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# Sex differences in intestinal morphology and increase in diencephalic neuropeptide Y gene expression in female but not male Pekin ducks exposed to chronic heat stress

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## Abstract

The impact of heat stress (HS) on production is intricately linked with feed intake. We investigated the effects of HS on intestines and diencephalic genes in Pekin ducks. One hundred and sixty adult ducks were allocated to two treatment rooms. The control room was maintained at 22°C and the HS room at 35°C for the first 10 h of the day then reduced to 29.5°C. After 3 weeks, 10 hens and 5 drakes were euthanized from each room and jejunum and ileum collected for histology. Brains were collected for gene expression analysis using qRT-PCR. Intestinal morphology data were analyzed with two-way ANOVA and diencephalic gene data were analyzed with Kruskal–Wallis test. There was an increase in villi width in the ileum ($p = .0136$) and jejunum ($p = .0019$) of HS hens compared to controls. HS drakes showed a higher crypt depth (CD) in the jejunum ($p = .0198$) compared to controls. There was an increase in crypt goblet cells (GC) count in the ileum ($p = .0169$) of HS drakes compared to HS hens. There was higher villi GC count ($p = .07$) in the jejunum of HS drakes compared to controls. There was an increase in the crypt GC density ($p = .0054$) in the ileum, not jejunum, of HS drakes compared to HS hens. Further, there were no differences in the proopiomelanocortin gene expression in either sex but there was an increase in the expression of neuropeptide Y (NPY) gene in HS hens ($p = .031$) only and a decrease in the corticotropin releasing hormone gene in the HS drakes ($p = .037$) compared to controls. These data show that there are sex differences in the effect of HS on gut morphology while the upregulation in NPY gene may suggest a role in mediating response to chronic HS.

**KEYWORDS**
anorexigenic, goblet cells, heat stress, intestinal morphology, Pekin ducks

## 1 | INTRODUCTION

Heat stress (HS) has been demonstrated to have adverse effects on the physiology, behavior, and overall welfare of poultry.[1–3] The intestine is an essential organ for digestion, absorption of nutrient, and provides protective barrier against invasive pathogens.[4,5] HS leads to reduced intestinal integrity by decreasing villi height (VH), villi height to crypt depth (VH:CD) ratio, mucin concentration and tight junction proteins in poultry.[6,7] Impairments in intestinal morphology can lead to a decrease in nutrient

There was no clinical trial involved in this work.

absorption and gut leakage that increases the risk of diseases and thereby decreases production performance. However, sex differences in the effects of HS on intestinal morphology in poultry is largely unexplored.

Birds experiencing HS reduce their feed intake as a strategy to lower heat production associated with the processes of ingestion, digestion, and absorption.[8,9] The decline in performance observed in heat exposed birds is closely connected to reduction in feed intake.[10] One of the mechanisms involved in regulating feed intake includes regulatory neuropeptides and studies have shown that HS alters the expression of these neuropeptides.[11,12] Examples of these neuropeptides include neuropeptide Y (NPY) and Agouti gene-related protein; both categorized as orexigenic neuropeptides while proopiomelanocortin (POMC) and cocaine-amphetamine related transcripts are considered anorexigenic (reviewed by reference 13). During chronic HS when metabolic homeostasis is significantly disrupted, the body activates various systems to adapt. In addition to the role of NPY on feed intake, studies indicate that NPY plays a vital role in adaptation to stress, countering the effects of corticotropin releasing hormone (CRH), a hormone released by the hypothalamus paraventricular nucleus (PVN) during stress.[14–16] In birds, CRH and a second neurohormone, arginine vasotocin (AVT), stimulate the release of adrenocorticotropic hormone (ACTH) and thus glucocorticoids to maintain homeostasis.[17] Therefore, this indicates that NPY may have a central role in stress response beyond feeding regulation.

NPY, a 36 amino acid peptide, is a part of the pancreatic polypeptide family that includes peptide YY and pancreatic polypeptide. NPY, which has been found to have both orexigenic and anxiolytic effects, has a multifunctional role in feed intake, homeostasis, and stress resilience.[18–21] NPY carries out its biological functions by interacting with distinct receptors, namely Y1, Y2, Y4, Y5, Y6, and Y7.[22–24] On the other hand, POMC is a precursor of several polypeptide hormones including adrenocorticotropic hormones in the anterior pituitary which stimulates the secretion of glucocorticoid from the adrenal cortex during stress.[25] POMC is locally expressed in the hypothalamus and pituitary gland with higher expression in the pituitary. In the diencephalon POMC has an anorexigenic effect on feed intake and shows catabolic effects on energy balance.[26–28] POMC peptides exert their effects through melanocortin receptors (MCR1-5).[29]

