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Expression of relaxin-3 and its receptors in the hypothalamic-pituitary-ovary axis in layers and broiler breeders

Kahina Boukherroub [a,*], Sunantha Kosonsiriluk [a], Pitchaya Santativongchai [a], Jasna Chelikandam [a], Lillian Ehresmann [a], Kristen Diehl [b]

[a] Department of Animal Science, University of Minnesota, Saint Paul, MN 55108, USA
[b] Animal Biosciences and Biotechnology Laboratory, BARC, ARS, USDA, Beltsville, MD 20705, USA

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ABSTRACT

Layers can regulate their feed intake and maintain well-organized ovaries. Conversely, broiler breeders are prone to overeating, leading to disorganized ovaries. Relaxin-3 (RLN3) is a neuropeptide hormone involved in metabolism, energy balance, and reproduction in mammals. In chickens, RLN3 was identified in the ovary, pituitary gland, and hypothalamus. This study aimed to explore the potential role of RLN3 in feeding behavior and reproduction. Expression of RLN3 and relaxin family peptide receptors (RXFP1 and 3) were examined in ovarian follicles, the pituitary gland, and the hypothalamus between layers (L) and broiler breeders (BB). Gene expression analysis revealed *RLN3* was highest in the granulosa cells (GC) of 9-12 mm follicles compared with other follicles, with BB had the highest overall expression. In the pituitary, *RLN3* was higher in L compared with BB and minimally expressed in follicle theca tissue (TH), cortex, and hypothalamus. Expression of *RXFP1* was highest in TH of all follicles without a significant difference between L and BB. The expression of *RXFP3* was highest in the cortex without a significant difference between L and BB. The hypothalamus had the highest expression of *RXFP3* across all tissues in both L and BB. Immunofluorescence staining for RLN3, RXFP1, and RXFP3 revealed their presence in the GC, TH interna and externa of 3-12 mm follicles in both L and BB. Interestingly, RLN3 was localized in small vesicles in the ooplasm of 3-12 mm follicles. Within the cortex, RXFP1 was localized in the GC and TH of cortical follicles while RXFP3 was exclusively localized in the stromal and muscle cells. Surprisingly, RXFP3 was also localized in the nucleus. Overall, RLN3 and its receptors were differentially expressed across the hypothalamic-pituitary-ovarian axis and between layers and broiler breeders. This suggests potential role in nutrition and ovarian dysregulation. Further, RLN3 action is likely mediated through autocrine and paracrine effects. Modulating RLN3 could lead to novel strategies of regulating feed intake in broiler breeders.

Introduction

The poultry industry developed two specialized types of commercial chickens, hens that lay table eggs (layers) and hens that lay fertilized eggs to be raised as meat birds (broiler breeders). For both strains, high egg-laying efficiency is important (Siegel, 2014). Layers are genetically selected to produce a maximum number of eggs with minimal feed intake (Gilbert, 1971; Etches and Schoch, 1984; Bain et al., 2016). Layers can regulate their feed intake and even though feed is offered *ad libitum* they do not overeat. Consequently, they can maintain a highly organized ovary structured into a size and maturity hierarchy of follicles. This hierarchy includes pre-recruitment follicles (1-8 mm), a single recently recruited follicle (9-12mm), and 4 to 7 pre-ovulatory follicles (12-40 mm) (Gilbert et al., 1983; Johnson, 2015; Ghanem and Johnson, 2019). Broiler breeders are genetically selected for fast growth and high feed intake. Unrestricted access to feed leads to overeating. This in turn, causes the ovary to exhibit significant disorganization in the follicle hierarchy with the occurrence of one or more follicles of the same size. This abnormal development of the follicular hierarchy leads to a higher incidence of internal ovulation, double-yolk eggs, soft-shelled eggs, irregular laying patterns, and low hatchability (Dunnington and Siegel, 1985; Hocking et al., 1987; Hocking, 2014). To address this issue, the current practice is to restrict access to feed and closely monitor the weight gain of these birds (Zakaria et al., 2005; Tahamtani et al., 2020;

* Corresponding author.
E-mail address: kahina@umn.edu (K. Boukherroub).

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Hocking, 2014). This practice is labor-intensive, costly, and not entirely effective.

The link between high feed intake, high body weight, and ovarian dysregulation in broiler breeders is well documented. Interestingly, layers can also be selected for disorganized ovaries, these birds also tend to exhibit high feed intake and larger body weight (Abplanalp et al., 1977; Hocking, 2014; Korver, 2023). Currently, the physiological/hormonal mechanism that connects ovarian follicle regulation and feed intake is not well understood. Despite existing knowledge, the physiological differences that allow layers to be fed *ad libitum* while maintaining organized ovaries, in contrast to broiler breeders, remain unclear. Addressing this gap could lead to strategies for better controlling feed intake in broiler breeders. This, in turn, could optimize production efficiency and reduce labor and associated costs.

Recently, the orexigenic (appetite-increasing) neuropeptide hormone relaxin-3 (RLN3) was discovered to be expressed in the granulosa cells (GC) of ovarian follicles and the pituitary gland in chickens. The receptors for relaxin-3, relaxin family peptide receptors (RXFP1 and RXFP3), are G-protein-coupled receptors (GPCRs). They were found to be expressed in theca tissue (TH) and the ovarian cortex, respectively (Ghanem and Johnson, 2021). In a separate study, RLN3 was found to be more highly expressed in the pituitary of female chickens compared to male chickens and co-localized with gonadotropic hormone genes (Lv et al., 2022). The same study also reported the expression of the receptors RXFP1 and RXFP3 in the hypothalamus. As the hypothalamus also contains nuclei that regulate feed intake (Sohn, 2015), RLN3 may be the missing link explaining overnutrition and ovarian dysregulation in broiler breeders.

The cellular localization of RLN3 and its receptors in the ovary, as well as their physiological functions in chickens, remain unknown. The aim of this study was to investigate the gene and protein expression of RLN3, RXFP1, and RXFP3 within the cellular compartments of ovarian follicles, the pituitary gland, and the hypothalamus. Additionally, the study sought to determine whether there are differences in expression between layers and broiler breeders.

Materials and methods

Experimental animals

Hy-Line W-80 laying hens and Aviagen Ross 708 breeder hens at 34–40 weeks of age ($n = 4$ / group) were used for the study. They were raised in conventional commercial conditions to maximize reproductive performance. The laying hens were subjected to a photo period of 16 h of light and 8 h of darkness (16L:8D) and given access to a commercial crumble layer diet (Home Fresh Extra Egg Layer, Kent Foods, IA) consisting of 16 % protein and water *ad libitum*. The broiler breeders were subjected to 17L:7D and given access to water *ad libitum*. They were fed a diet based on the Aviagen Ross 708 Parent stock nutrition specifications of 14 % crude protein (Aviagen, 2021a) their feed intake and weight were monitored to follow (Aviagen, 2021b). The animals and protocol used in the study were approved by the University of Minnesota Institutional Animal Care and Use Committee (protocol # 2112-39665A).

Tissue collection

Formalin-fixed tissues

Ovaries were removed and rinsed with chilled phosphate-buffered saline (PBS), pH 7.4. Pre-ovulatory follicles (F1-F5) were trimmed out and discarded. Follicles were segregated based on the sizes (9-12 mm, 6-8 mm, and 3-5 mm) and gently rolled over a paper towel to remove connective tissues. The follicles and ovarian cortex were fixed in 10 % neutral buffered formalin for 24 h at room temperature and transferred to 70 % ethanol and stored at 4°C until further tissue processing.

