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# Polymorphisms in the GH gene and their influence on milk production traits in local buffaloes

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## Abstract

This study was conducted in a private sector buffalo farm in Al-Qurna, located north of Basra Governorate, between December 2023 and November 2024. It covered the summer (July and August 2024), autumn (October and November 2024), and winter (December 2023, January and February 2024) seasons. The purpose of the experiment was to examine the impact of the mid-lactation stage on genetic polymorphism in the growth hormone (GH) gene members (E4, E5, E6, E8) in the blood of buffaloes, and their relationship with serum GH levels during mid-lactation. Thirty blood samples were collected from the same buffaloes (n=30). Polymerase Chain Reaction (PCR) was used to amplify the targeted genes, and nucleotide sequence analysis was performed to detect polymorphisms. The results were compared with GH gene genotypes in the GenBank database. Two genotypes of the GH gene were identified in the mid-lactation buffaloes. DNA sequencing of the GH genes (E4, E5, E6, E8) was carried out, and the sequence data were compared with two references from the NCBI database. The E4 gene showed no differences when compared with Acc. NC_059175, but several mutations were observed compared with Acc. HG738860. The E5 gene sequence revealed polymorphisms when compared with both Acc. KC107765 and Acc. NC_059175 references. Although the E6 gene sequence showed no polymorphisms, the E8 gene sequence displayed a single-point mutation. Additionally, the results indicated significantly higher ($P \leq 0.05$) serum growth hormone levels in genotype A compared to genotype B.

**Keywords**: GH, Mid Lactation, Buffaloes GH gene.

## 1. Introduction

Milk production in local buffaloes presents significant challenges for dairy farming, primarily due to poor environmental conditions, malnutrition, and limited genetic potential. Both environmental factors (such as herd size, season, lactation stage, and diet) and genetic factors contribute to the complex nature of milk production and its composition (Gebreyesus et al., 2016). The growth hormone receptor (GHR) gene plays a vital role in mediating the effects of growth hormone (GH), which is part of the type 1 cytokine/hematopoietin receptor family.

This family includes several domains, such as the signal sequence, the extracellular domain (which contains the GH-binding site), the transmembrane domain, and a long intracellular domain involved in GH signaling (Jiang and Lucy, 2001). In cattle, the GH gene is located on chromosome 20 and consists of 10 exons, with exon 1 being relatively small and containing non-coding sequences (Maj et al., 2004; Maj and Zwierzchowski, 2006). Various studies have investigated the GHR gene for potential polymorphisms, which could influence milk production and quality (Blott et al., 2003; Rahmatalla et al., 2011; Sanchez et al., 2016). A genome-wide association study (GWAS) identified quantitative trait loci (QTL) associated with milk traits, positioning GH as a key candidate gene. One notable polymorphism is a non-synonymous SNP in exon 8 (c.836T>A, p.Phe279Tyr), which causes a phenylalanine-to-tyrosine substitution in the GH protein's transmembrane domain. This substitution is associated with higher milk yield but with lower fat and protein content compared to the T allele (Viitala et al., 2006; Waters et al., 2011; Banos et al., 2008). Other studies have found that a silent mutation (c.463C>T, p.Leu155) in exon 6 of bovine GH is linked to better milk yield and quality, including higher protein, fat, and casein levels, as well as improved milk coagulation properties and lower somatic cell counts (Viale et al., 2017).

While many studies have identified QTLs and candidate genes that affect milk production and composition in cattle, research on such QTLs in water buffaloes is limited. A recent genome-wide search for mutations linked to economic traits in Murrah buffaloes identified 483 SNPs across 66 genes associated with milk traits. Of these, 35 SNPs were located in the GH locus, including mutations in the promoter, introns, and exons, with one significant mutation (c.381A>C) in exon 5, resulting in an arginine-to-serine substitution (Surya et al., 2019). However, the c.836T>A SNP, which has been linked to milk performance in other species, was not included in this study. Another study by Shi et al (Shi et al., 2012) confirmed the presence of this SNP in Indian and Chinese swamp buffaloes, but only the TT and AT genotypes were detected in a sample of 136 buffaloes, and its effect on milk traits was not explored. While previous research has investigated the role of noncoding SNPs in complex traits, non-synonymous SNPs are particularly important because they directly alter the protein sequence, potentially leading to phenotypic changes. This study aims to examine polymorphisms in exons E4 to E6 and E8 of the GH gene in local buffaloes, as these regions have shown polymorphisms in previous studies involving both cattle and buffalo. Although some SNPs in the GH gene have been identified in Indian buffaloes, their connection to milk performance remains underexplored. This study will address this gap by investigating how genetic variations in the GH gene influence milk yield, quality, and molecular changes in water buffaloes (El-Komy et al., 2020; El-Bayomi et al., 2018; EL-Magd et al., 2017). This study also aims to examine how genetic polymorphisms in exons E4 to E6 and E8 of the GH gene influence the expression, function, and milk performance traits during mid-lactation in local buffaloes.

