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Tissue-Specific Expression and Laying-Performance Responsiveness of *FXR1 PRL, GnRH2*, and *TFPI2* in Helmeted Guinea Fowl

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Abstract

Tissue-specific gene expression offers crucial insights into the molecular mechanisms underpinning neuroendocrine, reproductive, and metabolic processes in helmeted guinea fowls. However, tissue-to-tissue variations and laying group responses among candidate genes remain poorly understood. This study assessed the expression of four candidate genes Fragile X-related protein 1 (FXR1), gonadotropin releasing hormone 2 (GnRH2), Prolactin (PRL), and Tissue factor pathway inhibitor 2 (TFPI2) across the brain, liver, and ovary under two experimental conditions (T1: high layers; T2: low layers) using quantitative real-time PCR (qPCR) standardised against TATABOX as house-keeping gene. Across tissues and laying groups, the candidate genes exhibited distinct expression patterns. FXR1 demonstrated a significant main effect of tissue, driven primarily by brain versus liver differences. Prolactin (PRL) showed a significant overall group impact ($F = 10.980, P = 0.0041$), making as the most responsive gene to egg-laying performance with decreased expression under T2, particularly in the liver and ovaries. However, GnRH2 showed the largest tissue-associated effect among the candidate genes ($F = 9.931$, $P = 0.0012$), with brain expression considerably different from both liver and ovary while TFPI2 exhibited stable expression with no significant tissue, group or interaction effects. These findings emphasize the importance of tissue context when evaluating reproductive gene-expression patterns, highlighting PRL as a key target for laying

performance-associated reproductive responses. Further research integrating protein levels, circulating hormones, and reproductive phenotypes is necessary.

Keywords: Gene, Expression, Guinea Fowl, Reproductive, Physiology, Tissue.

Introduction

Neuroendocrine regulation, tissue-specific transcriptional activity, and metabolic processes interact intricately to influence poultry reproductive success (Nahashon *et al.,* 2020). Through endocrine and molecular routes, the brain, pituitary, ovary, and peripheral metabolic tissues govern follicular development, ovulation, steroidogenesis, and egg production species (Wadood and Zhang, 2024). Therefore, it is crucial to comprehend the molecular regulation of these processes in order to find potential molecular indicators and explain variation in reproductive function (Mandal *et al.,* 2020).

Investigating the molecular mechanisms behind physiological variations across tissues and experimental circumstances is made possible by gene-expression analysis. As long as normalization, reference-gene selection, experimental design, and statistical analysis are properly documented, quantitative real-time PCR (qPCR) is still one of the most popular methods for targeted transcript quantification (Shen *et al.,* 2021).

Four candidate genes with possibly comparable biological functions FXR1, GnRH2, PRL, and TFPI2 were the focus of this work.

FXR1 is a member of the fragile-X-related family of RNA-binding proteins and plays a role in translational, RNA metabolism, and post-transcriptional regulation. Depending on the physiological needs of a particular tissue, its expression may change (Wang *et al.,* 2023).

FXR1 showed notable tissue-associated variation in the current investigation, with liver expression being substantially different from brain expression. Because hypothalamic GnRH2 regulates ovarian function by stimulating pituitary gonadotropin release, GnRH2 is a key regulator of vertebrate reproduction. GnRH-related expression has also been shown in the chicken ovary, despite the hypothalamus being the primary site of reproductive GnRH2 signaling (Maddineni *et al.,* 2019).

The significance of tissue context when interpreting GnRH expression is supported by prior studies that showed significant changes in GnRH-I expression between the ovary and chicken hypothalamus (Santhana Kumar *et al.,* 2019).

A key endocrine regulator of avian reproductive physiology, PRL is especially linked to broodiness and incubation behavior (Da Silva *et al.,* 2022; Mo *et al.,* 2022). Therefore, alterations in prolactin signaling may have significant effects on laying persistence and reproductive activity. The only potential gene with a statistically significant overall treatment impact, according to the current analysis, was PRL. TFPI2 plays a role in the modulation of extracellular matrix and associated cellular functions. Because TFPI2 has been discovered as a candidate gene involved in egg-laying biology and has been linked to the regulation of GnRH2 release in hypothalamic neuronal cells, recent multi-tissue study on chickens is especially pertinent (Mo *et al.,* 2022). This offers a crucial justification for examining TFPI2 in reproductive organs alongside GnRH and PRL.