Fresh-frozen tissues

Follicles (9-12 mm, 6-8 mm, and 3-5 mm) were dissected from the ovary, gently rolled over a paper towel to remove surrounding connective tissues, and transferred to a petri dish filled with chilled PBS. The follicles were punctured with a scalpel blade, the granulosa layer was collected from the expressed yolk, as previously described by Tilly and Johnson, 1987. Granulosa and theca layers were rinsed free of yolk, snap-frozen in liquid nitrogen, and stored at −80°C for RNA extraction. Additionally, the hypothalamus and the pituitary gland were collected, snap-frozen, and stored at −80°C.

Tissue processing

Formalin-fixed tissues were processed through dehydration, clearing, and paraffin infiltration, using an Epredia™ STP 120 Spin Tissue Processor (Epredia, Kalamazoo, MI). Subsequently, the tissues were embedded in paraffin blocks using an Epredia™ HistoStar™ Embedding Workstation (Epredia, Kalamazoo, MI). The formalin-fixed paraffin-embedded tissue blocks were sectioned at a thickness of 5 μm using a Thermo Scientific Microm HM 325 microtome (Thermo Fisher Scientific, Waltham, MA), then mounted onto charged slides (VWR International, Radnor, PA), and stored at room temperature until further staining.

Hematoxylin and eosin (H&E) staining

The tissue sections were deparaffinized with xylene and rehydrated through a series of 100 % xylene, a mixture of 50 % xylene and 50 % ethanol, 100 % ethanol, 75 % ethanol, and distilled water. Tissues were stained with hematoxylin, Gill III (Sigma-Aldrich, St. Louis, MO) for 2 min, then the slides were quickly dipped twice in acidic alcohol (0.1 % HCl in 75 % ethanol) and rinsed with several changes of distilled water until the water became clear. Tissues were immersed in 75 % ethanol and stained with eosin for 2 min. Tissues were then dehydrated through a series of 75 % ethanol, 100 % ethanol, a mixture of 50 % xylene and 50 % ethanol, and 100 % xylene. Tissues were mounted with mounting medium, and coverslips were applied. Images were captured using an Olympus BX53 microscope (Olympus America Inc., Center Valley, PA, USA).

Immunofluorescence staining (IF)

The tissue sections were deparaffinized as described above. Antigen retrieval was performed using the heat-induced epitope retrieval method in sodium citrate buffer (pH 6.0, Abcam, Cambridge, Cambridgeshire, United Kingdom) for 15 min. After the slides cooled down, they were washed twice with PBS-Tween 20, pH 7.4 (PBS-T), rinsed with PBS, and blocked with 5 % normal serum in PBS for 1 h. Immunofluorescence staining was performed using RLN3 (Cat No. 26075-1-AP, 1:50) and RXFP1 (Cat No. 18419-1-AP, 1:100) antibodies purchased from Proteintech Group, Inc (Rosemont, IL, USA) and a custom chicken RXFP3 (1:500) antibody (Thermo Fisher Scientific, Rockford, IL). Slides were incubated overnight at 4°C and washed twice with PBS-T and rinsed with PBS. Fluorescein Isothiocyanate (FITC)–conjugated Affinipure Goat Anti-Rabbit IgG ($H + L$) was used as a secondary antibody at 1:100 dilution (Proteintech Group, Inc, Rosemont, IL, USA). Slides were incubated at room temperature for 1 h, washed twice with PBS-T, rinsed with PBS, and mounted with the anti-fade fluorescence mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Abcam, Boston, MA, USA). Images were captured using an Olympus BX53 microscope (Olympus America Inc., Center Valley, PA, USA). See Table 1 for a complete list of antibodies, concentrations, and dilutions.

Table 1
Primary and secondary antibodies used for immunofluorescence.

Primary antibody	Dilution	Secondary antibody	Dilution
RLN3 polyclonal antibody (Proteintech, 26075-1-AP), 300 μg/ml.	1:50	Donkey-anti rabbit FITC	1:100
RXFP1 polyclonal antibody (Proteintech, 18419-1-AP), 240 μg/ml.	1:100	Goat-anti rabbit FITC	1:100
Custom RXFP3 polyclonal antibody (Thermo Fisher Scientific), crude serum	1:500	Donkey-anti rabbit FITC	1:100

RNA extraction, reverse transcription, and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using RNAzol® reagent (MilliporeSigma, Burlington, MA, USA) and DNase-treated using DNase I (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The integrity of RNA was assessed using TapeStation 4200 (Agilent, Santa Clara, CA, USA). Samples with RNA integrity numbers of 7 and higher were used for RT-qPCR. Total RNA (1 μg) was reverse transcribed to cDNA using the SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. RT-qPCR was performed using QuantStudio™ 3 Real-Time PCR (Thermo Fisher Scientific, Waltham, MA, USA) using PowerTrack™ SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). All reactions were executed in duplicates. A no-template and no-reverse transcriptase controls were included for each gene. The running conditions were as follows: 95 °C for 2 min, then 40 cycles of 95°C for 5 s and 60°C for 30 s. Melting curves were analyzed after each run to confirm a single product. Standard curves were generated for each gene using synthetic double-stranded gBlocks to determine primer efficiencies. Efficiencies were 92 %, 86 %, 84 %, and 91 % for RLN3, RXFP1, RXFP3, and RLN3, respectively. Target gene expression was standardized to RPL19 using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Primers and gBlocks sequences can be found in Tables 2 and 3.

Statistical analysis

Data were checked for homoscedasticity using the Spearman's test and normality was assessed using diagnostic tests including Q-Q plot and the D'Agostino-Pearson's test. Data that did not pass the test were natural log (Ln) transformed to meet homoscedasticity and normality. Outliers were identified and removed using the GraphPad ROUT outlier test. After data transformation all the data met the homoscedasticity and normality requirements. Results are presented as mean ± SEM. Differences between and within groups were analyzed using a two-way analysis of variance (Two-Way ANOVA), with chicken strain (layers vs. broiler breeders) as one factor and follicle size or brain compartment as the second factor. Post hoc comparisons were conducted using Tukey's test. A p-value ≤ 0.05 was considered statistically significant, while $0.05 < p\text{-value} \leq 0.1$ was interpreted as a tendency toward significance. All statistical analyses were performed using GraphPad Prism Software, version 10.1.1.

Table 2
Gallus gallus-specific primer sequences.

Gene	NCBI accession	Primer Sequence	Product size
RLN3	NM_001113200	FWD 5′-TTCGCCAGCTAGAAGAGGCT REV 5′-AAGGGTAACTGCTGGGTCCT	78bp
RXFP1	XM_420385	FWD 5′- GTGGAGTGTGTTTGTGTGCC REV 5′- TCTGACAGCCTTCAGCAGTG	117bp
RXFP3	XM_004937174	FWD 5′- CTCCCTGGTGTATTCCGTGG REV 5′- CCACAGCCCAGAAGGGTAAG	170bp
RLP19	NM_001030929.1	FWD 5′-GTACCTGAAGGTCAAGGGTAAC REV 5′-AGAGCTTCTTGCGAGCTTT	95bp

Results

Histology of ovarian follicles

Fig. 1 illustrates a representative sample of 9-12 mm follicles, 6-8 mm follicles, 3-5 mm follicles, and ovarian stroma. Cellular compartments of the follicle are shown via H&E staining including the oocyte, granulosa cells (GC), theca interna (TI), and theca externa (TE). The ovarian stroma comprises primordial and primary follicles, blood vessels, ganglions, and smooth muscle fibers.

