Orexin gene expression analysis in two Nigerian indigenous and exotic chickens using quantitative polymerase chain reaction (qPCR)

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Abstract

This study investigated orexin gene expression patterns in two Nigerian indigenous chicken ecotypes (Fulani and Yoruba) compared to the exotic Cobb-500 breed using quantitative polymerase chain reaction (qPCR) technology. A total of 135 birds (45 per breed) were reared for four weeks, after which liver tissue samples were collected for RNA extraction and analysis. The orexin gene serves as a crucial regulator of appetite, energy balance, and stress responses in poultry, making it an important molecular marker for understanding breed-specific physiological adaptations. RNA was extracted using the Zymo RNA mini prep kit, followed by cDNA synthesis and qPCR analysis using Luna® Universal qPCR Mastermix. The TATA box binding protein served as the housekeeping gene for normalization. Gene expression was quantified using the $2^{-\Delta\Delta C}T$ method (Livak method) to determine fold changes between breeds. Results revealed significant inter-breed variations in orexin expression levels ($P < 0.05$). The Fulani ecotype demonstrated the highest expression (1.37-fold), followed by Cobb-500 broilers (0.35-fold), while Yoruba ecotype chickens showed the lowest expression (0.02-fold). Melt curve analysis confirmed primer specificity and amplification consistency across all samples. These findings suggest that elevated orexin expression in Fulani chickens may reflect superior physiological adaptability and energy regulation capabilities, supporting their resilience in variable environmental conditions. The differential expression patterns highlight orexin's potential as a molecular marker for selective breeding programs aimed at improving indigenous chicken productivity while maintaining genetic diversity and environmental adaptability in Nigerian poultry systems.

Keywords: orexin, gene, qPCR, expression, analysis.

Análise da expressão do gene da orexina em duas galinhas nativas nigerianas e em uma exótica utilizando reação em cadeia da polimerase quantitativa (qPCR)

Resumo

Este estudo investigou os padrões de expressão do gene orexina em dois ecótipos de galinhas nativas da Nigéria (Fulani e Yoruba) em comparação com a raça exótica Cobb-500, utilizando a tecnologia de reação em cadeia da polimerase quantitativa (qPCR). Um total de 135 aves (45 por raça) foi criado por quatro semanas, após as quais amostras de tecido hepático foram coletadas para extração e análise de RNA. O gene da orexina atua como um importante regulador do apetite, equilíbrio energético e respostas ao estresse em aves, tornando-se um marcador molecular essencial para compreender adaptações fisiológicas específicas de cada raça. O RNA foi extraído utilizando o kit Zymo RNA Mini Prep, seguido pela síntese de cDNA e análise por qPCR com o reagente Luna® Universal qPCR Mastermix. O gene TATA box binding protein foi utilizado como gene de referência para normalização. A quantificação da expressão gênica foi realizada pelo método $2^{-\Delta\Delta C}T$ (método de Livak) para determinar as variações relativas entre as raças. Os resultados revelaram diferenças significativas entre as raças nos níveis de expressão do gene da orexina ($P < 0,05$). O ecótipo Fulani apresentou a maior expressão (1,37 vezes), seguido pelos frangos Cobb-500 (0,35 vezes), enquanto o ecótipo Yoruba mostrou a menor expressão (0,02 vezes). A análise da curva de dissociação confirmou a especificidade dos primers e a consistência da

amplificação em todas as amostras. Esses achados sugerem que a elevada expressão da orexina nas galinhas Fulani pode refletir uma maior adaptabilidade fisiológica e capacidade de regulação energética, apoiando sua resiliência a condições ambientais variáveis. Os padrões diferenciais de expressão destacam o potencial da orexina como marcador molecular para programas de melhoramento seletivo voltados ao aumento da produtividade de galinhas nativas, mantendo a diversidade genética e a adaptabilidade ambiental nos sistemas avícolas nigerianos.

Palavras-chave: orexina, gene, qPCR, expressão, análise.