Even though these genes are biologically significant, little is known about how they express themselves simultaneously in many tissues under various physiological circumstances in chickens.

Because gene expression in one tissue may not reflect expression or biological activity in another, a multi-tissue approach is very beneficial.

In order to ascertain whether observed variation was primarily attributable to laying-group, tissue identity, or laying-group × tissue interaction, this study set out to characterize the expression profiles of FXR1, GnRH2, PRL, and TFPI2 in guinea fowl brain, liver, and ovary under high-laying and low-laying conditions. The objectives of the study were to: determine the relative expression profiles of FXR1, GnRH2, PRL, and TFPI2 in the brain, liver, and ovary; assess how experimental group affects each candidate gene's expression; ascertain whether candidate-gene expression varies significantly between tissues; test whether laying-group responses vary depending on tissue; and identify candidate genes exhibiting the strongest responsiveness to the experimental group.

MATERIAL AND METHODS

Study area

The Directorate of University Farms Federal University of Agriculture Abeokuta (FUNAAB), situated in Ogun State, Nigeria, served as the study's site.

Ogun is surrounded to the north by the states of Oyo and Osun, to the south by Lagos, to the east by Ondo, and to the west by the Republic of Benin. It features woody savanna in the northwest and is primarily covered by tropical rain forest.

One of Nigeria's fastest-growing states, Ogun State is located in the southwest of the country between latitudes 6.2 0 N and 7.8 0 N and longitudes 3.0 0 and 5.0 0 E east of the Greenwich Meridian. In particular, Abeokuta is located roughly 100 kilometers (62 miles) northwest of Lagos. Geographically, it is situated beside the Ogun River, which has historically offered benefits for irrigation and transportation. The physical coordinates of Abeokuta are roughly 7.15° N latitude and 3.35° E longitude.

Experimental birds

A total of 120 guinea fowl keets were obtained from FUNAAB- Alpha PEARL Hatchery. At one day, the keets were weighed and give wing-tagged for individual identification. To distinguish males from the females, the birds were through their vents at 14 weeks and again at 28 weeks of age, 72 female guinea fowl were selected in individual cages under uniform management and feeding conditions. After 52 weeks, birds were ranked into two egg production groups: high egg production group (laid > 4 eggs per week); and low egg production group (laid < 2 eggs per week), based on the number of eggs laid weekly. Sixteen (16) apparently healthy females were randomly selected for the experiment; 8 birds were selected from each of the two egg-production groups (high-layers and low-layers). Birds were sampled between 52 and 55 weeks of age, corresponding to their active laying period.

Experimental design

A comparative experimental design was adopted for this study. Helmeted guinea fowl (*Numida meleagris*) were reared under an intensive production system and classified into two physiological groups based on their laying status: high-laying and low-laying birds.

Four candidate genes, Prolactin, FXR1, TFPI2 and GnRH2, were evaluated for gene expression studies on three biological tissues such as brain, liver and ovary.

Candidate genes and Primers

FXR1 (fragile-X-related protein 1), GnRH2 (gonadotropin-releasing hormone), Prolactin (PRL) and Tissue Factor Pathway Inhibitor 2 (TFPI2) were studied.

These genes were chosen due to their significance in RNA control, neuroendocrine reproductive regulation, and molecular processes linked to tissue/egg formation.

According to Bustin (2020), specific primers were employed to amplify FXR1 and GnRH2 using Primer-BLAST to cover a 120–250 base pair (bp) region of the chosen genes based on the sequence available on GenBank and documented primer design processes. Table 1 displays specific PCR primer design details for the candidate genes GnRH2, prolactin, TFPI2 and FXR1.