Antibody validation for immunofluorescence staining

Fig. 2 demonstrates a successful immunofluorescence staining of RLN3, RXFP1, and RXFP3 antibodies, along with their negative controls and isotype controls. Fluorescence signals were observed in ovarian follicles co-incubated with the antibodies. No fluorescence signal was observed in negative controls when primary antibodies were omitted or isotype controls when normal rabbit serum was used instead of primary antibodies. Additionally, the validation of custom chicken RXFP-3 development is shown in **Supplementary Fig. 1**, where anti-RXFP-3 antibody were developed at Day 56 and Day 72 post-immunization.

Localization of RLN3, RXFP1, and RXFP3 in Ovarian Follicles

The distribution of RLN3, RXFP1, and RXFP3 appeared to be consistent between chicken layers and broiler breeders. Localization of RLN3 was found in the GC, TH interna, and TH externa of 9-12 mm (Fig. 3A), 6-8 mm (Fig. 3B), and 3-5 mm (Fig. 3C) follicles. No RLN3 was detected in the cortical follicles (Fig. 3D). Interestingly, RLN3 was also present in the ooplasm as vesicle-like structures, predominantly in 6-8 mm and 3-5 mm follicles. The receptor RXFP1 was localized in the GC, TH interna, and TH externa of 9-12 mm (Fig. 4A), 6-8mm (Fig. 4B), and 3-5 mm (Fig. 4C) follicles, as well as cortical follicles, but was not localized in the ovarian stroma (Fig. 4D). The receptor RXFP3 was localized in the GC, TH interna, and TH externa of 9-12 mm (Fig. 5A), 6-8mm (Fig. 5B), and 3-5 mm (Fig. 5C) follicles and the ovarian smooth muscle cells (Fig. 5D). RXFP1 was localized to the surface and cytoplasm of cells, whereas RXFP3 was found on the cell surface and in the nucleus.

Gene expression of RLN3, RXFP1, and RXFP3 in follicle compartments of broiler breeders and layers

RLN3 in the GC

The two-way ANOVA analysis revealed no significant interaction between strain (layer vs broiler breeder) and follicle size in the expression of RLN3 mRNA. A significant main effect of follicle size on RLN3 mRNA expression was observed, $F\ (3, 23) = 291.1$, $p < 0.0001$. The RLN3 mRNA expression in 9-12 mm GC follicles was significantly higher in broiler breeders compared to layers ($p = 0.05$). In layers, there was no significant difference in *RLN3* expression between the GC of 9-12 mm follicles and 6-8 mm follicles. Conversely, in broiler breeders, RLN3 mRNA levels were significantly higher in the GC of 9-12 mm follicles compared with the GC of 6-8 mm follicles ($p = 0.05$). In both layers and broiler breeders, GC from 3 to 5 mm and the cortex expressed significantly less RLN3 mRNA compared to the larger follicles (Fig. 6A).

RLN3 in the TH

The two-way ANOVA analysis revealed no significant interaction between strain (layer vs broiler breeder) and follicle size in the expression of RLN3 mRNA. A significant main effect of follicle size on RLN3 mRNA expression was observed, $F\ (2, 16) = 7.110$, $p = 0.0062$. The expression levels of *RLN3* in TH cells of follicles were minimal. However, in the layers group, the *RLN3* expression in TH cells of 6-8 mm

Table 3
Sequence of the gBlock used to determine primer efficiencies.

gBlock	Sequence
RLN3_RXFP1_RXFP3_RPL19	TTCGCCAGCTAGAAGAGGCTGCTCAGGGGGATGGGGGTGGAGCAGGAGTAGCCCCCTTAGGACCCAGCAGTTACCCTTA GCTAGCTGTGGAGTGTGTTTGTGTGCCAAGGAGCGGGAGAGAGAGACCCAGTGCAAAGGAGAGCAGGC GAGGAAGCCAAGGGAGAGAAGGGAGCTTGTTTCATGCA CTGCTGAAGGCTGTCAGAAGC

follicles was significantly higher than that in 3-5 mm follicles ($p = 0.03$) (Fig. 6A).

RXFP1 in the GC

The expression levels of RXFP1 mRNA in the GC of all follicles and the context were low in both the layers and broiler breeders. The two-way ANOVA analysis revealed no significant interaction between strain (layer vs broiler breeder) and follicle size in the expression of RXFP1 mRNA. A significant main effect of follicle size on RXFP1 mRNA expression was observed, F (3, 16) = 64.85 $p < 0.0001$. In both layers and broiler breeders the expression of RXFP1 mRNA was significantly higher in the cortex compared to rest of the follicles ($p < 0.0001$ and $p < 0.0001$, respectively) (Fig. 6B).

RXFP1 in the TH

The expression levels of RXFP1 mRNA in the TH of all follicles and the context were moderate in both the layers and broiler breeders. The two-way ANOVA analysis revealed no significant interaction between strain (layer vs broiler breeder) and follicle size in the expression of RXFP1 mRNA. A significant main effect of follicle size on RXFP1 mRNA expression was observed, F (2, 18) = 1.471, $p = 0.2561$. Within broiler breeders, the mRNA expression of RXFP1 was significantly higher in the 3-5 mm TH compared with the 9-12 mm TH ($p = 0.0420$) (Fig. 6B).

RXFP3 in the GC

The expression levels of RXFP3 mRNA in the GC of all follicles and the context were low in both the layers and broiler breeders. The two-way ANOVA analysis revealed a significant interaction between strain (layer vs broiler breeder) and follicle size in the expression of RXFP3 mRNA, F (3, 22) = 4.257, $p = 0.0163$. A significant main effect of strain was observed, F (1, 22) = 4.817, $p = 0.0390$. The expression of RXFP3 mRNA was significantly higher in the 6-8 mm GC of layers compared with broiler breeders ($p = 0.001$). A significant main effect of follicle size on RXFP3 mRNA expression was observed, F (3, 22) = 126.9, $p < 0.0001$. The expression of RXFP3 mRNA in the cortex was significantly higher than the rest of the follicles in both layers and broiler breeders ($p < 0.0001$ and <0.0001, respectively. (Fig. 6C).

RXFP3 in the TH

The expression levels of RXFP3 mRNA in the TH of all follicles and the context were low in both the layers and broiler breeders. The two-way ANOVA analysis revealed no significant interaction between strain (layer vs broiler breeder) and follicle size in the expression of RXFP3 mRNA. A significant main effect of follicle size on *RXFP3* mRNA expression was observed, F (2, 14) = 13.43, $p = 0.0006$. In layers and broiler breeders the mRNA expression of RXFP3 in TH of 3-5 mm follicles were significantly higher than 9-12mm follicles ($p = 0.003$, and 0.03 respectively). The RXFP3 mRNA expression in the GC of 3-5 mm follicles