1. Introduction

Poultry farming holds a significant position in the agricultural landscape, providing a vital source of nutrition and livelihood for millions of people (Ajala et al., 2020). The poultry agro-business in Nigeria is the most commercialized among all agricultural sub-sectors and has contributed massively to Nigeria's economy (Adene and Oguntade, 2006). Poultry farming in Nigeria accounts for over 180 million birds yearly and yields an annual production of approximately 454 billion tonnes of meat and 3.8 million eggs (FAO, 2018). Among the diverse poultry population in Nigeria, indigenous chickens constitute a significant majority, approximately 80 percent of the chicken population in rural Nigeria (FAO, 2018b).

The Fulani and Yoruba chickens represent the most popular and distinct breeds of Nigerian indigenous chickens, each with unique characteristics deeply rooted in the country's cultural and agricultural heritage. Understanding the physiological factors and mechanisms of these chickens is of utmost importance to animal nutrition and biotechnology. The Fulani chickens originate from the tough and weathered regions of Nigeria. The Fulani people who raise them live in small groups and maintain genetic isolation, which prevents interbreeding with other native chickens (Oyeniran et al., 2022). The Yoruba ecotype has drawn attention due to its potential as an egg-type chicken, attributed to its distinctive morphology (Osaiyuwu et al., 2011). This ecotype exhibits high genetic variation, indicating its ability to cope with environmental changes and stressors (Ige et al., 2014). The breed has proven its resilience by thriving in backyard systems across villages, towns, and cities, showcasing remarkable adaptation to stressful and harsh conditions (Sola-Ojo et al., 2012).

These indigenous ecotypes not only reflect Nigeria's cultural and agricultural heritage but also serve as invaluable genetic resources (Lasagna et al., 2020). Understanding the physiological and genetic factors that contribute to the unique characteristics of these breeds is crucial for their preservation and improvement. Recent advances in molecular biology techniques, particularly quantitative polymerase chain reaction (qPCR), have enabled precise analysis of gene expression patterns in poultry species, providing insights into the genetic basis of adaptive traits.

Orexin receptors are G protein-coupled receptors found primarily in the brain, particularly in regions associated with appetite control. Their discovery has ushered in a new era in neurobiology by revealing the molecular mechanisms underlying sleep-wake regulation, energy balance, and appetite control (Sakurai, 2007). These receptors are activated by orexin neuropeptides, namely orexin-A and orexin-B, which are synthesized by specific neurons located in the lateral hypothalamus. Through their intricate signaling pathways, orexin receptors play crucial roles in modulating wakefulness, arousal, and overall behavioral states (Scammel and Winrow, 2011).

The significant role of orexin in chickens has been demonstrated, where it regulates appetite and stress responses (Arcamone et al., 2014). Ohkubo et al. (2003) demonstrated orexin's role in regulating feeding behaviors in poultry. The orexin system extends to reproductive behaviors and has practical applications in poultry farming, informing feeding schedules and stress management strategies (Neethirajan, 2025). Studying orexin in chickens provides insights into avian biology and serves as a comparative model for understanding similar systems in other animals (Lassiter et al., 2015).

The study contributes to conservation efforts for Nigerian indigenous chicken genetic resources by documenting their unique molecular characteristics. This information supports the development of breeding strategies that preserve valuable adaptive traits while improving productivity. The research also establishes baseline data for orexin gene expression in Nigerian chickens, facilitating future comparative studies and breeding program evaluations. Therefore, this study was carried out to analyze and compare orexin gene expression levels in two Nigerian indigenous chicken ecotypes (Fulani and Yoruba) and one exotic breed (Cobb-500) using quantitative polymerase chain reaction (qPCR) technology.

2. Materials and Methods

2.1 Experimental site

The experimental aspect was carried out at the Layers Unit, Teaching and Research Farm, Ladoke Akintola University of Technology, Ogbomoso. Ogbomoso is located in the derived Savanna Zone that lies on longitude 4 º 10' East of the Greenwich Meridian and latitude 8º 10' North of the equator. The altitude ranges from 300m and 600 m above sea level, while the mean temperature and annual rainfall are 27 ºC and 1247mm, respectively (Google Earth Map, 2024).

The laboratory aspect of the study was carried out in Acutig Genetic Laboratory, behind MS house, Asero Estate, Abeokuta. The laboratory region receives an annual rainfall ranging from 1,270 mm to 1,800 mm, with temperatures typically between 22°C and 33°C. The relative humidity is generally high, averaging around 80%. It lies between latitude 7º 10' N and 7º 15' N and longitudes 3º 17' E and 3º 26' E (Ufoegbune et al., 2008).