Table 1: Detail Information of Primers for GnRH2, PRL, TFPI2 and FXR1 Candidate Genes

Gene	ID	Primers	Annealing temperature	Expected Amplicon Size
PRL	XM_021399287.1	F5'CAGGGACACGCTTTCTCCAT3' R5'ATGGAGAAAGCGTGTCCCTG3'	54℃	96bps
GnRH2	AY895154.1	F 5' CTTCCACTGTTCGGCCTCAT3' R5'ATGAGGCCGAACAGTGGAAG3'	54℃	242bps
TFPI2	XM_015281937.4	F 5' GTGTGGATCACTGTCTGCCA3' R 5' CACGTCCCAGAAGCATCACT3'	54℃	219 bps
FXR1	110397383	F5'GGTGTTCTCCTCAGGTTGGAG3' R 5' GCTCTGGCTGCCAATTGTTT3'	54℃	143 bps
TATA BOX BP	NM_205103.1	F5'ATCAAGCCAAGAATTGTTCTGC3' R5'CTTCGTAGATTTCTGCTCGAACT3'	60^{O}C	85 bps

Sample Collection and Tissue Preparation

Four samples of guinea fowl hens were chosen at random from each egg-laying group (high and low) after they had been laying for 90 days. They were then put to death by cervical dislocation and tissues from their liver, ovaries, and hypothalamus were harvested. The brain tissue was carefully dissected to include the hypothalamic region responsible for reproductive hormone regulation. Liver samples were excised from the central lobe, while ovarian samples were collected from the active follicular region. Each tissue sample was rinsed with sterile phosphate buffered saline (PBS) to remove excess blood and contaminants. Every sample was kept at -20°C after being immersed in RNA later (Invitrogen, Carlsbad, CA) for the entire night.

RNA Extraction using TRizol Method

Total RNA was extracted using the TRIzol method. Approximately 50 mg of tissue was crushed and homogenized in 1 ml of ice-cold TRIzol reagent. The homogenate was incubated at room temperature for approximately five minutes to allow complete dissociation of nucleoprotein complexes.

Chloroform was added, and the samples were mixed thoroughly and centrifuged at approximately 12,000 × g using the micro-centrifuge shown in plate 1. The aqueous phase containing RNA was transferred to a new RNase-free tube. RNA was precipitated with ice-cold isopropanol, incubated at 4°C, and centrifuged to obtain an RNA pellet.

The pellet was washed with 75% ethanol, centrifuged, briefly air-dried, and dissolved in RNase-free water. The Q-bit 2.0 Fluorometer was used to measure the RNA purity and concentration. Samples with an A_{260}/A_{280} ratio of approximately 1.8–2.0 were considered suitable for downstream analysis.

Complementary DNA (cDNA) Synthesis using Luna-Script RT Super Mix

First-strand cDNA was synthesized from total RNA extracted from brain, liver and ovary tissues using Luna Script® RT Super Mix (New England Biolabs, USA). The reverse-transcription reaction was performed in a final volume of 20 µL containing 4 µL of 5X Luna Script RT Super Mix and the appropriate amount of RNA template, with nuclease-free water added to obtain the final reaction volume. The Luna Script RT Super Mix contains random hexamer and oligo-dT primers, dNTPs, reverse transcriptase and RNase inhibitor.

The reverse-transcription reactions were incubated at 25°C for 2 min for primer annealing, followed by 55°C for 10 min for cDNA synthesis and 95°C for 1 min for reverse transcriptase inactivation. The resulting cDNA was subsequently used as template for two-step RT-qPCR amplification of GnRH2, FXR1 and TFPI2 using the Luna Universal qPCR Master Mix.

The synthesized cDNA samples were stored at −20°C until use for quantitative real-time PCR analysis.

Quantitative Real-Time PCR Amplification of Prolactin, GnRH2, FXR1 and TFPI2

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to determine the expression levels of prolactin, gonadotropin-releasing hormone 2 (GnRH2), fragile X-related protein 1 (FXR1), and tissue factor pathway inhibitor 2 (TFPI2) in the brain, liver and ovary tissues of helmeted guinea fowl. Gene-specific primers were used to amplify the target transcripts. The primer sequences, expected amplicon sizes and other primer characteristics are presented in Table 1.

The qRT-PCR reactions were performed using Luna® Universal qPCR Master Mix (New England Biolabs, USA; M3003), a dye-based master mix containing the components required for DNA amplification and fluorescence-based quantification, except primers and template. The reactions were prepared in a final volume of 20 µL containing 10 µL of 2× Luna Universal qPCR Master Mix, 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, an appropriate volume of cDNA template, and nuclease-free water to make up the final reaction volume. The final concentration of each primer was 0.25 µM. The manufacturer's recommended reaction conditions were followed.