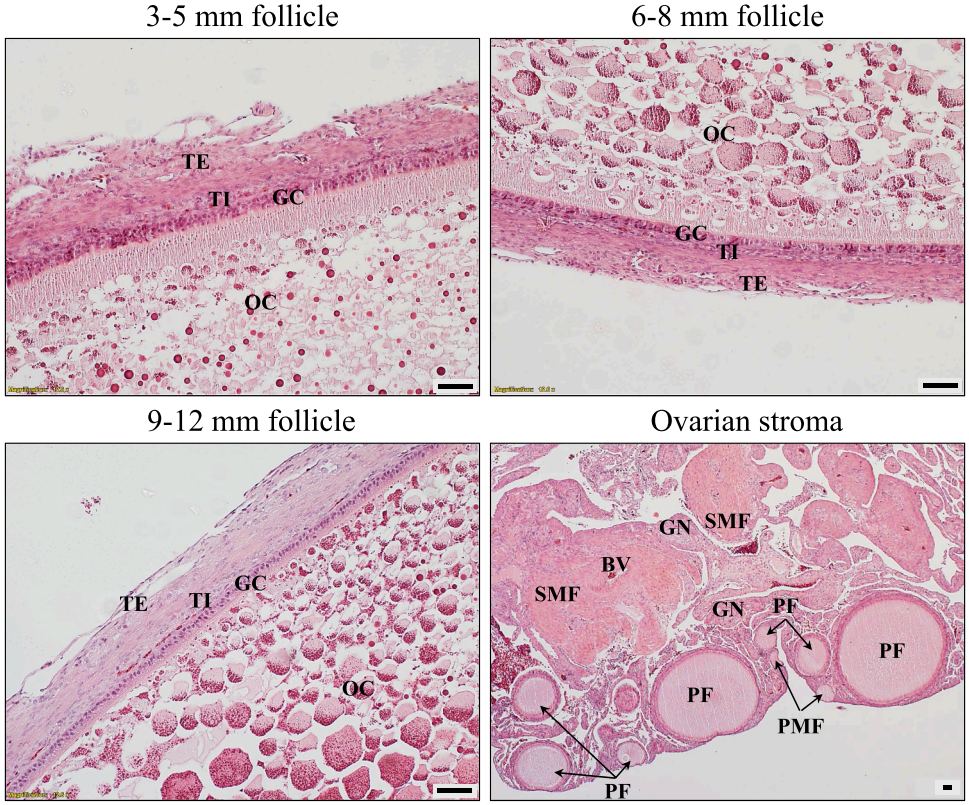

Fig. 1. Histology of chicken ovarian follicles by hematoxylin and eosin (H&E) staining. Close-up of a representative 3-5 mm follicle, 6-8 follicle, 9-12 follicle, and ovarian stroma. Scale bars = 50 μm. Abbreviations: BV – Blood Vessel, GC - Granulosa Cells, GN – Ganglion, OC - Oocyte, PF – Primary Follicle, PMF – Primordial Follicle, SMF – Smooth Muscle Fiber, TE - Theca Externa, TI - Theca Interna.

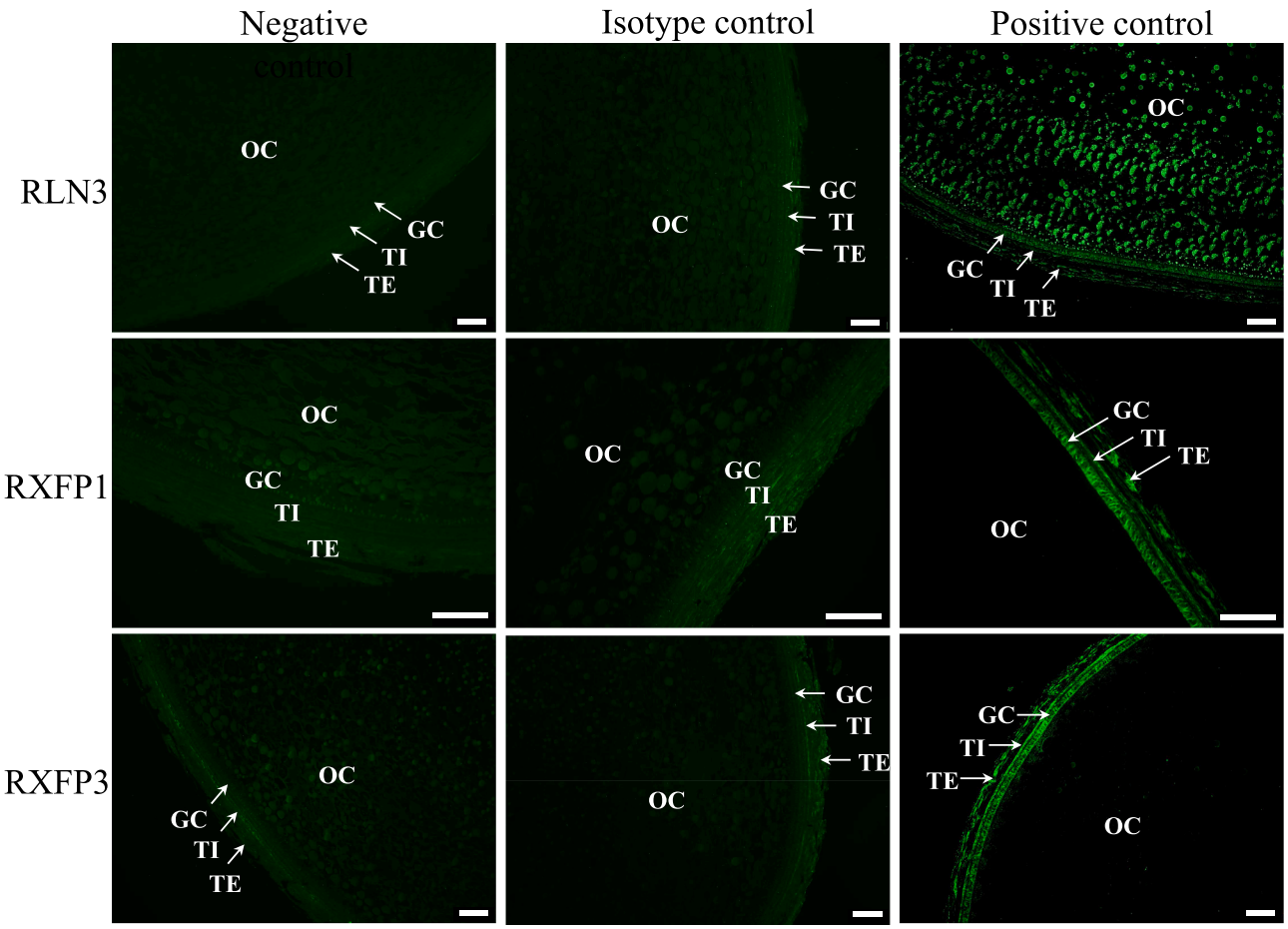

Fig. 2. Antibody validation for immunofluorescence staining. Representative images demonstrate successful immunofluorescence staining using the validated relaxin 3 (RLN3), relaxin family peptide receptor 1 (RXFP1), and relaxin family peptide receptor 3 (RXFP3). Left panel: negative control with the omission of the primary antibodies, showing minimal background fluorescence. Middle panel: isotype control using normal rabbit IgG replacing the primary antibodies. Right panel: Positive staining in 3-5 mm follicles of broiler breeders, confirming antibody specificity. Results are consistent across all follicle sizes. Scale bars = 100 μm. Abbreviations: GC - Granulosa Cells, OC - Oocyte, TE - Theca Externa, TI - Theca Interna.

was significantly higher in layers compared to broiler breeders (p = 0.04). (Fig. 6C).

Gene expression of RLN3, RXFP1, and RXFP3 in pituitary gland and hypothalamus of broiler breeders and layers

RLN3 in pituitary and hypothalamus

The two-way ANOVA analysis revealed no significant interaction between strain (layer vs broiler breeder) and brain compartment in the expression of *RLN3*. A significant main effect of strain was observed, F (1, 12) = 6.219, p = 0.0282. The mRNA expression of RLN3 in the pituitary and hypothalamus tended to be higher in layers compared with broiler breeder (p = 0.1, 0.08, respectively). A significant main effect of brain compartment was also observed, F (1, 12) = 154.1, p < 0.0001, with the RLN3 mRNA expression significantly higher in the pituitary compared with the hypothalamus in layers and broiler breeders (p < 0.0001 and = 0.0001, respectively) (Fig. 7A).