In prior research conducted in our laboratory,[30] adult Pekin duck breeders were exposed to chronic HS for 3 weeks. Our findings indicated that chronic HS adversely affected the welfare of the ducks and led to increased physiological signs of stress in a sex-dependent manner. Specifically, the HS ducks exhibited elevated levels of serum corticosterone and cortisol with more pronounced response from the hens. Additionally, HS decreased body weight, egg production and quality significantly in hens. We also observed an increase in cortisol levels in the egg albumen following the 3-week HS.[30] Furthermore, eggs collected during the last 3 days of the experimental period displayed reduced fertilization rates and hatchability.[30] The changes in hypothalamic appetite-related genes under chronic cyclic HS condition and the differential effect of chronic HS on intestinal morphology are unknown, particularly in ducks. Therefore, the objective of the current study was to assess the effect of chronic cyclic HS on gut morphology and diencephalic NPY, POMC and CRH genes expression. We collected intestinal and brain samples from ducks from that previous study.[30] Our result showed that HS altered intestinal morphology in a sex dependent manner with hens showing more pronounced damage and the expression of NPY gene was upregulated in the hens while CRH was downregulated in the drakes. There were no significant differences observed in POMC gene expressions during HS.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental design

One hundred and sixty adult Pekin ducks (80 ducks/room; 60 hens to 20 drakes) were obtained from Maple Leaf Farms and housed at Purdue Animal Science Research Farm. The control room was maintained at a temperature of 22°C while the HS room was exposed to a cyclic temperature of 35°C for the first 10 h of the day and reduced to 29.5°C for the remaining 14 h for 3 weeks. The ducks were given access to feed, water, and light according to industry standards. All procedures were approved by the Purdue Animal Care and Use Committee (PACUC # 2109002195). At week 3 post HS, 10 hens and 5 drakes were randomly selected and euthanized with pentobarbital (FatalPlus, 396 mg/mL/kg) from each room, jejunum and ileum samples were collected for histology. Whole brain was collected, snap frozen on dry ice and stored at −80°C for gene expression analysis using quantitative real-time polymerase chain reaction (qRT-PCR). This study focuses on intestinal morphology and brain neuropeptide expression, the production performance, welfare, and glucocorticoid data has been published elsewhere.[30]

### 2.2 | Intestinal morphology

The intestinal samples collected were flushed with 0.9% saline, stapled to cardboard at both ends, fixed inside 10% formalin and stored at 4°C until samples were processed. The samples were sent to the histology lab at Purdue University (Purdue University, West Lafayette, IN, USA) for embedding in paraffin wax. Three sections of jejunum and ileum from each sample were taken and subsequently immersed in paraffin wax for embedding. The embedded samples were sliced into 5 μm thickness using a rotary microtome (Mercedes Scientific, FL, USA), floated in water bath to spread out, and two of the desired sections were placed on a glass slide. A duplicated slide was made for all samples. The slides were placed on trays and allowed to dry for at least 24 h. After drying, the slides were then subjected to heat at a temperature of 60°C for 15 min for proper adhering of tissues to the slide. The slides were prepared in duplicate, one set undergone staining with hematoxylin and eosin (H&E) to assess villi height to crypt depth (VH:CD) ratio, villi height (VH), and crypt depth (CD), and villi width (VW). The second set of slides were subjected to Alcian blue stain to visualize goblet cells (GCs). These staining procedures were carried out following established protocols and standard techniques. A microscope with an electronic camera (National Optical and

Scientific Instruments, Inc., Schertz, TX, USA) was used to visualize and capture images of samples, only the intact and whole villi were considered, and at least six images per sample were measured. The obtained images were analyzed using ImageJ macro (ImageJ open-source software version 1.8). Villus height was determined by measuring the distance from the mouth of the crypt to the tip of the villus while the villi width was measured at the basal and median part of each villus and the values averaged. Similarly, crypt depth was measured by assessing the distance from the basement membrane to the base of the villus. Additionally, the ratio of VH:CD was calculated. GC per villus and crypt was counted, and the average count was determined. The villi GC density was calculated by dividing the average villi GC count by the average villi height per duck. Similarly, the crypt GC density was calculated by dividing the average crypt GC count by the average crypt depth per duck.