## 2. Materials and Methods

This study was conducted in the molecular biology lab (MBL), College of Veterinary Medicine, Basrah University. The study protocol was reviewed and approved Basrah University Animal and Ethics Committee with an ethical approval number of 46/37/2024.

**Blood Sample Preparation:** Blood samples (5 mL/animal) were collected from the jugular veins of all animals (n = 400) into EDTA-coated tubes (for DNA extraction).

### Detection of Genomic DNA

The Thermo Scientific NanoDrop OneC was used to detect the concentration and purity of the DNA, which is assessed absorbance at 260 nm and 280 nm (260/280) normalized to 10 mm pathlength. A ratio of 1.8 is generally accepted as pure for DNA.

**primers**

In this study four primers were used to find the relationship between the physiological characteristics of blood and milk samples during the lactation period, the isolated DNA was amplified in 50µl (Go Taq green Master Mix). The used primers are in Table (1). The primer used and reaction mixtures condition according to (EL-Magd et al., 2017).

Table 1. Primer pairs sequence and amplicon size.

| Primers | Primer sequence | PCR Product |
|---|---|---|
| GHR.E4 | F: AGGACCATCCATTACCCTCCTGATTT<br>R: TCCATTCCCATCACTGCATGAC | 265bp |
| GHR.E5 | F:AGGAGCTGGCACCTTATATGCAGT R:<br>CCCCGCTTATGTAATCTAAAGCCATGT | 472bp |
| GHR.E6 | F:ACTGATTCTCTGCTGAAATGCACAGT R:<br>CCATTTTCCACTGGGTCTCATTCAGT | 205bp |
| GHR.E8 | F: CTTTGGAATACTTGGGCTAG<br>R: CACTTCACTCAGGATTCAC | 166bp |

DNA detection was conducted at Macrogen Company in Korea, where 48 samples were sequenced. The obtained sequences were then subjected to BLAST analysis to accurately identify and compare genetic information."

**2.7.2 Phylogenetic tree**

By aligning GHR gene sequences, the MEGA X program was used to construct the phylogenetic tree of *Bubalus bubalis* isolates from this study and strains from GenBank.

**Determination of Growth Hormones Growth hormone concentration measurement:**

In this test, the buffalo's GH was measured in the plasma by the solid-phase sandwich ELISA (ELK Biotechnology, China). The ELISA micro-plate wells were pre-coated by the target-specific antibody.

The samples, standards, and controls are added into the wells with a buffalo's GH specific biotin-conjugated antibody. Later, the Horseradish Peroxidase (HRP) was added to wells. After the incubation, a substrate solution was added to color the wells that contained the GH. Finally, the reaction was terminated by adding a sulphuric acid solution. The color change was measured by the spectrophotometer at a wavelength of 450nm ± 10nm, and the GH concentration was calculated by comparing the OD of the samples to the standard curve, (Elmasry, et al., 2013).

**Analysis of Statistics**

Analysis was done on the results (El-Magd et al., 2013). To ascertain the significance of the shift from control, the findings were statistically evaluated using the t-test in SPSS software (El-Magd et al., 2014).

# 3. Results

## Polymerase chain reaction of genes

The milk yield of buffaloes is lower compared to foreign buffaloes due to various environmental, nutritional, and genetic factors. One major reason for the subpar milk traits is the lack of effective marker-assisted selection (MAS) practices for valuable traits. Therefore, genetic improvement through MAS is crucial for enhancing production traits. This study is part of a larger project focused on the genetic improvement of water buffaloes. Previous research has screened genes such as IGF1, IGF1R, IGF2, IGF2R, and Cyp19A1 for polymorphisms and analyzed their associations with growth and fertility traits. In this study, we expanded our research to investigate genetic variations in the GHR gene and assess their potential impact on milk production traits.