RXFP1 in the pituitary and hypothalamus

The two-way ANOVA analysis revealed no significant interaction between strain (layer vs broiler breeder) and brain compartment in the expression of *RXFP1*. A significant main effect of brain compartment was observed, F (1, 11) = 876.2, p < 0.0001. The RXFP1 mRNA expression was significantly higher in the hypothalamus compared with the pituitary in layers and broiler breeders (p = 0.0011 and = 0.0005, respectively) (Fig. 7B).

RXFP3 in the pituitary and hypothalamus

The hypothalamus had the highest expression of RXFP3 across all tissues (pituitary, cortex, granulosa, and theca). The two-way ANOVA analysis revealed no significant interaction between strain (layer vs broiler breeder) and brain compartment in the expression of *RXFP3*. A significant main effect of brain compartment was observed, F (1, 12) = 107.3, p < 0.0001. The RXFP3 mRNA expression was significantly higher in the hypothalamus compared with the pituitary in layers and broiler breeders (p = 0.0001 and 0.0001, respectively) (Fig. 7C).

Discussion

The aim of the present study was to compare the protein localization and gene expression of RLN3 and its receptors (RXFP1 and RXFP3) in the ovary, pituitary gland, and hypothalamus of layers and broiler breeders. Results confirmed the expression of RLN3 mRNA is highest in the GC of 6–12 mm follicles compared with 3-5 mm and cortical follicles, in both layers and broiler breeders. These findings align with previous gene expression analyses in chickens and Japanese quail (Ghanem and Johnson, 2021; Hoang et al., 2023). Results further demonstrate that RLN3 mRNA expression to be higher in the GC of 9-12 mm follicles of broiler breeders compared with layers. In killifish, RLN3 was demonstrated to regulate steroidogenesis (Wilson et al., 2009), GC of broiler breeders are known to have excessive steroidogenic activity (Weaver and Ramachandran, 2020). Higher expression of *RLN3* in broiler breeders could potentially be mediating this activity. Future studies will

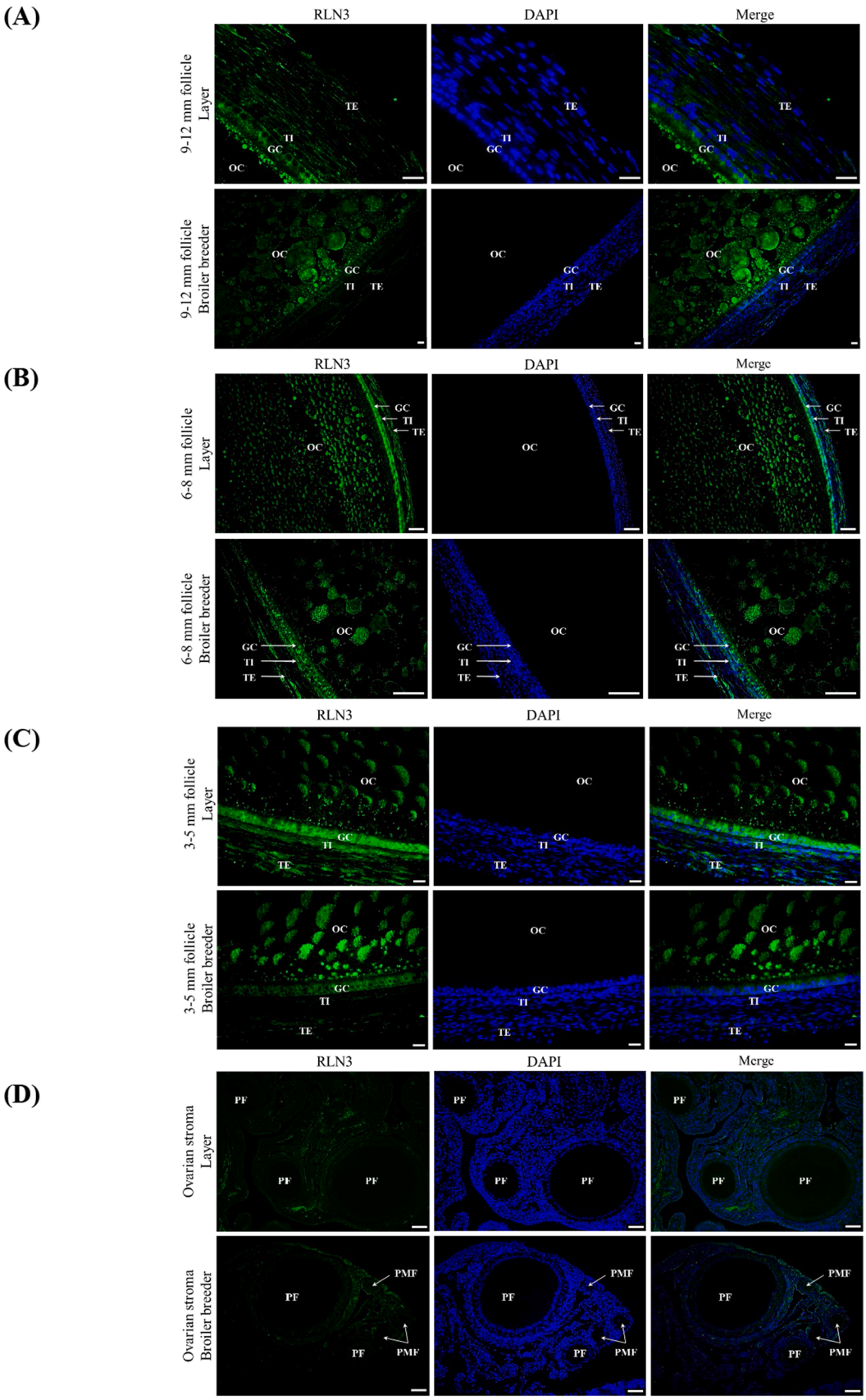

Fig. 3. Immunofluorescence localization of relaxin (RLN3) in chicken ovarian follicles of layers and broiler breeders. (A) 9-12 mm follicles. Scale bars = 20 μm. **(B)** 6-8 mm follicles. Scale bars = 100 μm. **(C)** 3-5 mm follicles. Scale bars = 20 μm. **(D)** Ovarian Stroma. Scale bars = 50 μm. Abbreviations: GC - Granulosa Cells, OC - Oocyte, PF – Primary Follicle, PMF – Primordial Follicle, TE - Theca Externa, TI - Theca Interna.

