hens ($p < 0.001$) beginning at day 1 and continued through the length of the experiment. Cortisol only elicited an increase in HLR for drakes ($p < 0.05$) compared to controls but only at day 1 after implants. In drakes, there were no notable increases in HLR with the corticosterone treatment. Figure 3 illustrates these results.

3.3 Morphometrics

There were no significant effects of corticosterone or cortisol treatments on body weights of either hens ($p = 0.32$) or drakes ($p = 0.11$). The relative spleen weight in the hens is higher in the corticosterone group ($p < 0.01$) compared to the cortisol but not the control group. Both treatments showed no significant difference in the relative liver weight for hens and the relative spleen and liver weights for drakes as shown in Table 1. Due to the age of the ducks, bursa and thymus were regressed thus we were not able to evaluate them in this study.

3.4 Egg quality

Results of GC treatment on egg quality are presented in Table 2. No significant effects on egg quality were observed following either GC treatment compared to controls.

TABLE 1 Effect of a 2-week chronic stress stimulation on immune organ parameters in breeder ducks.

	Drake		Hen		
Treatment	Spleen (g/kg)	Liver (g/kg)	Spleen (g/kg)	Liver (g/kg)	Follicle (#)
Control	0.61 ± 0.068	13.3 ± 0.93	0.41 ± 0.041[ab]	21.9 ± 1.80	4.4 ± 0.68
Corticosterone	0.57 ± 0.034	14.3 ± 0.50	0.52 ± 0.038[b]	25.0 ± 0.80	4.6 ± 0.51
Cortisol	0.54 ± 0.101	13.8 ± 1.71	0.33 ± 0.025[a]	23.8 ± 1.47	5.4 ± 0.51
p-value	0.80	0.84	<0.01	0.34	0.45

Data shown are means ± SEM, n = 5/sex/treatment.
Different letter coding within parameter is significantly different ($p < 0.05$).
Values of spleen and liver are relative to the body weight (g/kg).

TABLE 2 Effect of a 2-week chronic stress stimulation on egg quality parameters in breeder ducks.

Time group	Egg Weight(g)	Shell weight(g)	Haugh unit	Yolk weight(g)	Shell strength(N)	Vitelline membrane strength(N)
Control 0	91.0 ± 1.44	8.3 ± 0.09	98.8 ± 1.59	26.9 ± 0.45	51.9 ± 2.22	3.02 ± 0.359
Control 1	90.6 ± 2.20	8.1 ± 0.14	95.9 ± 1.45	25.6 ± 0.68	48.2 ± 2.45	1.94 ± 0.222
Control 2	90.1 ± 2.99	8.2 ± 0.24	96.5 ± 1.36	24.9 ± 1.07	49.5 ± 1.50	2.70 ± 0.257
Control 3	93.4 ± 2.62	8.6 ± 0.16	94.2 ± 1.97	26.1 ± 0.88	50.9 ± 2.33	2.04 ± 0.235
Corticosterone 0	87.5 ± 1.80	8.3 ± 0.30	98.6 ± 1.16	26.9 ± 0.82	52.2 ± 1.28	2.39 ± 0.268
Corticosterone 1	90.6 ± 2.00	8.3 ± 0.25	99.4 ± 1.41	27.0 ± 0.65	48.8 ± 1.59	2.58 ± 0.270
Corticosterone 2	93.3 ± 1.62	8.5 ± 0.19	100.0 ± 1.53	27.9 ± 0.73	50.5 ± 1.44	3.11 ± 0.432
Corticosterone 3	93.3 ± 3.28	8.5 ± 0.19	102.0 ± 1.96	27.6 ± 0.92	40.9 ± 6.97	3.75 ± 0.463
Cortisol 0	95.8 ± 1.28	9.0 ± 0.12	100.0 ± 1.58	29.7 ± 0.43	49.6 ± 2.78	3.21 ± 0.351
Cortisol 1	93.2 ± 1.43	8.7 ± 0.14	97.9 ± 1.13	28.5 ± 0.47	48.0 ± 3.12	2.70 ± 0.242
Cortisol 2	96.2 ± 1.02	8.9 ± 0.14	95.9 ± 1.49	28.5 ± 0.58	51.9 ± 1.68	2.92 ± 0.300
Cortisol 3	95.2 ± 1.55	9.1 ± 0.16	92.9 ± 1.65	28.2 ± 0.66	52.1 ± 3.32	2.28 ± 0.386
p-value	0.46	0.95	0.06	0.39	0.25	0.10

Data shown are means ± SEM, n = 15/time-period/treatment.

3.5 Mass spectrometry for albumen GCs

We found no measurable levels of corticosterone in eggs from any of the treatment groups similar to that previously described by our lab and others (Caulfield and Padula, 2020; Oluwagbenga et al., 2022). Albumen cortisol levels (Figure 4) were increased on day 7 ($p < 0.05$) and day 14 ($p < 0.01$) in the cortisol treatment group compared to both corticosterone and control groups.