2.2 Experimental birds and management

Forty-five (45) birds each from the Fulani and Yoruba ecotypes of Nigerian Indigenous Chickens, and the exotic chicken (Cobb-500) were procured at 4 weeks of age, totaling 135 birds. The birds were chosen from diverse geographic locations representing the respective ecotypes. The birds were reared over a period of 4 weeks and fed without restriction for the duration of the experiment.

2.3 RNA extraction procedure

2.3.1 Tissue collection

After the experiment, tissue samples, specifically liver, were collected from the selected birds after slaughtering. Samples were immediately stored in RNA later to preserve RNA integrity. Storage with RNA later is essential to preserve the integrity of RNA, as RNA is susceptible to degradation by ribonucleases, which can become active when tissues are exposed to higher temperatures. High-quality RNA is critical for accurate gene expression analysis. Improper storage can lead to RNA degradation, resulting in unreliable results. Storage in RNA later minimizes the risk of degradation and guarantees that the samples represent the in vivo RNA status.

2.3.2 RNA extraction

Total RNA was extracted from the liver tissues using the Zymo RNA mini prep kit, following the manufacturer's instructions.

2.3.3 Sample preparation

25mg of the tissue was submerged in 600µl of RNA lysis buffer, centrifuged at 14000g (relative centrifugal force) for 30s, and the supernatant was transferred into a nuclease-free tube.

2.3.4 Total RNA extraction

All procedures were performed at room temperature, as room temperature enhances cell lysis and protein denaturation, critical for releasing intact nucleic acids (Lehman; Kreipe, 2001). The content from the nuclease-free tube was transferred into the Spin-Away™ filter in a collection tube and centrifuged at 16,000g for 30s. This is to remove the majority of the genomic DNA from the flow through. 600µl of 96% ethanol was added and mixed well by vortexing. The mixture was then transferred into the Zymo-Spin IIICG1 column in a collection tube and centrifuged at 16000g for 30s. The flow through was discarded.

2.3.5 DNase I treatment

The column was then washed with 400 µL RNA Wash buffer and centrifuged at 16000 g for 30 s; the flow-through was discarded. 5 µL DNase I, 75 µL DNA digestion buffer were added into a nuclease-free tube, mixed, and the mixture was then added directly into the column matrix and incubated for 15min at 30 °C. 400 µL of RNA prep buffer was added to the column after incubation, centrifuged, and the flow-through was discarded. 700 µL of RNA wash buffer was added to the column and centrifuged at 16000 g for 30 s, and the flow-through

was discarded. 400 µL of RNA wash buffer was added and allowed to stand for about 60 s to ensure complete removal of the wash buffer. The column was then transferred into a nuclease-free tube. 100 µL of DNase/RNase-free water was added directly to the column matrix and centrifuged at 14000 g for 30 s. The flow-through was stored for conversion to cDNA.

2.3.6 Complementary deoxyribonucleic acid (cDNA) synthesis

Complementary DNA was synthesized using the LunaScript® RT SuperMix kit. A total reaction volume of 20 µL was prepared according to the manufacturer's instructions. The components were prepared according to (Table 1).

Table 1. Components for cDNA synthesis cocktail.

Components	**Initial volume (mL)**	**Initial volume (µL)**	**Final volume (µL) for 25 Rxns (Vol µL/25)**
NO-RT mix	0.1	100	4
RT- super mix	0.1	100	4
Nuclease-free water	1.5	150	25
Template (RNA)	-	-	6
Total	-	-	25

Source: Authors, 2025.

After preparations, the setup was then run in the thermal cycler using the conditions in (Table 2).

Table 2. Cycling conditions for cDNA synthesis.

Stage	**Temperature (°C)**	**Time (min)**
Primer annealing	25	2
cDNA Synthesis	55	10
Heat Inactivation	95	1

Source: Authors, 2025.

2.4 Quantitative polymerase chain reaction (qPCR)

Quantitative Polymerase Chain Reaction assay was performed using the Luna® Universal qPCR Mastermix (M3003), following the manufacturer's protocol. A qPCR cocktail was first prepared, before being aliquoted into 20 µL reaction volumes and run in the qPCR machine. The cocktail was prepared by adding all the components of the master mix except the template, as seen in (Table 3). The primers used for this work were the OREX primer and the TATA box BP as the housekeeping gene, as presented in (Table 4).