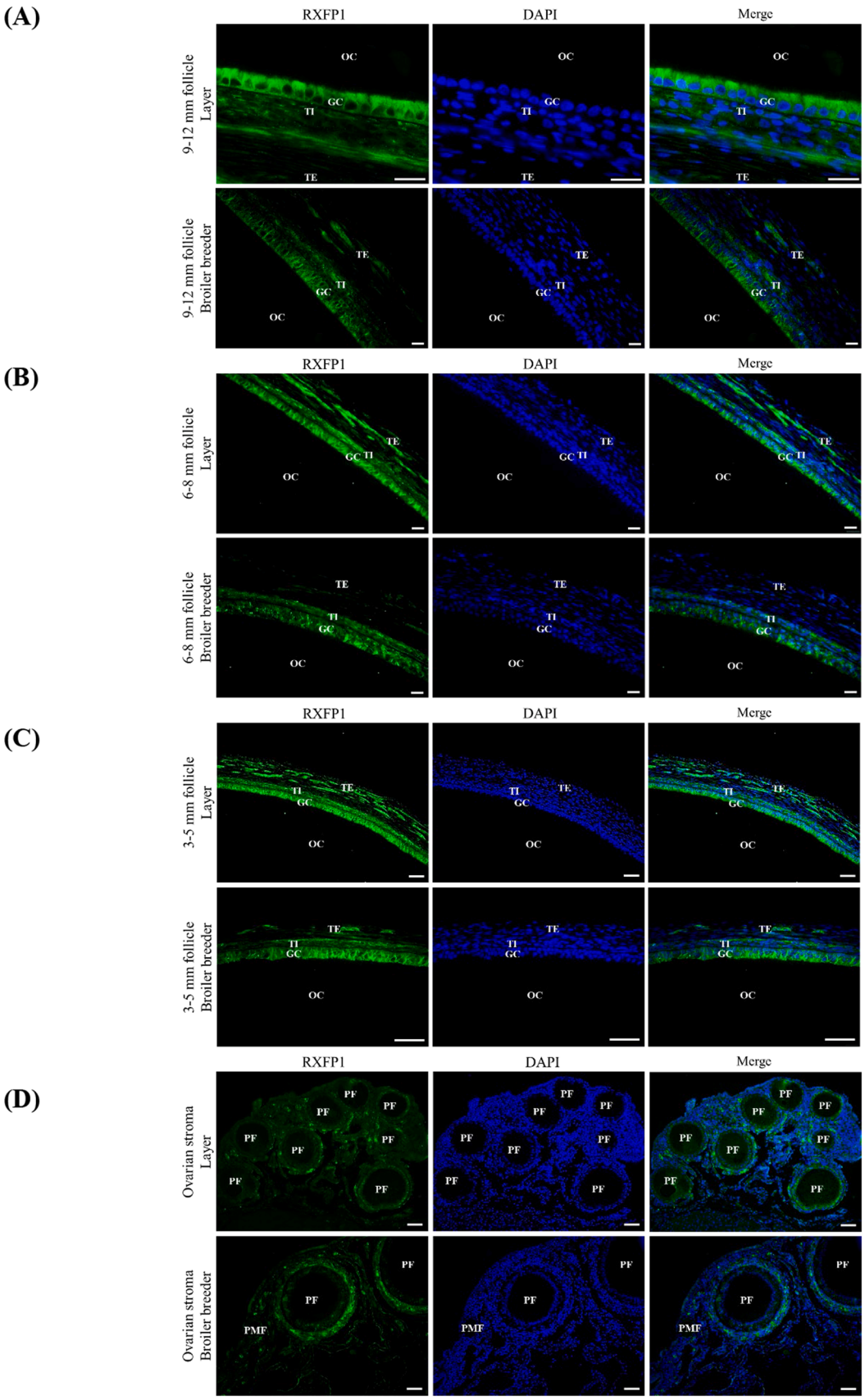

Fig. 4. Immunofluorescence localization of relaxin family peptide receptor 1 (RXFP1) in chicken ovarian follicles of layers and broiler breeders. (A) 9-12 mm follicles. Scale bars = 20 μm. **(B)** 6-8 mm follicles. Scale bars = 20 μm. **(C)** 3-5 mm follicles. Scale bars = 50 μm. **(D)** Ovarian Stroma. Scale bars = 50 μm. Abbreviations: GC - Granulosa Cells, OC - Oocyte, PF – Primary Follicle, PMF – Primordial Follicle, TE - Theca Externa, TI - Theca Interna.

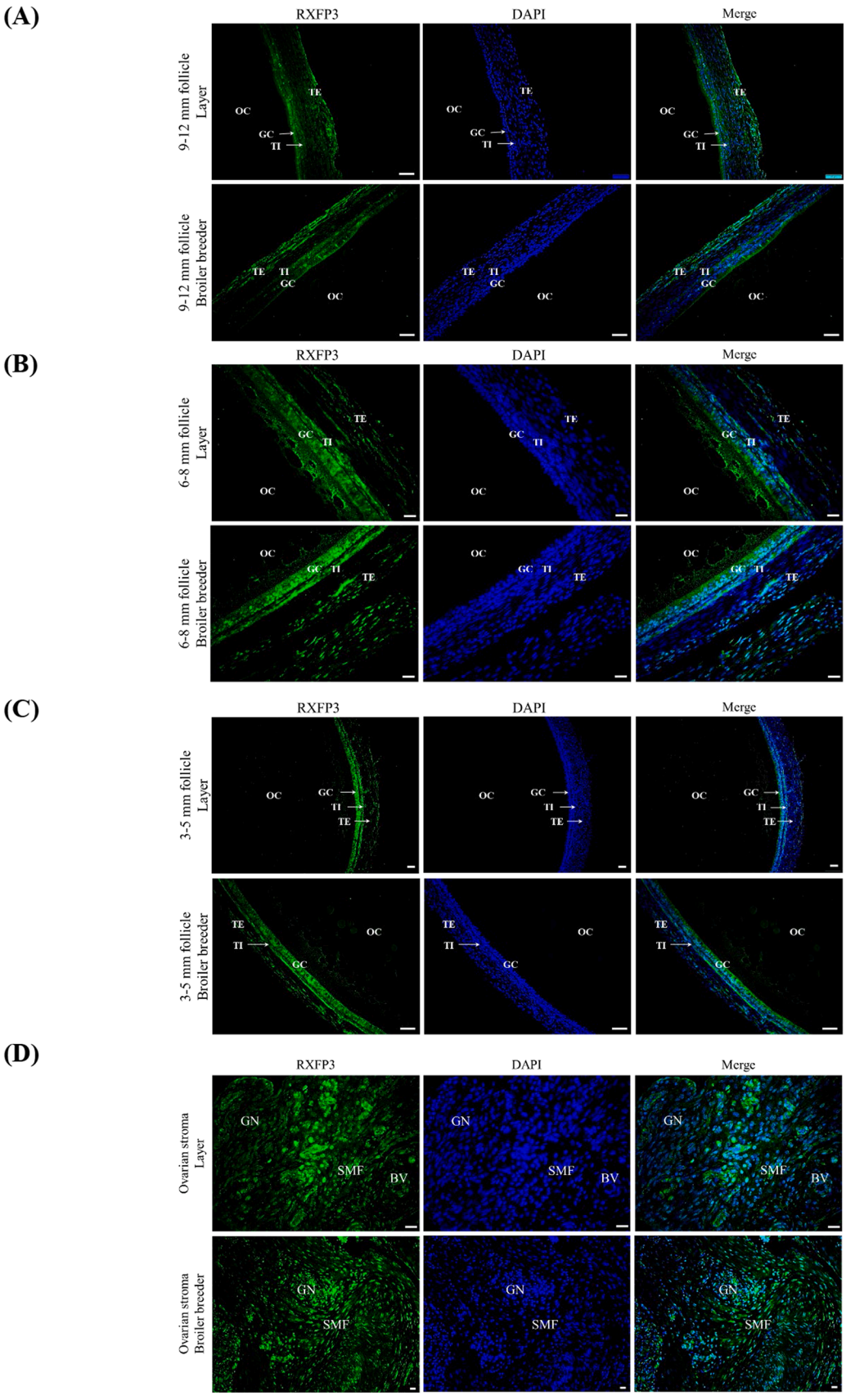

Fig. 5. Immunofluorescence localization of relaxin family peptide receptor 3 (RXFP3) in chicken ovarian follicles of layers and broiler breeders. (A) 9-12 mm follicles. Scale bars = 50 μm. **(B)** 6-8 mm follicles. Scale bars = 20 μm. **(C)** 3-5 mm follicles. Scale bars = 50 μm. **(D)** Ovarian Stroma. Scale bars = 20 μm. Abbreviations: BV – Blood Vessel, GC - Granulosa Cells, GN – Ganglion, OC - Oocyte, PF – Primary Follicle, PMF – Primordial Follicle, SMF – Smooth Muscle Fiber, TE - Theca Externa, TI - Theca Interna.