The qRT-PCR was performed using a real-time PCR instrument programmed for an initial denaturation at 95°C for 60 s, followed by 40–45 cycles of denaturation at 95°C for 15 s and extension at 60°C for 30 s, with fluorescence acquisition at the end of the extension step. A melt-curve analysis was subsequently performed from 60°C to 95°C to assess amplification specificity.

Each target gene and reference gene was analyzed using appropriate technical replicates. No-template controls (NTCs) were included to monitor contamination, while no-reverse-transcription controls were used where appropriate to assess possible genomic DNA contamination. Amplification curves and melt-curve profiles were examined to confirm successful and specific amplification of the target genes.

The quantification cycle (Ct) values obtained for prolactin, GnRH2, FXR1 and TFPI2 were normalised using TATABOX as the endogenous reference gene.

RNA Expression Analysis and qPCR

Relative transcript abundance was calculated using the $2^{-\Delta\Delta Ct}$ method. ΔCt values were calculated from the difference between target-gene and reference-gene cycle thresholds.

High-laying group was designated as the calibrator condition.

$\Delta Ct = Ct(target) - Ct(reference)$

$\Delta\Delta Ct = \Delta Ct(low\text{-}layers) - \Delta Ct(high\text{-}layers)$

Relative expression $= 2^{-\Delta\Delta Ct}$

The statistical analysis was conducted using ΔCt values, while fold changes were presented as transformed measures of relative expression.

Quality Control and Outlier Handling

Extreme observations were screened using an interquartile-range criterion before statistical inference. The purpose was to reduce the influence of extreme observations on estimates of treatment and tissue effects.

Statistical Analysis

Two-way factorial ANOVA was performed separately for each gene using:

ΔCt = Laying group + tissue + laying group × tissue + error

The model evaluated the main effect of laying group (high layer vs low layer); the main effect of tissue; and the laying group × tissue interaction.

Where a significant tissue effect was detected, Tukey's HSD test was used for post-hoc comparisons. Within each tissue, Welch's two-sample t-test was used to compare high layers and low layers. Benjamini–Hochberg correction was applied to the 12 tissues × gene comparisons to control the false discovery rate. Statistical significance was evaluated at $P < 0.05$.

RESULTS

Relative Expression Profiles

Table 1, Figure 1 and Figure 2 revealed the observed differences in relative expression among genes, tissues and treatments. For FXR1, the low-layer/high-layer fold-change estimates were approximately 2.58-fold in brain, 16.29-fold in liver and 5.61-fold in ovary. GnRH2 showed approximately 3.05-fold relative expression in brain, while its relative expression was lower in liver and ovary. PRL showed a markedly different pattern. The calculated fold changes were approximately 0.79-fold in brain, 0.009-fold in liver and 0.004-fold in ovary, indicating substantial decreases in relative expression under low-layer, particularly in peripheral tissues. TFPI2 showed approximately 1.24-fold, 0.058-fold and 0.53-fold relative expression in brain, liver and ovary, respectively.

Table 2: Relative Expression Changes from High to Low-layers

Tissue	Target Gene	$\Delta C_T T_1$	$\Delta\Delta C_T$ (T2-T1)	(T2-T1)	Fold change ($2^{-\Delta\Delta CT}$)	Raw P-value	Adjusted P.adj., BH	LS
Brain								
	FXR1	10.69	9.32	-1.37	2.58	0.63	0.85	Ns
	GnRH2	2.49	0.88	-1.61	3.05	0.06	0.28	Ns
	PRL	0.02	0.35	0.33	0.79	0.83	0.91	Ns
	TFPI2	6.22	5.91	-0.31	1.24	0.93	0.93	Ns
Liver								
	FXR1	5.84	1.82	4.03	16.29	0.09	0.28	Ns
	GnRH2	-2.24	-0.40	1.84	0.28	0.36	0.72	Ns
	PRL	-3.45	3.42	6.87	0.01	0.08	0.28	Ns
	TFPI2	1.50	5.62	4.12	0.05	0.27	0.65	Ns
Ovary								
	FXR1	8.85	6.36	-2.49	5.61	0.54	0.84	Ns
	GnRH2	-3.01	-2.13	0.88	0.54	0.56	0.84	Ns
	PRL	-4.69	3.14	7.83	0.00	0.03	0.28	Ns

Tissue	Target Gene	$\Delta C_T T_1$	$\Delta\Delta C_T$ (T2-T1)	(T2-T1)	Fold change ($2^{-\Delta\Delta CT}$)	Raw P-value	Adjusted P.adj., BH	LS
	TFPI2	3.02	3.95	0.93	0.53	0.84	0.91	Ns

CT= Cycle threshold, T2 and T1 are low and high layer respectively, Δ=change, BH=Benjamini-Hochberg, LS =level of significance.