### 2.3 | RNA isolation, reverse transcription, primer validation, and efficiency

The whole brain samples were dissected into diencephalon regions, then homogenized and placed in 2 mL tubes. Any surplus tissue was stored in cryovial tubes at −80°C for future use. RNA was isolated from these samples using Trizol reagent and following manufacturer's protocol. The purity and concentration of the extracted RNA were assessed using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Samples with concentrations falling between 500 and 2000 ng/μL were deemed acceptable and any samples with values surpassing 2000 ng/μL were appropriately diluted with nuclease-free water. The RNA samples were evaluated based on a 260/280 ratio falling around 2.0 and a 230/260 ratio between 2.0 and 2.2. RNA was treated with DNase using the Turbo DNase Kit from Invitrogen by Thermo Fisher Scientific, with slight modifications to the manufacturer's instructions. The integrity of the RNA was assessed using 1% agarose gel electrophoresis. Denatured RNA samples were reanalyzed, utilizing excess homogenized samples that were stored. The total RNA was then transcribed to cDNA using the High-Capacity Reverse Transcription Kit from Applied Biosystems by Thermo Fisher Scientific. Reverse transcription was carried out using a thermocycler at 25°C for 10 min, followed by 37°C for 120 min, 85°C for 5 min, and then held at 4°C. Primers were validated using cDNA pool and primer specific master mix containing SSO advanced Sybr green with annealing temperatures ranging from 57.5 to 62°C to identify temperature with clean melt curve and no decrease in threshold cycle (CT) values. Primer efficiency was done by serial dilution and samples were run in duplicate. CT values were exported to excel worksheet, and efficiency was calculated and maintained between 90% and 110%.

### 2.4 | Quantitative real-time polymerase chain reaction

The qRT-PCR was conducted to analyze the CRH, POMC and NPY genes expression, utilizing the Bio-Rad CFX thermocycler (Bio-Rad, Temecula, CA, USA) in a 15 μL reaction volume with the SYBR qPCR master-mix. The qRT-PCR cycling conditions were 95°C for 3 min, followed by 40 amplification cycles structured as follows: 95°C for 10 s, specific primer annealing temperature for 30 s, 65°C for 5 s, and 95°C for 5 s. Subsequently, the melt curves were examined for each gene and absence of amplification in the no-template wells was confirmed to ensure no contamination. The coefficient of variation was calculated, and samples with values exceeding 1 were re-run. The annealing temperatures and primers utilized are listed in Table 1. Relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method,[31] normalized against housekeeping (HK) gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the control group serving as the reference.

### 2.5 | Statistical analyses

The duck was considered the experimental unit. Intestinal morphology data were analyzed with two-way ANOVA using MacJMP SAS (JMP Pro 15). NPY, POMC, and CRH genes data do not follow a normal distribution as shown by Shapiro–Wilk test and were analyzed using Kruskal–Wallis test. Post hoc analyses were done by Tukey Kramer pairwise comparison test. A $p < .05$ was considered significant and $p > .05 < .1$ was considered a trend toward significance.

## 3 | RESULTS

### 3.1 | Intestinal morphology

The representative images of jejunal and ileal tissue stained with Alcian blue are shown in Figure 1. Villi width was significantly higher in the ileum ($p = .0136$) and jejunum ($p = .0019$) of HS hens compared to the controls however no significant differences were observed in either intestinal section for drakes (Figure 2A). We observed a significant increase in crypt depth in the jejunum ($p = .0198$) of HS drakes compared to the controls with no differences in the ileum of drakes and in either intestinal section for hens (Figure 2B). Both hens and drakes showed no significant differences in villi height (Figure 2C) and VH:CD ratio (Figure 2D).

Crypt GC count was significantly higher in the ileum ($p = .0169$) of HS drakes compared to HS hens but no significant differences in the jejunum of drakes and hens (Figure 3A). There was a trend for higher villi GC count in the jejunum ($p = .07$) for HS drakes compared to the controls however no differences in the ileum of drakes and either intestinal section for hens (Figure 3B). There were no significant differences in villi GC density for both sexes (Figure 3C). Crypt GC density was significantly higher in the ileum ($p = .0054$) of HS drakes compared to HS hens but no significant difference in the jejunum of both sexes (Figure 3D).