4 Discussion

The purpose of our study was to explore the effects of prolonged exposure to increased circulating levels of corticosterone or cortisol on egg quality, HLR, and albumen GC levels. To accomplish this, we treated adult drakes and hens with each GC for 2 weeks using subcutaneous implants. We found no effects on any variable associated with egg quality. We reported a sex difference in the HLR whereas hens showed increased HLR following both GC treatments while drakes had a transient increase in HLR following cortisol. We found an increase in cortisol in albumen following cortisol treatment that suggests either a selective transport of cortisol into the albumen by oviduct tissues or GC-mediated *de novo* synthesis of cortisol in ovarian or oviduct tissues. Regardless, our data suggest that albumen cortisol may be an indicator of welfare or of at least chronic stress.

We have now consistently observed a sex difference in the HLR response in several studies from our lab. Tetel et al. (2022a), Tetel et al. (2022b) reported a sex-dependent increase in HLR in Pekin ducks treated with intramuscular injections of ACTH. Similarly, numerous studies have reported sex differences in the levels of GC in mammals in that female levels are higher than in males. Further, Owen et al. (2005) reported a sex difference in leukocyte composition of breeding southwestern willow flycatcher. Shini et al. (2008) reported an increase in HLR response following oral administration of corticosterone in broilers. Further, the exposure to

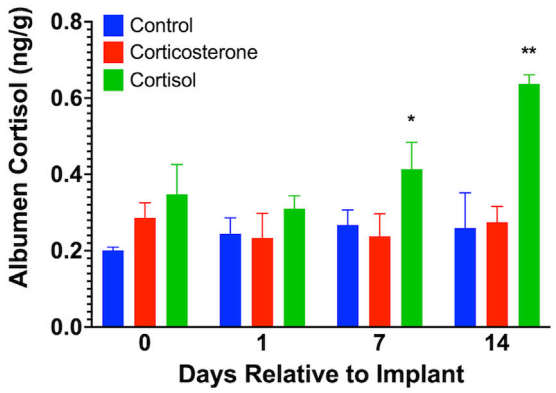

FIGURE 4
Egg albumen concentration of cortisol. Corticosterone was not detected in any samples. Albumen cortisol levels were significantly increased at 7 and 15 days after cortisol implant, but not corticosterone, treatment compared to controls. * = $p < 0.05$, ** = $p < 0.01$.

exogenous corticosterone elevated HLR response in Japanese quail (Nazar et al., 2012) and Eurasian kestrel nestlings (Müller et al., 2011) compared to controls. It has been established that GC influences the production of lymphocytes from lymphoid tissues and cells (Weston et al., 1972; Pardue and Thaxton, 1984; Claman, 2010). In addition, several studies reported that the increase in HLR in response to various stressors can be attributed to the elevated GC levels (reviewed by (Scanes, 2016)). Therefore, sex differences in HLR ratio might be positively correlated to sex differences in the circulating levels of GC. Madison et al. (2018) demonstrated sex differences in hippocampal responses to stressors in zebra finches. These authors further stated that females showed upregulated hippocampal mineralocorticoid receptors while males downregulated both mineralocorticoid and GC receptors in response to social stressors. Sex differences could be explained by relative circulating levels of gonadal hormones. Estradiol is a circulating gonadal steroid that exerts modulating effects on HPA responsiveness and sensitivity to stress and GC negative feedback respectively (Young, 1995a; Young, 1995b). Other authors have also demonstrated that estradiol affects GC receptor effectiveness and stimulates the HPA axis (Kitay, 1963; Leśniewska et al., 1990; Xiao et al., 1994) reviewed by (Kudielka and Kirschbaum, 2005). These observations help to clarify why hens have a higher circulating level of GC and in turn HLR response to stressors and this confirms our result that showed a greater HLR in hens in response to cortisol and corticosterone administration compared to drakes.

Stress has been shown to have adverse effects on egg production and the quality of laying hens. A study from our lab showed that exposure to heat stress decreased daily egg production and decreased the shell and yolk weight of pekin ducks (Oluwagbenga et al., 2022). Both acute and chronic stress reduces egg weight and shell strength and weight, albumen deposition, yolk weight and Haugh unit (Mashaly et al., 2004; Ebeid et al., 2012; Ma et al., 2014; El-Tarabany, 2016; Barrett et al., 2019). This decrease in egg quality observed in heat-stressed ducks was attributed to the reduction in feed intake, respiratory alkalosis, and reduction in blood flow to the oviduct (Ma et al., 2014). Ebeid et al. (2012) demonstrated that heat stress leads to respiratory alkalosis due to hyperventilation and the increased blood pH reduces the amount of Ca^{+} that is essential for shell formation. The decreases in yolk and albumen quality were attributed to compromised oviduct and ovary (El-Tarabany, 2016). However, others have shown no effects of GC on egg or albumen weight (Wolfenson et al., 2007; Deng et al., 2012). Interestingly, Kim et al. (2015) studied the effect of dietary corticosterone on egg quality and reported no effects on egg weight, shell strength or weight. In our study, we found no GC effects on egg quality. Our GC treatments elicited a significant increase in circulating GC levels, but at relatively low levels indicative of GC levels following prolonged, chronic stressors. Our study did not elicit the immediate high levels of GC typical to the onset of a stressor. However our low, albeit significant, levels of GC may not have been sufficient to elicit changes in egg quality. We have previously suggested that ACTH may have extra-adrenal actions (Tetel et al., 2022b; Tetel et al., 2022a). Thus, ACTH may act directly on the oviduct or ovarian tissues to alter egg quality which was not possible in our study due to negative feedback of GCs on the diencephalon and the anterior pituitary secretion of ACTH. ACTH receptor distribution has been shown to go beyond the adrenal gland. The melanocortin two receptor (MC2R) and melanocortin five receptor (MC5R) are recognized as ACTH receptors in the adrenal gland where they regulate GC and mineralocorticoid production (Nimura et al., 2006; Mountjoy, 2010). The MC2R mRNA has been identified in the brain and blood cells of fetal mice, as well as in the brain of teleost fish (Klovins et al., 2004; Nimura et al., 2006) and in the ovary and testes of rainbow trout fish (Aluru and Vijayan, 2008). It is suggested that the MC2R found in teleost fish brains may play a role in regulating ACTH secretion through a negative feedback mechanism (Klovins et al., 2004; Nimura et al., 2006). The effect of GC on egg quality may be mediated by other receptors such as MC2R and MC5R or may be dependent on the direct action of ACTH on the oviduct. Our findings suggest that perhaps ACTH can act on oviduct tissues to impair egg quality, as evidenced by the increase in cortisol in albumen.