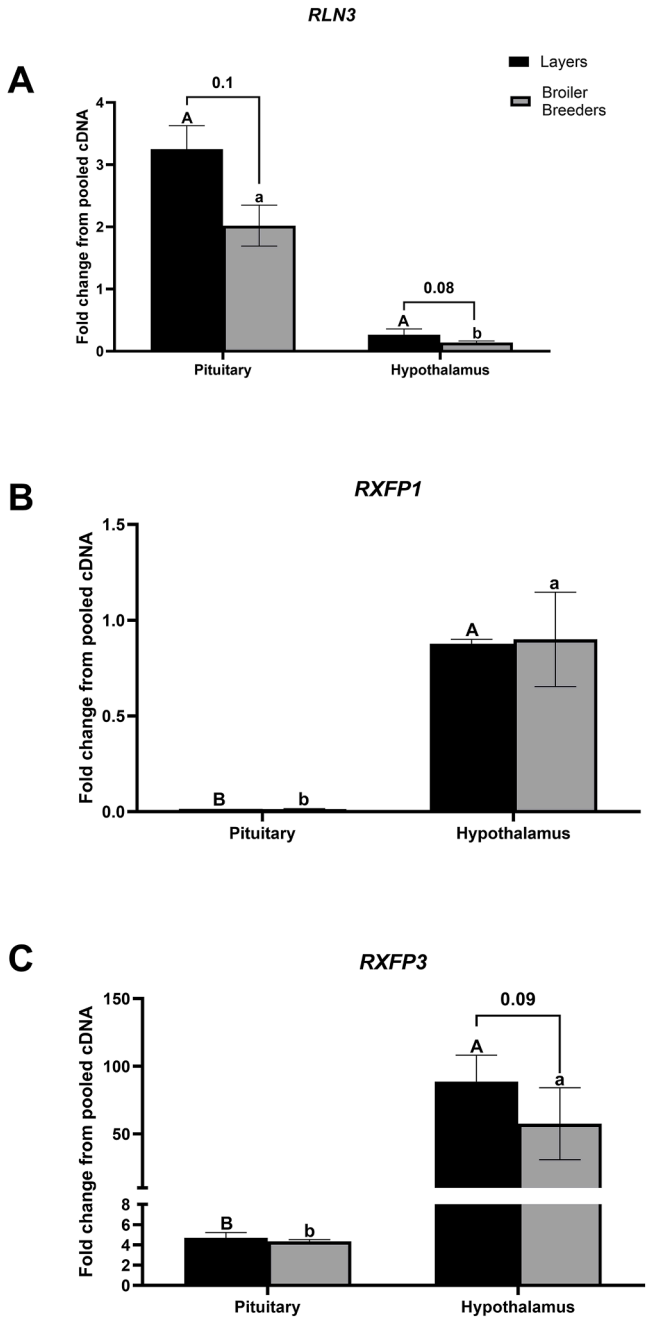

Fig. 6. Comparative mRNA expression of relaxin (RLN3), relaxin family peptide 1 (RXFP1), and relaxin family peptide 3 (RXFP3) mRNA between layers and broiler breeders in ovarian cortex and follicles. (A) RLN3 mRNA expression in granulosa cells (GC) along with the cortex (Left) and theca cells (Right) of the 9-12 mm, 6-8 mm, and 3-5 mm follicles. **(B)** RXFP1 mRNA expression in granulosa along with the cortex (Left) and theca cells (Right). **(C)** RXFP3 mRNA expression in granulosa cells along with the cortex (Right) and theca cells (Left). The data are presented as fold changes relative to pooled cDNA. Values are expressed as mean ± SEM analyzed by two-way ANOVA (n = 4 / hen type / tissue type). Values with different letters are significantly different ($p \leq 0.05$) (Capital letters for layers and lowercase for broiler breeders). Outliers were removed from 3 to 5 mm GC from broiler breeder # 2, 9-12 mm TH from layer # 3-, and 6-8-mm TH from layer # 3.

focus on elucidating this connection.

Immunostaining confirmed RLN3 localization in the GC of 3-12 mm follicles. RLN3 was also localized to the TH interna and externa of all follicles. An intriguing finding of this study was the localization of RLN3 in small vesicles in the ooplasm of 3-5 mm and 6-8 mm follicles, but not obvious in 9-12 mm follicles. This pattern of deposition mirrors yolk deposition in follicles and supports that RLN3 is a secreted protein (Gilbert et al., 1983). In immature pigs, relaxin was detected in the follicular fluid of small and large follicles and was observed to increase with increased follicle size and was speculated to play a role in follicle development (Ohleth et al., 1998). The finding that RLN3 was localized in the ooplasm of large white follicles and small yellow follicles in chickens draws a parallel consistent with the above study. Further investigation to determine the role RLN3 positive ooplasm vesicles in follicle development is needed.

This study also aimed to address a knowledge gap regarding the specific follicular compartments in which RLN3 receptors, RXFP1 and RXFP3, are localized. Immunostaining results showed both receptors localized in the GC, the TH interna, and TH externa of 3-12 mm follicles. This expression pattern suggests that RLN3 is most likely signaling through paracrine and autocrine actions as previously speculated (Wilson et al., 2009; Ghanem and Johnson, 2021). This hypothesis is strengthened by the localization of RLN3 in TH externa and interna, despite the very low levels of mRNA detected in those cells. Suggesting that the RLN3 detected through staining is likely bound to receptors.

Immunostaining also detected RXFP1 in the GC and TH of some but not all cortical follicles. This suggests that RLN3 could be playing a role in follicle development within the cortex through RXFP1 signaling. By contrast, RXFP3 appeared to be expressed mainly in stromal and smooth muscle cells as opposed to cortical follicles, indicating a potential role for RLN3 that may go beyond steroidogenesis. Unexpectedly, RXFP3 appeared to be localized to the nuclei or cell membrane depending on the stage of development of the follicles, cell type within the follicles and cortex. This pattern was consistent across layers and broiler breeders. Only the GC and TH interna of 9-12 mm follicles did not have any RXFP3 localized to the nucleus. There is mounting evidence that in addition to G-protein coupled receptors (GPCRs) signaling from the cell surface, they can signal from intracellular membrane compartments including nuclear membranes (Gobeil et al., 2006; Crilly and Puthenveedu, 2021). Furthermore, nuclear GPCR signaling can trigger a signaling cascade distinct from that of its plasma membrane counterparts (Jong et al., 2009). The localization of RXFP3 to the cell surface of GC and TH interna of 9-12 mm follicles could be indicative that RLN3 may be signaling differently during cyclic recruitment though the RXFP3 receptor.

The mRNA expression of RXFP3 was 5-fold higher in layers compared with broiler breeders in the GC of 6-8mm pre-recruitment follicles. *In vitro* studies have demonstrated that prior to cyclic recruitment, cAMP production by GC of pre-recruitment follicles is actively inhibited (Johnson and Bridgham, 2001; Woods et al., 2005). This in turn, prevents 6-8 mm follicles from prematurely transitioning to the preovulatory stages and disrupting the ovarian hierarchy. Given that binding of RLN3 to RXFP3 inhibits cAMP *in vitro* (Liu et al., 2003), it is possible that RXPF3 signaling could be playing a role in keeping GCs undifferentiated and therefore preventing the premature recruitment of 6-8 mm follicles to the preovulatory stage. The predominant localization of RXFP3 to the nuclei of 6–8 mm GC cells suggests that RXFP3 may play a role in regulating follicular overgrowth observed in broiler breeders through nuclear signaling.