### 3.2 | Diencephalic NPY, POMC, and CRH genes expression

There was a significant increase in the relative gene expression of NPY in HS hens ($p = .031$) compared to controls but no difference in

**TABLE 1** Sequence and annealing temperature of primers used for qRT-PCR.

| Primers | Forward | Reverse | Temperature (°C) |
|---|---|---|---|
| GAPDH | TGATTAACGGGAATGCCATC | TCATGGTTCACACCCATCAC | 60 |
| NPY | GAGGCACTACATCAACCTCATCAC | TGTTTTCTGTGCTTTCCCTCAA | 58 |
| POMC | AGCAGCAAATGCCAGGAC | ATTTGTTCCAGCGAAAGTGG | 62 |
| CRH | GGAGATCATCGGGAAGTGAA | CGTGTTTCTGCACTTGGTAGG | 59 |

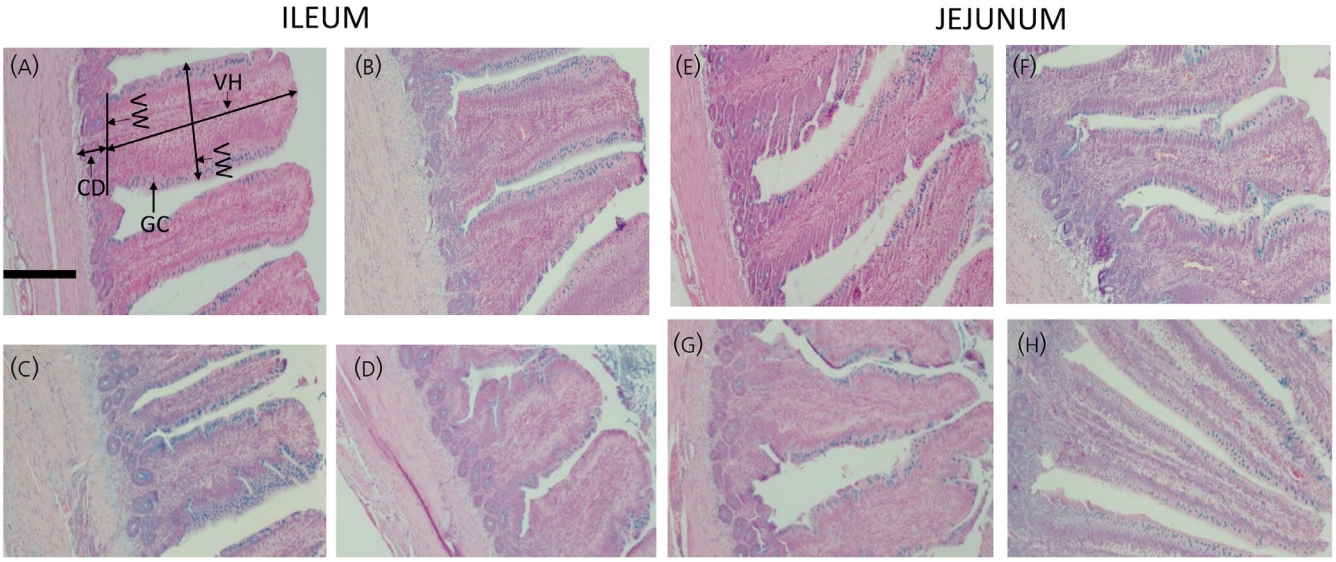


**FIGURE 1** Ileum of control drake (A), heat stress drake (B), control hen (C), and heat stress hen (D), Jejunum of control drake (E), heat stress drake (F), control hen (G), and heat stress hen (H). Representative images of ileum and jejunum tissue stained with Alcian blue from drakes and hens exposed to cyclic heat stress or control. Bar = 100 μm. CD, crypt depth; GC, goblet cells; VH, villi height; VW, villi width.

POMC and CRH genes expression (Figure 4A). The means and standard error of means for hen's NPY (control = 1.03 ± 0.090, HS = 1.28 ± 0.040), POMC (control = 1.56 ± 0.445, HS = 1.21 ± 0.314), and CRH (control = 1.05 ± 0.117, HS = 1.02 ± 0.044).

Drakes in HS group showed a significant decrease in the relative gene expression of CRH ($p = .037$) compared to controls with no difference in POMC and NPY genes expression (Figure 4B). The means and standard error of means for drake's NPY (control = 1.05 ± 0.166, HS = 1.03 ± 0.061), POMC (control = 2.73 ± 1.400, HS = 1.92 ± 1.150), and CRH (control = 1.04 ± 0.149, HS = 0.73 ± 0.030).