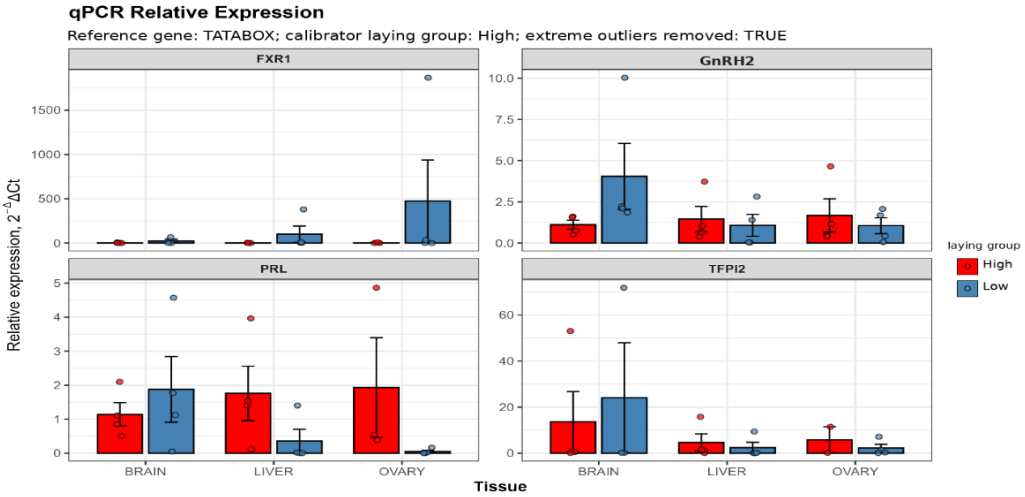


Figure 1: qPCR Relative Expression, $2^{-\Delta\Delta Ct}$

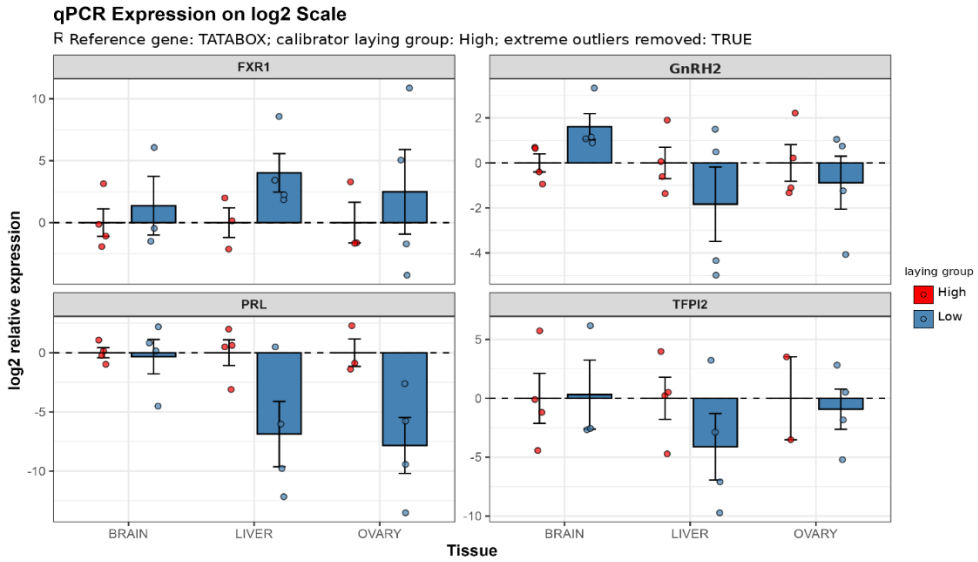


Figure 2: qPCR Expression on Log2

Two-way ANOVA

Table 2 depict the variation in gene expression across laying group, tissues and laying group x tissue interaction effects. The factorial analysis demonstrated different regulatory patterns among the four genes as shown below:

FXR1 Expression

There was a substantial tissue-associated variance in FXR1 (P = 0.0357). The direction of the estimated expression change showed increased expression under low-layer in all three tissues; however, experimental group alone did not meet the standard 5% significance threshold (P= 0.0912).