Table 3. Quantitative Polymerase Chain Reaction cocktail reaction set up.

Component	**Volume per reaction (µL)**	**Volume per MM (µL)**	**Final concentration**
Mastermix	170	170	90
Forward primer	8.5	5.1	2.7
Reverse primer	8.5	5.1	2.7
Nuclease-free water	85	91.8	48.6

Component	Volume per reaction (µL)	Volume per MM (µL)	Final concentration
Template	4	4	4

Source: Authors, 2025.

Table 4. Contents of primer sequences.

Content of primer	Sequence
OREX F	CTTGGCCACCTGAAGACAC
OREX R	GGGTCACCGTAGGCTGAGT
TATA BOX BP F	ATCAAGCCAAGAATTGTTCTGC
TATA BOX BP R	CTTCGTAGATTTCTGCTCGAACT

Source: Authors, 2025.

2.5 Statistical analysis

Livak method ($2^{-\Delta\Delta C}T$) was used to analyze fold change, which is the level of expression of the Orexin gene in the different chicken breeds, and means were separated using *Duncan's* multiple range test of SAS (2003).

3. Results

3.1 Amplification of the orexin gene

The melt curve confirmed specific amplification of the orexin gene across all samples. Individual melt curve peaks showed minimal variation, with the highest fluorescence intensity (FI) recorded in a Cobb-500 sample (84.67 FI) and the lowest in a Yoruba ecotype sample (81.82 FI) (Figure 1).

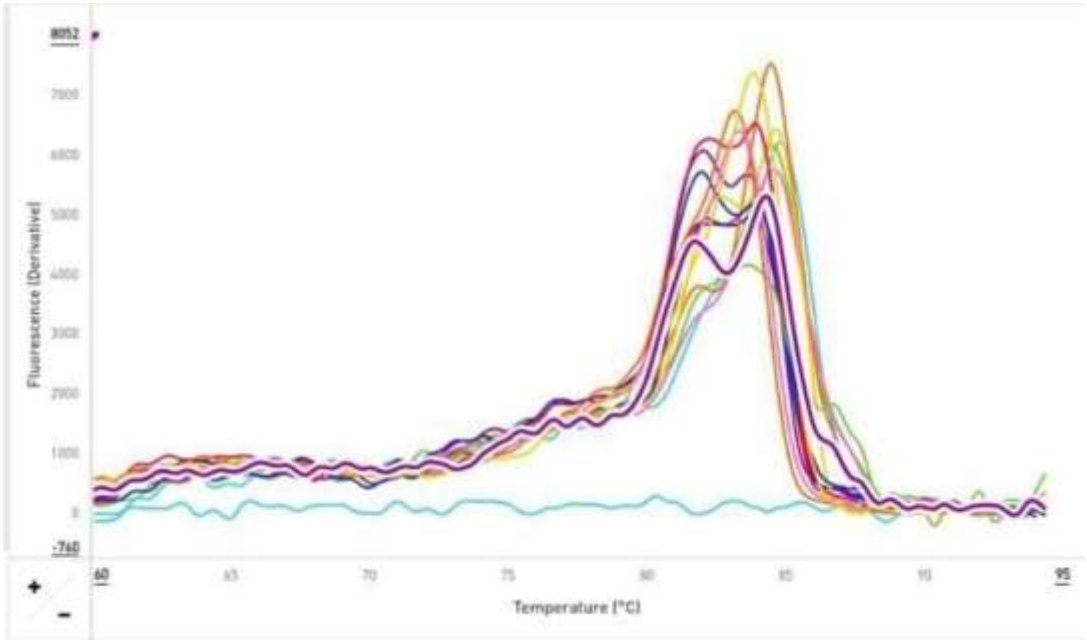

Figure 1. shows individual melt curves, color-coded by breed. Exotic (Cobb-500): Red, orange, yellow, pink, purple. Yoruba ecotype: Light blue, dark blue, light green, dark green, cyan. Fulani ecotype: Light brown, dark brown, violet, magenta, deep pink. The average melt curve values were Cobb-500: 83.68 FI, Yoruba ecotype: 83.61 FI, Fulani ecotype: 83.61 FI. Source: Authors, 2025.