PCR products from *GHR.E4, E5, E6, and E8* locus were genotyped and the obtained results revealed only one banding pattern (Fig. 1). The results indicated the presence of all these genes in investigated samples.

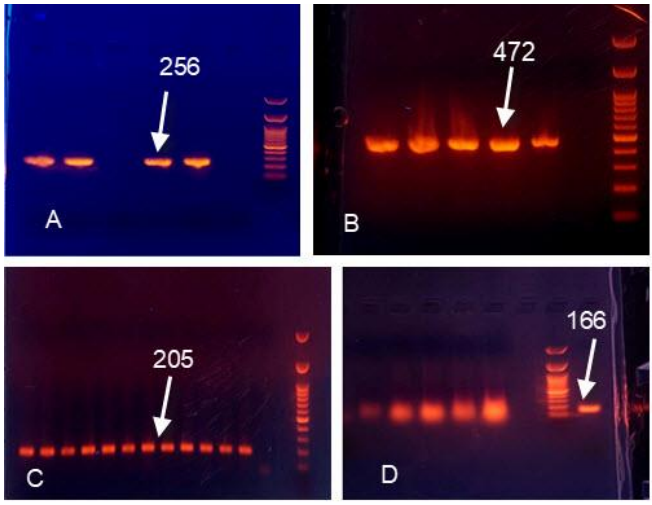


Figure 1. Agarose Gel Electrophoresis Image Shows PCR Product Analysis of *GHR genes*. A: GHR4, 256bp; B: GHR4, 472bp; C: GHR E6, 205bp; D: GHR E8, 166bp.

## Results of DNA Sequencing of GH genes:

DNA sequencing for the growth hormone genes (E4, E5, E6, E8) was applied. The sequence analysis was compared with two references from NCBI database. E4 gene showed no differences in comparison with Acc. NC_059175 but there are several mutations appear in comparison with the reference Acc. HG738860 (Table 2). The sequence of E5 gene showed polymorphisms in comparison with both references of the data database, Acc. KC107765 and Acc. NC_059175 (Table 3). Although the sequence of E6 gene showed no polymorphisms the sequence of E8 gene has only a single point mutation (Table 4, 5). The results indicate the presence of genetic variations in this locus. This was additionally confirmed by sequencing. A lack of polymorphisms in buffalo *GHR.E6* was also reported in other studies.

Table 2. Mutation in E4 gene in comparison with two accession No. of NCBI

| sample No. | Mutation in comparison with Acc. NC_059175 | Mutation in comparison with Acc. HG738860 |
|---|---|---|
| 1 | non | C/T, -/C, -/T, G/A, G/A, A/T, T/C, T/C, G/A |
| 2 | non | C/T, -/C, -/T, G/A, G/A, A/T, T/C, T/C, G/A |
| 3 | non | C/T, -/C, -/T, G/A, G/A, A/T, T/C, T/C, G/A |
| 4 | non | C/T, -/C, -/T, G/A, G/A, A/T, T/C, T/C, G/A |
| 5 | non | C/T, -/C, -/T, G/A, G/A, A/T, T/C, T/C, G/A |
| 6 | non | C/T, -/C, -/T, G/A, G/A, A/T, T/C, T/C, G/A |
| 7 | non | C/T, -/C, -/T, G/A, G/A, A/T, T/C, T/C, G/A |
| 8 | non | C/T, -/C, -/T, G/A, G/A, A/T, T/C, T/C, G/A |
| 9 | non | C/T, -/C, -/T, G/A, G/A, A/T, T/C, T/C, G/A |
| 10 | non | C/T, -/C, -/T, G/A, G/A, A/T, T/C, T/C, G/A |
| 11 | N/A | N/A |
| 12 | non | C/T, -/C, -/T, G/A, G/A, A/T, T/C, T/C, G/A |