The distinction between the brain and liver was the main cause of the tissue effect. A significant comparison between the liver and the brain was found using Tukey's HSD (adjusted P = 0.0311). The brain and liver did not significantly differ from the ovary.

The results imply that the experimental group had less of an impact on FXR1 transcriptional variation than tissue identity.

GnRH2 expression

Of the genes analyzed, GnRH2 showed the strongest tissue-specific pattern. Laying group (P = 0.6507) and laying group × tissue interaction (P = 0.2203) was not significant, whereas the tissue effect was highly significant (P= 0.0012). While there was no substantial difference between liver and ovary, brain expression differed significantly from both.

The brain and hypothalamus play a key role in GnRH-mediated reproductive regulation, which is scientifically compatible with this pattern. However, since the study measured transcript abundance rather than GnRH production or circulating hormone levels, the current results should be taken as tissue-expression variations rather than direct proof of endocrine function.

PRL Expression

Compared to the other potential genes, PRL showed a different response pattern. The effect of laying group was statistically significant (F = 10.980, P = 0.0041). The relationship between tissue and laying group was not significant.

In terms of description, PRL expression significantly dropped under low-layer, especially in the liver and ovary. The estimated fold-changes for the liver and ovary were roughly 0.009 and 0.004, respectively. PRL was the most treatment-responsive candidate gene in the current investigation, according to the findings.

Nevertheless, after Benjamini–Hochberg adjustment, the tissue-specific Welch comparisons lost their significance. Therefore, rather than asserting statistically verified pairwise differences in each individual tissue, the proper interpretation is that low-layer was linked to an overall decrease in PRL expression.

TFPI2 Expression

TFPI2 did not exhibit a significant tissue effect (P = 0.4410), laying-group × tissue interaction (P = 0.6250), or treatment effect (P= 0.4850). However, significant variance was found across individual samples, especially in the assessments of relative expression.

Although the very small sample size and high biological variation restrict the robustness of this conclusion, the lack of statistical significance indicates that TFPI2 was rather stable under the experimental settings tested.

Multiple-comparison Analysis

For every gene in every tissue, Welch's t-tests were run. A number of raw P-values, such as brain GnRH2 (P = 0.0667), liver FXR1 (P = 0.0951), liver PRL (P = 0.0829), and ovary PRL (P = 0.0374), approached or exceeded the standard significance level.

Nevertheless, none of the 12 comparisons were still statistically significant at $P < 0.05$ following Benjamini–Hochberg adjustment.

Table 3: Variation in Gene Expression by Laying-group, Tissue and their Interaction

Target Gene	Parameters	F-value	P-value	Significance
FXR1				
	Laying-group	3.25	0.09	Ns
	Tissue	4.19	0.03	*
	Laying-group x Tissue (Interaction)	0.19	0.82	Ns
GnRH2				
	Laying-group	0.21	0.65	Ns
	Tissue	9.93	0.001	**
	Laying-group x Tissue (Interaction)	1.64	0.22	Ns
Prolactin				
	Laying-group	10.98	0.001	**
	Tissue	0.09	0.91	Ns
	Laying-group x Tissue (Interaction)	2.63	0.10	Ns
TFPI2				
	Laying-group	0.51	0.48	Ns
	Tissue	0.86	0.44	Ns
	Laying group x Tissue (Interaction)	0.48	0.62	Ns

FXR1 = Fragile X-Related Protein 1, GnRH2=Gonadotropin Releasing Hormone 2, TFPI2=Tissue Factor Pathway Inhibitor 2, * = P<0.05, ** = P<0.01, Ns =Not significant.

DISCUSSION

The present study demonstrated clear differences in the expression patterns of FXR1, GnRH2, PRL and TFPI2 across tissues and between high- and low-layer groups. Although not all observed differences were statistically significant after correction for multiple testing, the magnitude and direction of the fold changes provide useful biological information about the possible involvement of these genes in reproductive regulation.