It was previously thought that corticosterone, produced by the adrenal cortex, is the primary plasma GC in birds (deRoos, 1961). Injecting corticosterone subcutaneously was found to increase corticosterone in the egg, providing a potential non-invasive method for measuring stress in chickens (Downing and Bryden, 2008). However, a recent study from our lab, showed that cortisol, not corticosterone, is deposited in the albumen (Oluwagbenga et al., 2022) as suggested by previous study (Caulfield and Padula, 2020). Further, we are unable to localize any glucocorticoid in the yolk (Oluwagbenga, et al., 2022). Others have demonstrated that antibody-based assays for glucocorticoids may actually cross-react with gestagens and/or pregnenalone that may elicit false-positive results (Rettenbacher et al., 2009). Steroidogenic enzymes were studied in the Bursa of Fabricius and thymus, and it was found that steroidogenic pathways within these organs lead to the synthesis of cortisol, not corticosterone (Lechner et al., 2001). Those authors further noted that cortisol has a higher affinity to the GC receptor than corticosterone in both the bursa and thymus of chickens. The increase in circulating cortisol in our study following corticosterone treatment could be explained by the corticosterone-stimulated

synthesis and release of cortisol by adrenal or extra-adrenal tissues. However, confirmation of this exciting possibility requires more research. Our findings support the concept that cortisol is a key part of the stress response in ducks and that it can be selectively deposited in egg albumen, potentially serving as a non-invasive indicator of welfare.

In conclusion, our findings further confirm that there are sex differences in the HLR response of ducks to subcutaneous GC implantation where hens exhibit a greater response. We also determined that cortisol is selectively found in the egg albumen but not the yolk following implantation. Our findings suggest that GCs have diverse effects, and although corticosterone is generally considered to be the main GC in birds, cortisol may provide important insights for improving their welfare. Finally, the measurement of cortisol in egg albumen could serve as a non-invasive marker of stress, however more research must be done to affirm this possibility.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by Purdue Animal Care and Use Committee, Purdue University, West Lafayette, IN, United States.

Author contributions

All authors contributed to study design, implementation, data collection, analyses and interpretation, as well as writing of manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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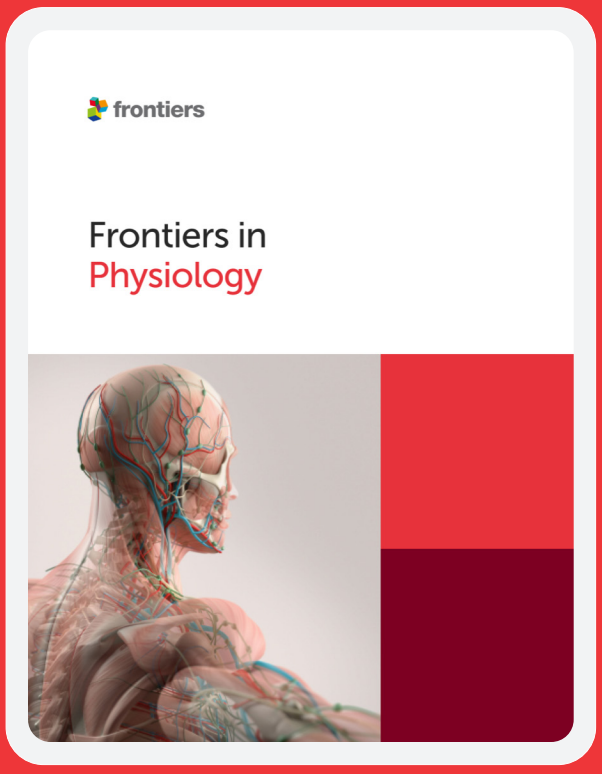

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