Table 3. Mutation in E5 gene in comparison with two accession No. of NCBI

| sample No. | Mutation in comparison with Acc. KC107765 | Mutation in comparison with Acc. NC_059175 |
|---|---|---|
| 1 | T/C, G/T, C/T, T/C, T/C | A/G, G/T, C/A |
| 2 | T/C, G/T, C/T, T/C, T/C | A/G, G/T, C/A |
| 3 | T/C, G/T, C/T, T/C, T/C | A/G, G/T, C/A |
| 4 | N/A | N/A |
| 5 | N/A | N/A |
| 6 | T/C, G/T , C/T, T/C, T/C | A/G, G/T, G/T, C/A |
| 7 | T/C, , C/T, T/C, T/C | A/G, G/T, C/A |
| 8 | T/C, A/G, G/T, C/T, T/C. A/C, T/C | none |
| 9 | T/C, G/T, T/C, T/C | A/G, G/T, C/A |
| 10 | T/C, A/G, G/T, C/T, T/C | none |
| 11 | T/C, G/T, C/T, T/C, T/C | A/G, G/T, C/A |
| 12 | T/C, G/T, C/T, T/C, T/C | A/G, G/T, C/A |

Table 4 . Mutation in E6 gene in comparison with two accessions No. of NCBI

| sample No. | Mutation in comparison with Acc. NC_059175 | Mutation in comparison with Acc. EF207441 |
|---|---|---|
| 1 | None | None |
| 2 | None | None |
| 3 | None | None |
| 4 | None | None |
| 5 | None | None |
| 6 | None | None |
| 7 | None | None |
| 8 | None | None |
| 9 | None | None |
| 10 | None | None |
| 11 | None | None |
| 12 | None | None |

Table 5. Comparison of mutations in buffalo samples with reference sequences Acc. NC_059175 and Acc. MF490255.

| Sample No. | Comparison with Acc. NC_059175 | Comparison with Acc. MF490255 |
|---|---|---|
| Sample 1 | None | A/G |
| Sample 2 | None | A/G |
| Sample 3 | None | A/G |
| Sample 4 | None | A/G |
| Sample 5 | None | A/G |
| Sample 6 | None | A/G |
| Sample 7 | None | A/G |
| Sample 8 | None | A/G |
| Sample 9 | None | A/G |
| Sample 10 | None | A/G |
| Sample 11 | None | A/G |
| Sample 12 | None | A/G |

## Phylogenetic analysis of investigated genes:

The DNA sequences of the hormone growth genes were analysed against previous sequences in the database to investigate the phylogenetic relation between the sequences. The sequence of E4 showed a very close relation with the *Bubalus bubalis* no. 160015118507 breed Murrah

chromosome 19 and they showed a somewhat distinct relation with Bubalus bubalis partial GHR gene for Growth hormone receptor, exon4. Both are from India (Fig 2). Similar results were obtained for E5 gene which was found closely related to previous animals from India and nearly to *Bubalus bubalis* growth hormone receptor (GHR) gene investigated from Egypt (Fig 3).

The sequences labeled "GHRE4" (e.g., 01 GHRE4, 02 GHRE4, etc.) are grouped closely together, indicating a high degree of similarity among them. Two reference sequences, NC_059175.1 and HG738860.1, are included. These represent previously recorded genetic data for Bubalus bubalis GHRE4.

Most of the sequences (e.g., 12 GHRE4, 08 GHRE4, etc.) are closely related to NC_059175.1, suggesting minimal divergence from this reference. Some sequences (e.g., 01 GHRE4, 05 GHRE4) show closer relationships to HG738860.1, which could indicate genetic variations or mutations. The branch lengths (indicated by the numerical values) show the genetic distances. Sequences with "0.000" branch lengths have no observable mutations compared to their immediate common ancestor, while sequences with non-zero branch lengths (e.g., 0.014) show slight divergence.

Bootstrap Values: The tree demonstrates a close genetic relationship among the GHRE4 sequences analyzed in this study, with most sequences aligning closely to NC_059175.1.

However, there is some genetic variation observed in sequences like 01 GHRE4 and 05 GHRE4, which cluster more closely with HG738860.1. This suggests the presence of polymorphisms within the GHRE4 gene in the sampled buffalo population.

These findings highlight potential genetic diversity in the GHRE4 gene within the studied buffaloes.

The phylogenetic analysis could be further used to explore evolutionary relationships or identify genetic markers associated with specific traits, such as milk production.