## 4 | DISCUSSION

The purpose of our study was to determine whether HS would exert differential effect on intestinal morphology and alter diencephalic CRH, NPY and POMC gene expression. To achieve this, we exposed adult Pekin ducks to cyclic HS or control for 3 weeks. We observed that HS upregulated the relative expression of NPY in hens but not drakes. There was a downregulation of CRH gene in drakes but not in hens however no differences were observed for relative POMC genes expression in both drakes and hens. HS also exerts a differential effect on intestinal integrity by increasing the count and density of GC in the small intestine of drakes but not hens. Our data shows that there are sex differences in the effects of HS on intestinal morphology while upregulation of relative NPY gene expression during chronic HS may play a central role in response to HS. Production performance, welfare, and glucocorticoid data of this study has been published elsewhere.[30]

The integrity of the intestinal barrier in poultry is very important and any compromise can lead to impaired digestion and nutrient absorption, increased intestinal permeability to toxins and pathogens which all then can lead to poor performance and diseases.[1,32,33] VH:CD ratio, VH, VW, and CD are often utilized as markers of intestinal health and function. Shorter villi indicate poor absorptive capacity while deeper crypt indicates higher proliferative capacity which can lead to poor growth performance.[34] Studies have shown that HS alters intestinal morphology by decreasing the VH:CD ratio, VH, and increases CD in broilers and ducks exposed to HS thereby leading to poor nutrient absorption and passage of large particles.[6,35–37] Another study reported an increase in villi width following HS in broiler chickens.[38] Although there was no difference in VH:CD ratio in our study, there was an increase in villi width in both ileum and jejunum of HS hens but not drakes, a significantly deeper jejunal crypt in HS drakes but not hens. Wider villi indicate increased absorptive area of the villi[39] which is most likely a compensatory mechanism to increase

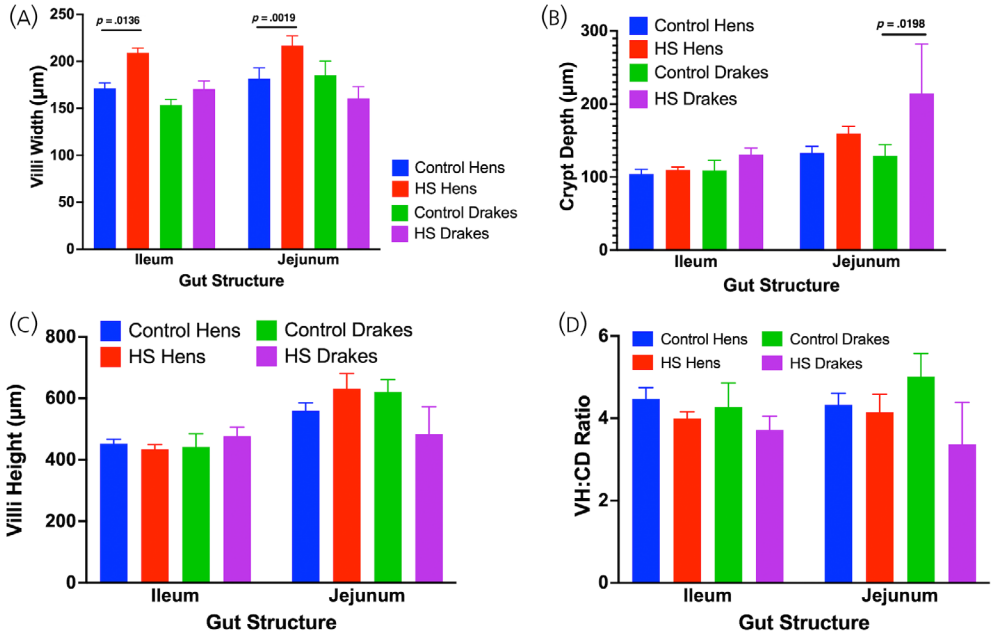


**FIGURE 2** Villi width (A), crypt depth (B), villi height (C), and villi height to crypt depth ratio (D) in the ileum and jejunum of hens and drakes exposed to cyclic heat stress or control. Data were analyzed by two-way ANOVA and shown as Means ± SEM, $n = 10$/treatment for hens and 5/treatment for drakes. Heat stress hens showed a significant increase in the ileal and jejunal villi width compared to the controls with no difference observed for drakes. Drakes exposed to heat stress showed a significantly deeper crypt in the jejunum only compared to the control with no differences observed for hens. There was no significant difference observed in the villi height and villi height to crypt depth ratio for both sexes.