One of the primary outcomes of this study was that tissue identity greatly altered expression of FXR1 and GnRH2 which corresponds with thee report of Chen *et al.* (2019) This finding demonstrates the importance of tissue context when evaluating candidate genes involved in reproductive physiology. FXR1 revealed significant tissue diversity, with liver expression different from brain expression. Tissue-dependent FXR1 regulation may reflect variations in cellular RNA-processing requirements because the liver is a highly metabolically active organ with considerable transcriptional and post-transcriptional activity (Chen *et al.,* 2021). Recent research has demonstrated that FXR1 participates in mRNA regulation, translation and the organisation of cytoplasmic messenger ribonucleoprotein complexes. George *et al.* (2021), for example, demonstrated that FXR1 can bind regulatory regions of mRNA and influence translation by interacting with components of the translation-initiation machinery. More recent work by Chen *et al*. (2024) further demonstrated that FXR1 can form an mRNA–protein network that functions as a signalling scaffold. This suggests that FXR1 is not simply a passive RNA-binding protein but can participate in the organisation of cellular signalling processes.

Consequently, the significant tissue effect observed in the guinea fowl may indicate that FXR1 has different regulatory functions depending on the physiological characteristics of the tissue. The especially high numerical increase observed in the liver could be related to the metabolic function of that tissue, although this interpretation remains hypothetical because the present experiment did not directly measure FXR1 target transcripts, protein abundance or metabolic pathways.

Zhang *et al.* (2026) reported that FXR1 expression peaked around the primordial follicle stage in their mouse model.

However, this evidence should not be directly equated with an effect on egg production in guinea fowl. The present study only demonstrates differences in FXR1 transcript abundance, not causality. Therefore, the appropriate conclusion is that the strong tissue effect suggests tissue-specific regulation of FXR1, with the liver showing particularly marked numerical variation between laying groups.

The tissue effect of GnRH2 was significantly more pronounced. The expression of the brain differed greatly from that of the liver and ovary. This outcome is in line with GnRH's acknowledged pivotal function in the hypothalamic control of reproduction. GnRH-related expression is not consistent throughout reproductive tissues, as evidenced by earlier studies on chickens that showed variations in GnRH-I expression between hypothalamus and ovarian tissues (Millar *et al*., 2022; Qin *et al*., 2021).

This finding is biologically important because GnRH is central to neuroendocrine regulation of reproduction. The strong brain-specific expression observed here is consistent with the expected involvement of GnRH-related signalling in the neural control of reproduction.

The present results also agree conceptually with recent work demonstrating an important reproductive function for GnRH2 in vertebrates. Marvel *et al.* (2021) demonstrated that GnRH2 is involved in reproductive regulation in zebrafish, including effects on gonadotropin production, gametogenesis and reproductive output. Their study showed that alterations in GnRH2 activity were associated with changes in reproductive performance under nutritional stress.

Although the zebrafish model is different from guinea fowl, the study supports the broader biological interpretation that GnRH2 can participate in the neuroendocrine regulation of reproductive activity.

The strong tissue effect in the present study therefore suggests that GnRH2 expression is primarily associated with neural/reproductive regulatory tissues rather than being uniformly expressed across peripheral tissues. The low expression observed in liver and ovary may indicate that these tissues are not the principal sites of GnRH2 transcript abundance in the experimental animals.

Importantly, the absence of a significant treatment effect means that the present results do not demonstrate that GnRH2 expression differs significantly between high and low layers. Rather, they demonstrate that GnRH2 expression differs strongly according to tissue. This distinction is particularly important because it prevents overinterpretation of the 3.05-fold numerical difference.

The results are also consistent with the concept that egg production involves coordinated signalling through the hypothalamic-pituitary-ovarian axis. Wang *et al*. (2024) demonstrated that reproductive traits in chickens involve interactions between hypothalamic signalling, pituitary gonadotropin regulation and ovarian processes. They also identified GnRH-related regulatory pathways among the molecular systems associated with laying performance.

Thus, the present finding of significantly greater GnRH2 expression in brain provides molecular evidence that the neural component of reproductive regulation is strongly tissue-dependent in guinea fowl. Nevertheless, because circulating GnRH, LH and FSH were not measured, the result should not be interpreted as direct evidence that increased GnRH2 transcript abundance caused differences in egg production.

For PRL, the most notable treatment-related finding was noted. Prolactin showed a statistically significant main effect of laying-group (P = 0.0041), in contrast to FXR1, GnRH2, and TFPI2. Under low-layers, the expression change was primarily downward, with reductions in the liver and ovary being especially noticeable.