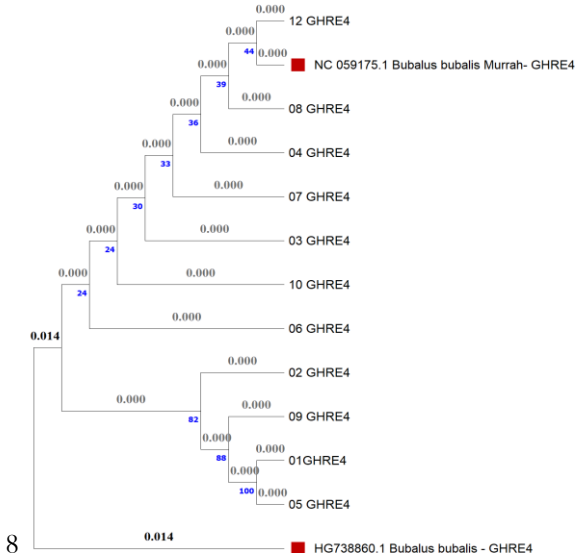


Figure 2. Phylogenetic tree analysis of GHRE4 showing relation with two previously registered strains.

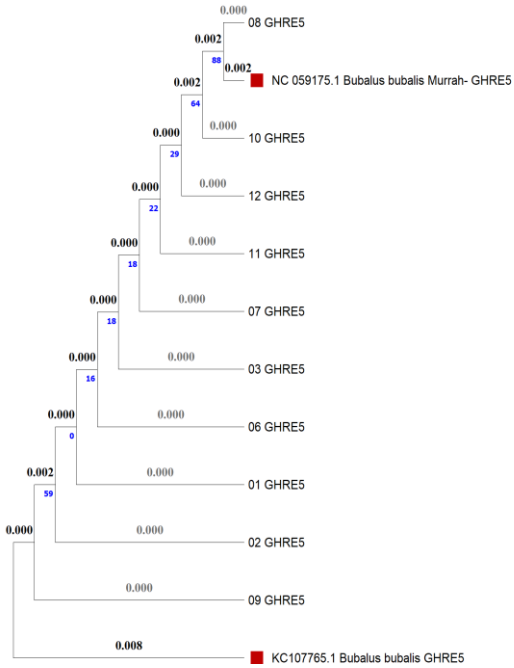


Figure 3. Phylogenetic tree analysis of GHRE5 showing relation with two previously registered strains.

The phylogenetic analysis of E6 gene showed no difference with other animals retrieved from database (Fig 4). In contrast, the E8 gene of samples showed high similarity with Indian isolates and slight differences from the *Bubalus bubalis* growth hormone receptor (GHR) gene, exon 8 investigated in Egypt (Fig 5).

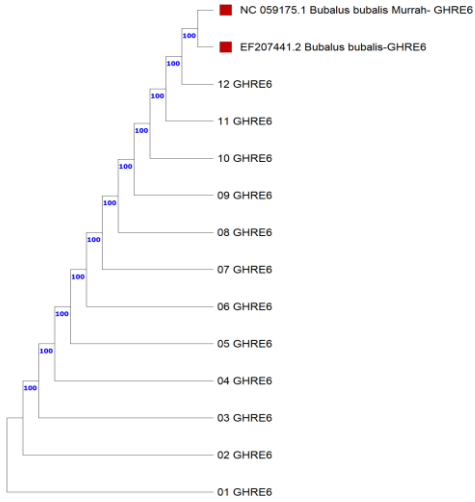


Figure 4. Phylogenetic tree analysis of GHRE6 showing relation with two previously registered strains.

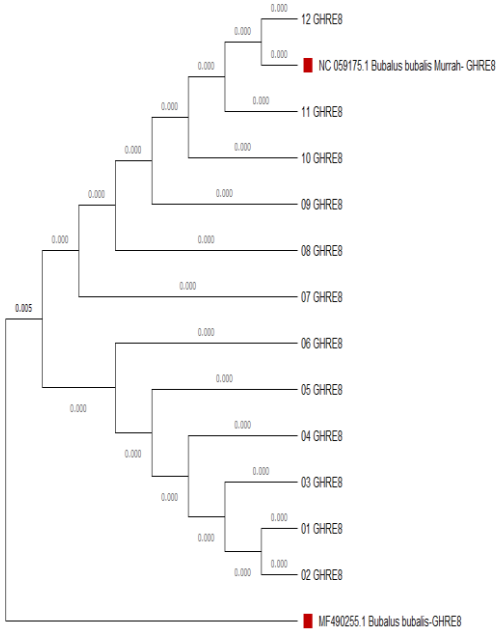


Figure 5. Phylogenetic tree analysis of GHRE8 showing relation with two previously registered strains.