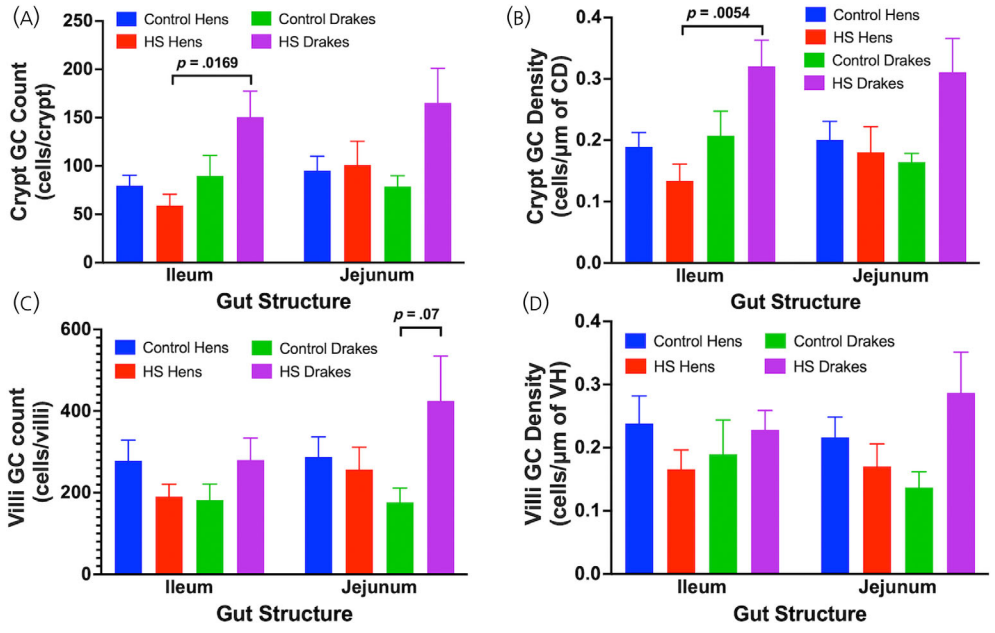


**FIGURE 3** Crypt goblet cells count (A), crypt goblet cells density (B), villi goblet cells count (C), and villi goblet cells density (D) in the ileum and jejunum of hens and drakes exposed to cyclic heat stress or control. Data were analyzed by two-way ANOVA and shown as Means ± SEM, $n = 10$/treatment for hens and 5/treatment for drakes. Drakes exposed to heat stress showed a significant increase in ileal crypt goblet cells compared to heat stress hens however no difference observed in the jejunum. Heat stress increased ileal crypt goblet cell density for drakes compared to heat stress hens with no differences were observed in the jejunum. Heat stress showed a tendency to increase jejunal villi goblet cell count for drakes compared to the controls, with no differences observed in the ileum. No significant differences observed for villi GC density.