In mammals, RLN3, RXFP1, and RXFP3 are present in the hypothalamic paraventricular nucleus, a region with a well-characterized role in regulating energy balance and modulating reproductive function (McGowan et al., 2008; van der Westhuizen et al., 2008; Bathgate et al., 2013; Patil et al., 2017). To explore a potentially similar role in chickens, the differences in the expression of RLN3 and its receptors in the hypothalamus and pituitary between layers and broiler breeders were compared. Expression of RLN3 mRNA was higher in both the pituitary and the hypothalamus of layers compared with broiler breeders. These results were unexpected, as it was anticipated that broiler breeders, given their higher weight and higher rate of feed intake, would exhibit higher RLN3 expression. A potential explanation for this discrepancy is that the broiler breeders used in this study were restricted fed. Typically,

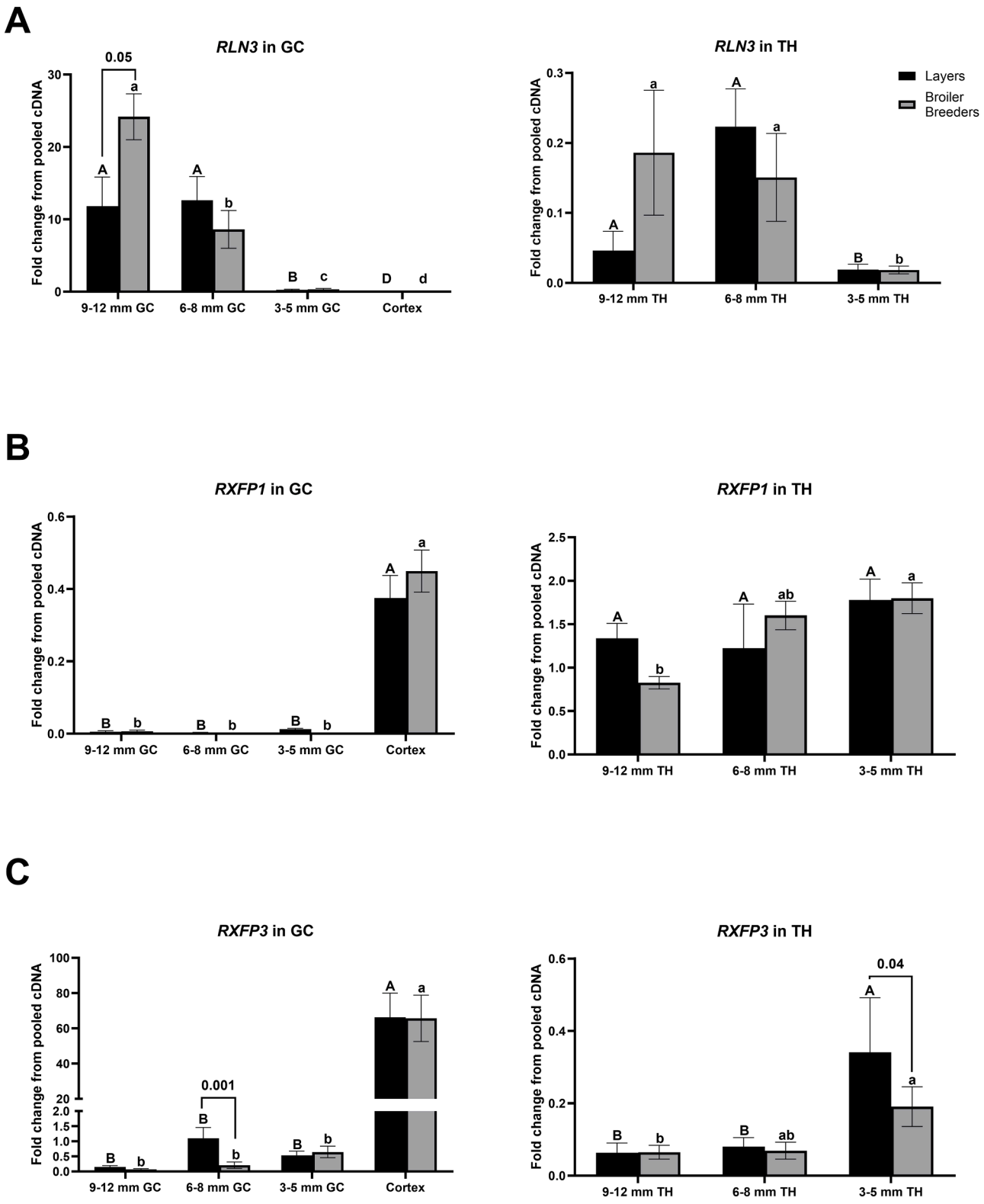

Fig. 7. Comparative mRNA expression of relaxin-3 (RLN3), relaxin family peptide 1 (RXFP1), and relaxin family peptide 3 (RXFP3) in the pituitary and hypothalamus of layer and broiler breeder. (A) RLN3 mRNA expression in the pituitary and the hypothalamus. **(B) RXFP1 mRNA** expression in the pituitary and the hypothalamus **(C)** RXFP3 mRNA expression in the pituitary and the hypothalamus. The data are presented as fold changes relative to pooled cDNA. Values are expressed as mean ± SEM analyzed by two-way ANOVA ($n = 4$ / hen type / tissue type). Values with different letters are significantly different ($p \leq 0.05$).

broiler breeders spend a considerable amount of time fasting between feedings. Restricted feeding causes increased stress and pecking behavior in broiler breeders compared to layers (Renema and Robinson, 2004). Enhanced expression of RLN3 was observed in cultured chicken pituitary cells treated with corticotropin-releasing hormone (CRH) (Lv et al., 2022). Hence, it can be speculated that stress can alter RLN3 expression in chickens. A transcriptional analysis of the hypothalamus of fasted chicks found RLN3 gene was upregulated during fasting and incidentally was involved in a network that plays an important role in feeding and metabolism (Higgins et al., 2010). It remains unclear whether the increased expression of RLN3 is induced by fasting itself or the stress associated with fasting. Furthermore, as the studies primarily focused on RLN3 transcripts, it is essential to validate the corresponding protein changes resulting from fasting before drawing any definitive conclusions.

The mRNA expression of RXFP1 and RXFP3 in the hypothalamus being higher compared with the pituitary, is indicative of a potential feedback mechanism between the hypothalamus and the pituitary. Layers had higher levels of RXFP3 mRNA in the hypothalamus compared with broiler breeders. Indicating that potential differences in RLN3 signaling between layers and broiler breeders could be mediated via RXFP3 signaling. The exact source of RLN3 and RXP3 in the hypothalamus will offer even more insight on its potential role but has yet to be determined.

This study provided new insights into the potential role of RLN3 and its signaling on feeding behavior and reproductive function, highlighting differences between layers and broiler breeders. However, further investigation is necessary to address several limitations, including the protein quantification of RLN3 and its receptors, precise localization of RLN3 and its receptors in the hypothalamus and pituitary, comparisons between restricted feeding and various feeding levels in broiler breeders, and functional studies to explore the physiological impact of RLN3 signaling on reproductive function and feeding behavior. Studies are underway to address these questions.

Conflict of interest

The authors authors declare no conflict of interest.

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Supplementary materials

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