This consistency indicated high specificity of primers and no off-target amplification, as indicated in (Figure 2).

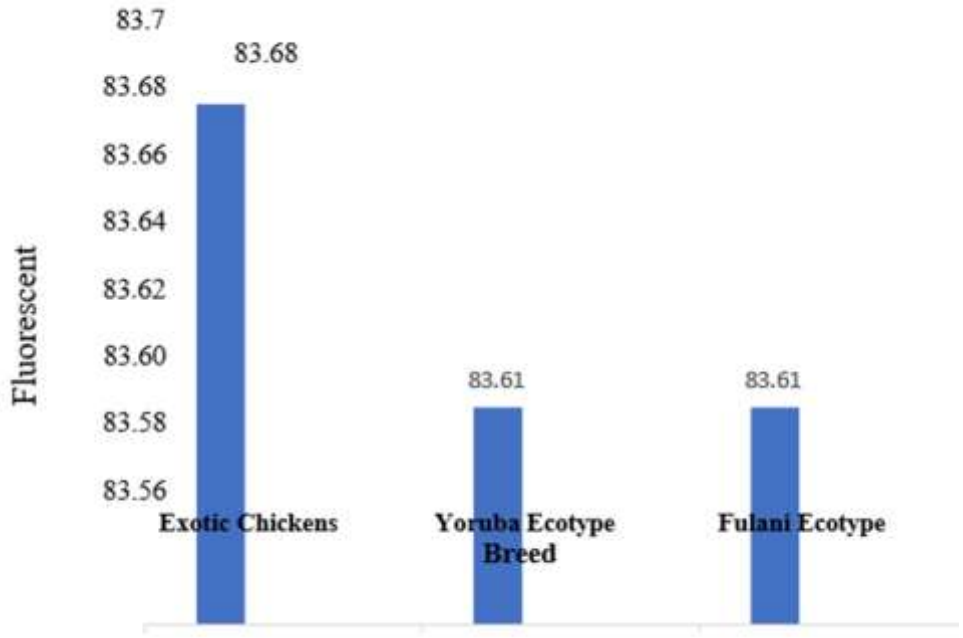

Figure 2. Cycle Threshold (CT) and Fold Change Analysis. Source: Authors, 2025.

Quantitative PCR data for orexin (ORX) and the TATA box binding protein (HKG) were analyzed using the $2^{-\Delta\Delta C}T$ method. Table 5 presents the raw CT values, $^{\Delta C}T$, $^{\Delta\Delta C}T$, and calculated fold changes for individual samples. The Cobb-500 sample BT3, with the lowest $^{\Delta C}T$ value (-16.32), was used as the control.

Table 5. Fold change value for each breed using the Livak method ($2^{-\Delta\Delta C}T$).

Sample	Target	^{C}T Value	HKG	$^{\Delta C}T$	$^{\Delta\Delta C}T$	$2^{-\Delta\Delta C}T$ (Fold change)
BT1	**ORX**	31.24	37.78	-6.54	9.78	0.01
BT2	**ORX**	23.46	38.00	-14.54	1.78	0.29
BT3	**ORX**	21.62	37.94	-16.32	0.00	1.00
BT4	**ORX**	24.95	37.88	-12.93	3.39	0.10
BT5	**ORX**	24.54	37.92	-13.38	2.94	0.13
YE1	**ORX**	28.10	37.78	-9.68	6.64	0.01
YE2	**ORX**	29.02	38.00	-8.98	7.34	0.01
YE3	**ORX**	24.66	37.94	-13.28	3.04	0.12
YE4	**ORX**	26.37	37.88	-11.51	4.81	0.04
YE5	**ORX**	28.14	37.92	-9.78	6.54	0.01
FE1	**ORX**	27.69	37.78	-10.09	6.23	0.01

FE2	**ORX**	22.36	38.00	-15.64	0.68	0.62
FE3	**ORX**	26.68	37.94	-11.26	5.06	0.03
FE4	**ORX**	19.93	37.88	-17.95	-1.63	3.09
FE5	**ORX**	20.80	37.92	-17.12	-0.80	1.75

Note: BT = Exotic Birds, YE = Yoruba Ecotype, FE = Fulani Ecotype. Source: Authors, 2025.