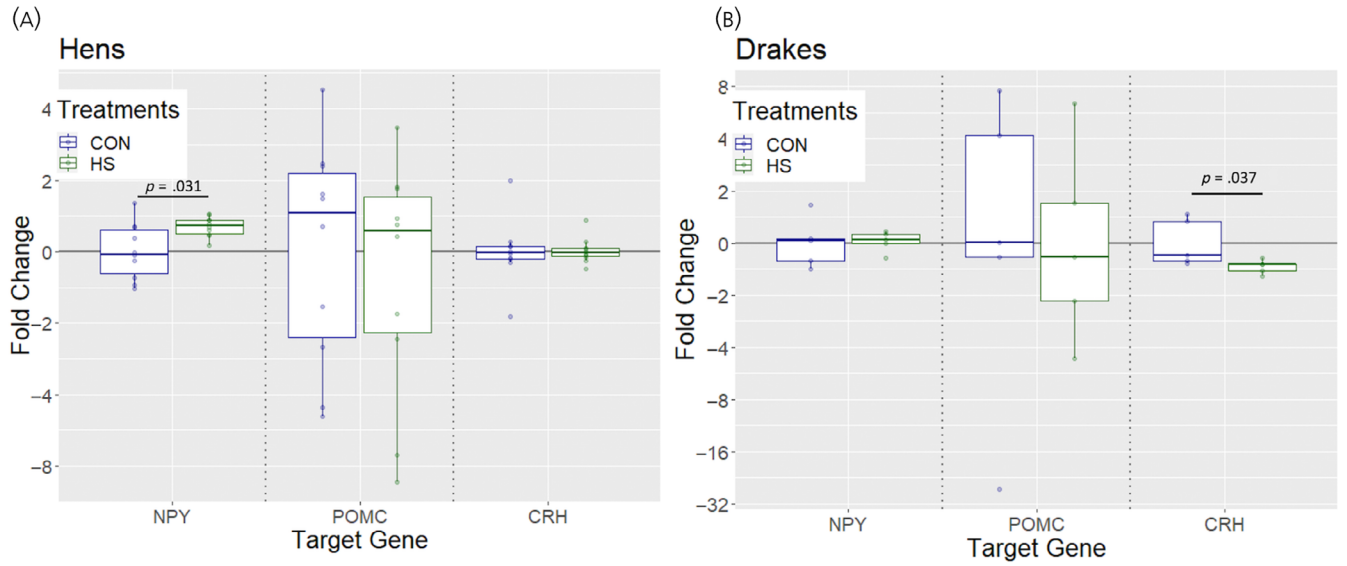


**FIGURE 4** Relative NPY and POMC gene expression in hens (A) and drake (B) exposed to cyclic heat stress or control. Data were analyzed by Kruskal–Wallis test and the whiskers represent the range of data within 1.5 times the interquartile range from the edges of the box, $n = 10$/treatment for hens and 5/treatment for drakes. Hens exposed to heat stress showed a significant increase in relative NPY gene expression with no difference observed for drakes. Heat stressed drakes showed a significant decrease in CRH gene expression compared to the controls, but no difference was observed for the hens. Both drakes and hens showed no differences in the relative expression of POMC gene.

nutrient absorption in response to sloughing of the villi. A deeper crypt is indicative of a more rapid turnover of tissue to facilitate villi renewal which compensates for the shedding and weakening of the villi.[40] The intestinal crypt contains continuously proliferating stem cells that divide to produce various epithelial cells, including enterocytes and GCs responsible for absorptive and secretive functions.[41] As enterocytes mature, they migrate to the surface of villi, where they function in nutrient absorption before being replaced by newly formed cells. The deeper crypt observed in the jejunum of HS drakes in this study could be a defense mechanism to regenerate the villi and increase digestive enzymes secretion for improved nutrient absorption. Studies have shown that glucocorticoid receptor (GR) is expressed in the intestinal mucosa of humans.[42] In addition, mineralocorticoid receptor, which binds both mineralocorticoid and glucocorticoid, and GR were expressed in intestinal mucosal of domestic ducks,[43] and chicks.[44] Further, the expression of corticotropin releasing hormone receptor and ACTH receptor, melanocortin receptor 2, was reported in the small and large intestine of rats.[45] Studies have also shown the extra-adrenal synthesis of glucocorticoid in the intestinal epithelial cells.[46] The regulatory action of glucocorticoid through its receptors on the proliferation, differentiation, and migration of crypt stem cells may explain the effect of glucocorticoid on intestinal morphology.[47]

Intestinal GCs are specialized glandular cells responsible for synthesizing and secreting mucin—a gel-like substance that forms a protective layer covering the mucous membranes of the epithelia.[48] Mucin provides a physical barrier between the luminal content and the epithelial cells in the intestine thereby preventing contact between large particles or pathogenic microorganism with the intestinal epithelium.[49] We observed a significant increase in ileal crypt GC count for HS drakes compared to HS hens, a tendency for higher jejunal villi GC count in HS drakes compared to the control, and a significant increase in the ileal crypt GC density of HS drakes compared to HS hens. Interestingly, studies have shown HS to decrease the number and density of goblet cells in heat exposed birds[7,50,51] whereas, other studies also reported an increase during HS in laying hens and broilers.[52,53] A study on the digestive tract of long-snouted seahorse shows that the GCs are also involved in digestion and absorption.[54] The increase in GC count and density in HS drakes observed in our study is likely a compensatory mechanism to restore intestinal homeostasis and increase nutrient absorption. These changes seem to correspond to the result in our previous study in which there was no difference in body weight of HS drakes but a significant decrease in the body weight of HS hens.[30]

Hypothalamic neuropeptides play an important role to regulate feed intake and energy, NPY is considered one of the most potent stimulants of feeding behavior.[55] Our study showed that chronic HS upregulated the expression of NPY in the hens but not in drakes while no differences was observed for POMC. NPY expression has been shown to be higher in ducks with high feed intake[56] and there was an increase in the feed intake of quails given intracerebroventricular injection of NPY.[57] Due to the increase in NPY expression during HS we would expect a constant or increase in body weight, attributable to higher feed intake. However, the hens used in this study lost weight presumably due to decreased feeding or increased metabolism[30] which is contrary to what is known about NPY and its role in feed intake. This poses the question if increased expression of NPY is always related to hunger, and this leads to other possibility that NPY is involved in stress response. Similar to our findings, studies have shown the upregulation in NPY gene expression post stress in