Prolactin has long been recognized as a crucial component of avian reproductive physiology, particularly in relation to broodiness and incubation behaviour. Recent work on Nigerian indigenous chickens has continued to show the importance of PRL in relation to broodiness and egg-production characteristics, demonstrating the continuing relevance of PRL as a candidate gene in poultry production biology (Babalola *et al*., 2025). Therefore, the current findings suggest that the experimental group changed reproductive endocrine control via a mechanism linked to PRL.

Nevertheless, the molecular evidence by itself cannot indicate that better reproductive performance resulted from decreased PRL transcription. Integration with phenotypic egg production measures, circulating prolactin concentrations, PRL receptor expression, and/or downstream reproductive pathways would be necessary for such a causal interpretation.

A more expansive interpretation in which intrinsic tissue physiology and laying performance responsiveness constitute somewhat different sources of transcriptional variation is further supported by the lack of interaction effects (Li *et al.,* 2021).

TFPI2 displayed a different pattern from the other genes. Expression increased slightly in the brain but decreased substantially in liver and ovary. Nevertheless, the two-way ANOVA demonstrated no significant laying group effect or laying group × tissue interaction.

At first sight, the absence of statistical significance might suggest that TFPI2 has little relevance to reproductive performance. However, a particularly important recent study by Wang et al. (2024) identified TFPI2 as a hub candidate gene associated with egg-laying performance in chickens. Their multi-omics analysis showed that TFPI2 was associated with egg production and, importantly, functional experiments indicated that TFPI2 could promote GnRH secretion in chicken hypothalamic neurons (Zhang *et al.,* 2022; Liu *et al.,* 2023). High-yield chickens also showed greater hypothalamic TFPI2 expression than low-yield chickens at several laying stages.

This finding provides an interesting comparison with the present guinea-fowl results. In the current study, TFPI2 did not significantly differ between treatments, despite considerable numerical variation among tissues. The difference between the two studies could be explained by species, age, laying stage, tissue sampled, environmental conditions, genetic background or sample size.

Furthermore, Li *et al.* (2025) demonstrated that TFPI2 is widely expressed in chicken ovarian follicles, particularly in granulosa cells, and that its expression can be regulated by FSH and LH. They also found that TFPI2 influenced granulosa-cell proliferation, steroid hormone synthesis and matrix metalloproteinase-related pathways.

These findings strengthen the biological rationale for including TFPI2 in the present study. However, the lack of significant expression differences indicates that TFPI2 should be considered a potentially important reproductive regulatory gene rather than a gene shown by this experiment to be differentially expressed between high and low layers.

CONCLUSIONS

Overall, the findings indicate that FXR1 and GnRH2 were predominantly influenced by tissue identity, PRL was the most laying performance-responsive gene, while TFPI2 remained statistically stable across the experimental conditions. The significant brain-specific expression of GnRH2 supports its potential involvement in neuroendocrine reproductive regulation, whereas the significant tissue effect of FXR1

suggests context-dependent regulation. The marked reduction in PRL in low-layers indicates an association between PRL transcription and laying status.

Recommendations for Further Research

Future studies should:

i. assess the levels of circulating prolactin and PRL expression; and examine downstream signaling pathways and the PRL receptor;
ii. investigate the relationship between candidate gene expression and characteristics related to egg production and quality;
iii. examine if metabolic and reproductive characteristics are linked to FXR1 expression; and look into the connection between GnRH2 regulation and TFPI2;
iv. include measures of hormone and/or protein levels; and
v. find potential biomarkers of reproductive performance, integrate multi-tissue molecular and phenotypic datasets.

Data Availability Statement

The underlying qPCR data were generated as part of the present research project. Subject to institutional and ethical restrictions, the dataset will be made available from the corresponding author upon reasonable request.

Ethics Statement

The study was approved by the Animal Research Ethics and Welfare Committee (AEWC) of the College of Animal Science and Livestock Production (COLANIM) of the Federal University of Agriculture (FUNAAB). The animals were handled strictly in accordance with the committee's guidelines, and their approved number was FUNAAB/COLANIM/AEWC 2025/10/001.

Conflict of Interest

The authors declare that there is no competing interest.

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