The results in Table (6) showed a significant (P≤0.05) increase in growth hormone concentrations during mid-lactation compared with dry buffaloes.

Table 6. Effect of Mid Lactation on Value of Serum Growth Hormone In Buffaloes (Mean±SD) (n=30)

| Parameters<br>Group | GH<br>( mg/l ) |
|---|---|
| Mid-Lactation | 20.14±0.l0a |
| Dry | 10.27±0.53b |

Values expressed as mean ± SD., n = 12 buffaloes, with different superscripts (a and b) in the column differ significantly (P≤0.05).

# 4. Discussion

The buffalo (*Bubalus bubalis*) is a domesticated species widely raised across various regions globally, with Iraq depending on buffaloes as a primary source of milk. However, buffaloes are often hindered by challenges such as slow growth rates (El-Magd et al., 2016) and delayed puberty (Yakan et al., 2018). Specifically, the Murrah breed in Iraq has been noted to reach puberty as late as 33 months (National Dairy Research Institute Annual Report, (El-Adawy et al., 2018). Growth rate is a critical trait that impacts the timing of puberty, conception, and the age at first calving.

Research indicates that continuous administration of the Growth Hormone-Releasing Factor (GRF) can stimulate the release of Growth Hormone (GH) without reducing its responsiveness over time. Studies have shown that daily subcutaneous (SC) injections in lactating

cows for six months (Sharawy et al., 2017), twice-daily SC injections in young dairy heifers for eight months (El-Magd et al., 2028), and intravenous (IV) injections in growing buffalo calves at 15-day intervals for nine months (El-Magd et al., 2013) all effectively promote GH release. However, GH secretion tends to decline with age in both humans (Corpas et al., 1993) and cattle (Shingu et al., 2002). A recent study found that GH responses to GRF were more pronounced in calves than in adult cows (Chen et al, 2017).

GH is an essential galactopoietic hormone in both cows and buffaloes (Do et al., 2019; Fontanesi et al., 2007), working alongside prolactin to support milk production (Fallin et al., 2001; Wyszynska-Koko et al., 2006; Bao et al., 2016). bGH was administered after 74 days of lactation to cows receiving a complete mixed ration ad libitum. Ten cows averaging 34.4 kg of milk per day were divided into two groups, one receiving daily subcutaneous bGH injections (51.5 IU/day) and the other a placebo. The bGH-treated group showed a 9.5% increase in milk yield, a 22.7% increase in milk fat yield, a 14.5% increase in lactose yield, and a 17.1% rise in milk energy secretion. Feed intake decreased slightly by 4.3%, though not statistically significant, and milk protein secretion and nitrogen balance remained unchanged. Serum GH levels in the treated group were maintained within the normal physiological range during the injection period, but 48 hours after the final injection, GH levels returned to baseline, and milk production decreased to pre-injection levels. This study highlighted the significant impact of GH administration on milk synthesis in high-yielding dairy cows (Lucy et al., 2009).

In the previous study (Lucy et al., 2009), buffaloes received intravenous bovine GRF (bGRF) at 10 µg/100 kg body weight or an equal volume of saline at 15-day intervals for nine months. Plasma GH responses were measured through blood samples taken on days 1, 90, 180, and 270, with additional samples collected at various time points relative to bGRF injection. Average growth rate (AGR) and feed conversion efficiency (FCE) were recorded at 15-day intervals. Plasma GH concentrations increased significantly following the bGRF injection, peaking 10-20 minutes after administration and returning to baseline by 180 minutes. No significant differences were observed in the peak GH levels or the area under the curve (AUC) in response to bGRF across the four sampling occasions. However, the treatment group showed significantly higher overall plasma GH concentrations, AGR, and FCE compared to the control group. Evaluation of Some Metabolic Parameters and gene polymorphisms were studied in the same region previously (Abbas, et al.,2024; Yousief, et al., 2023 a, b; Jaffar, et al., 2019; Othman, et al., 2020). This study concluded that long-term bGRF administration over nine months did not reduce GH responsiveness in buffalo heifers and that sustained GH release led to improved growth rates and feed conversion efficiency in buffaloes.

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## Conflict of interests

The authors affirm that they have no competing interests to disclose.

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