different brain regions, and this is dependent on the duration and intensity of the stressor (reviewed by reference 58). A study reported an increase in the expression of NPY and no change in POMC in layer chicks exposed to acute HS.[11] In contrast, there are studies that reported a decrease or no change in NPY expression of birds under HS.[12] These inconsistencies can be attributed to the differences in intensity and duration of heat exposure which has a great impact on the expression of the genes. Decrease in feed intake has been reported in birds exposed to HS[8,59,60] and this is one of the coping mechanisms in which poultry reduce thermogenesis during HS. Further, fasting, feed restriction or decrease in feed intake has been reported to stimulate the upregulation of NPY in broilers,[61] Japanese quails,[62] and rats.[63] This shows that the decrease in feed intake of birds exposed to HS could upregulate the expression of NPY. In agreement with our findings, there was no significant difference observed in relative expression of hypothalamic POMC in broilers exposed to either chronic[12] or acute HS.[64] POMC, a precursor of anorexigenic neuropeptides such as melanocortin and α-melanocyte-stimulating hormone induces satiety and increases energy expenditure.[65] A study by Garcia de Yebenes et al.[66] showed that NPY exhibits its anabolic effects by opposing the actions of POMC through Y2 receptor. This further confirms the upregulation of NPY gene and no change in POMC gene observed in this study.

In addition to its abundant expression in the hypothalamus,[24] NPY and its receptors are also expressed in other peripheral tissues such as adrenal gland where it stimulates the secretion of catecholamines[67] and in the gastrointestinal tract.[68] This indicates that NPY has an array of physiological functions in stress response beyond feed intake. A study showed that central administration of glucocorticoid, a hormone produced by adrenal cortex during stress, increased the expression of NPY mRNA expression in broiler chicks.[69] NPY has been reported to have a potent anti-stress and anti-anxiety effects and has been found to decrease immobilization time and corticosterone levels in rats exposed to stress.[70,71] Further, NPY has also been found to have thermoregulatory effects as chicks injected centrally with NPY and exposed to high temperature showed reduced body temperature, lower plasma corticosterone and epinephrine and increased expression of heat-shock proteins.[72] Another study reported that centrally administered NPY reduced rectal temperature, brain and plasma norepinephrine in heat exposed chicks[21] further establishing its role in thermoregulation and adaptation to stress.

In birds, CRH is synthesized primarily in the Nucleus of the hippocampal commissure but can also be found in the PVN of the hypothalamus.[73] Upon binding to its receptors on corticotropes located in the anterior pituitary, CRH triggers the secretion of ACTH which then stimulates the adrenal cortical cells to release glucocorticoids.[74] There is a second neurohormone in birds called AVT primarily found in the PVN of the hypothalamus which also stimulates the corticotropes in the anterior pituitary to release ACTH.[75] In our study, we found no significant difference in CRH gene expression in the hens but a significant decrease in drakes following chronic HS. CRH has been reported to be predominantly functional in the early phase of stress whereas AVT exhibits late activation and is involved in the later phase of stress.[73,76,77] We were unable to evaluate the expression of AVT gene expression in our study due to the unavailability of duck-specific primers for AVT—numerous attempts with different sets of primers designed for chickens were unproductive in the duck. We have consistently shown in our lab that there are sex differences in glucocorticoid response of ducks to stress with the hens being more responsive than the drakes.[30,78,79] The serum corticosterone level was significantly higher in the hens at week 1 relative to the onset of heat stress while no difference was observed for the drakes as reported in the first part of this study.[30] Cortisol concentration was also higher in the hens compared to the drakes.[30] This previous report corroborates the expression of CRH gene in this study since serum glucocorticoid level is dependent to a greater extent on CRH secretion. This could mean that drakes are not as sensitive to heat stress or recover faster when compared to the hens. Another mechanism that has been proven in mammals but not in avians is the interaction of estradiol with the HPA axis through the impairment of glucocorticoid dependent negative feedback in the female thereby leading to prolonged secretion of glucocorticoids.[80] Therefore, these findings and results from our study further validate that CRH may not be involved in sustaining stress response.

In conclusion, HS altered intestinal morphology in a sex-dependent manner with the hens showing more pronounced damage compared to the drakes. CRH gene expression was downregulated in drake during prolonged HS. There was also an increase in the expression of diencephalic NPY gene in hen but not drakes following chronic HS. However, the main purpose of NPY activation may be beyond feeding regulation and further studies are required to explore the central role of NPY during chronic HS.

## AUTHOR CONTRIBUTIONS

**E. M. Oluwagbenga:** Data curation; formal analysis; investigation; visualization; writing – original draft; writing – review and editing. **M. Bergman:** Investigation; methodology; writing – review and editing. **K. M. Ajuwon:** Conceptualization; supervision; writing – review and editing. **G. S. Fraley:** Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; supervision; writing – review and editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jne.13424.

## DATA AVAILABILITY STATEMENT

Data available on request from the corresponding author.

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