3.2 Mean fold change comparison by breed

The mean fold change expression of the orexin gene per breed is summarized in (Table 6). The Fulani ecotype had the highest average expression (1.35), followed by Cobb-500 (0.35), and the Yoruba ecotype (0.02).

Breed	**Orexin Expression**
Exotic (Cobb-500)	0.35^{ab}
Yoruba Ecotype	0.02^{b}
Fulani Ecotype	1.37^{a}

Source: Authors, 2025.

4. Discussion

The melt curve analysis confirmed the specificity and consistency of amplification across all samples. Minor variation in fluorescence intensity among breeds likely reflects breed-specific genetic differences in the orexin gene target region. The average melt curves were nearly identical across the three groups, indicating stable amplification conditions. This uniformity supports the technical reliability of the qPCR data, reducing the likelihood of primer-dimer formation or nonspecific amplification.

Fold change analysis using the $2^{-\Delta\Delta C}T$ method revealed clear inter-breed differences. Yoruba ecotype chickens showed the lowest orexin expression, averaging a fold change of 0.02. Cobb-500 broilers showed moderate expression (0.35). Fulani ecotype chickens recorded the highest expression level (1.37). These differences were statistically significant ($P < 0.05$) and suggest that orexin expression is not uniform across breeds.

The low expression in Yoruba chickens may be associated with lower appetite stimulation or reduced energy mobilization. This supports earlier findings that indigenous breeds often show reduced growth potential under controlled conditions due to their evolutionary adaptation to low-input systems (Mwacharo et al., 2013). In contrast, the Fulani ecotype, with the highest orexin expression, may be better adapted for active foraging, alertness, and physiological resilience. This pattern aligns with studies linking orexin signaling to metabolic activity and behavioral responsiveness (Sakurai, 2007). Greene et al. (2020) showed that orexin regulates hepatic lipogenesis in chickens, reinforcing its role in energy homeostasis. The Cobb-500 broiler, bred for rapid growth and feed efficiency under stable conditions, showed intermediate expression. This supports observations by Doe et al. (2021), who noted that commercially bred chickens exhibit reduced orexin signaling due to the lack of environmental stressors.

Breed-specific variation in orexin expression points to its potential as a candidate marker for selective breeding. Higher orexin levels may be advantageous in resource-limited or fluctuating environments, as observed in Fulani chickens. The gene's conserved expression in Cobb-500 and Yoruba chickens suggests a more stable selection profile. These findings provide a molecular basis for understanding physiological traits linked to breed adaptability. Orexin expression reflects underlying genetic regulation of energy balance and behavior, with implications for improving performance and resilience in indigenous poultry systems.

5. Conclusions

This study quantified orexin gene expression in three chicken breeds using qPCR. The results showed significant variation across the groups. The Fulani ecotype had the highest expression level, suggesting enhanced physiological adaptability. The Exotic breed showed moderate expression, consistent with its selective breeding

for controlled environments. The Yoruba ecotype recorded the lowest expression, indicating limited orexin activity. These differences highlight the potential of orexin as a molecular marker for selecting chickens with better energy regulation and environmental resilience. The Fulani ecotype's elevated expression supports its suitability for low-input or variable farming conditions.

The Fulani ecotype should be prioritized in breeding and conservation programs. Its elevated orexin expression indicates strong adaptability and energy regulation. Structured cross-breeding with selected traits from exotic breeds may improve productivity without losing genetic diversity. Programs should avoid replacing indigenous breeds with commercial lines. Instead, they should integrate functional gene traits into local populations. Future research should account for age, sex, and environmental factors to improve understanding of orexin regulation across diverse production systems.

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7. Authors' Contributions

Hameed Olayemi Salawu: project design, research, data processing, article writing, and publication. *Azeem Oladiran Ige*: research, data processing, and article writing. *Adeola Badhrat Ajibola*: raw data collection, data processing, and data analysis. *Abimbola Deborah Matt-Obabu*: project writing, data processing, article writing, and publication.

8. Conflicts of Interest

No conflicts of interest.

9. Ethics Approval

Yes applicable.

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