Polish Journal of Veterinary Sciences Vol. 28, No. 3 (2025), 389–402

DOI

Original article

Immunolocalization and immunoexpression levels of sibling peptides nesfatin-1 and ghrelin, and their potentially shared receptor in the gastrointestinal tract of Holstein-Friesian bulls

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Abstract

This study investigates the localization and immunoexpression levels of nesfatin-1 and ghrelin – two metabolically active peptides – and their putative shared receptor, growth hormone secretagogue receptor (GHSR), across the gastrointestinal tract (GIT) of Holstein-Friesian bulls. Recognized for their opposing roles in energy balance, nesfatin-1 and ghrelin are considered "sibling peptides" due to their complementary physiological functions and origin within the gastrointestinal system. The investigation encompassed both immature (calves) and mature (adult) cattle to assess developmental variation in the immunoexpression and localization of these peptides. Immunohistochemistry and ELISA were used to determine their localization patterns and quantify protein concentrations across distinct GIT segments. Nesfatin-1 was found broadly distributed in mucosal layers and the enteric nervous system (ENS), with a pronounced presence in the abomasum and duodenum. Notably, calves exhibited higher levels of nesfatin-1 across most GIT regions, suggesting age-related differences in metabolic regulation. Ghrelin was predominantly localized in the abomasum and, to a lesser extent, in other gastrointestinal regions, including the forestomachs and intestinal mucosa. Its presence in neuronal structures of the ENS, although less abundant, hints at potential neural roles beyond endocrine signalling. GHSR immunoexpression was restricted mainly to the enteric ganglia and selected epithelial cells, with significant levels observed in the duodenum, particularly in calves. The receptor was absent in the rumen, implying that ghrelin activity in this region might be mediated via systemic or paracrine pathways rather than local receptor binding. The findings reveal both overlapping and distinct localization patterns of these peptides and their receptor, showing complex interactions in GIT physiology. Elevated nesfatin-1 immunoexpression in young animals suggests a potential developmental role, while the conserved ghrelin distribution reinforces its established gastric functions. These results may contribute insights into the regulatory architecture of bovine metabolism and potentially inform strategies for optimizing cattle growth and health management, providing a relevant reference point for veterinary sciences.

Keywords: cattle, gastrointestinal tract, ghrelin, growth hormone secretagogue receptor, nesfatin-1

Introduction

The regulation of energy balance and metabolic processes in mammals relies on a complex network of peptides, that act across various physiological systems to maintain homeostasis (Wen et al. 2019). Understanding the roles and interactions of these molecules is especially relevant in livestock species, such as domestic cattle (*Bos taurus taurus*), where energy regulation directly impacts growth, reproduction and overall productivity (Yang et al. 2023, Muzemil and Buhari 2024). Among these regulatory peptides, nesfatin-1 and ghrelin have gained attention due to their distinct yet interlinked roles in appetite modulation and energy homeostasis (Kojima et al. 1999, Oh-I et al. 2006, Stengel and Taché 2010). While considerable research has explored these peptides in humans and experimental models, their localization and function in cattle, particularly at different developmental stages, remain inadequately characterized. As ruminants, cattle possess a multi-chambered stomach system that supports unique mechanisms of feed intake and nutrient absorption compared to other herbivores, making direct extrapolation from non-ruminant studies problematic. Consequently, a ruminant-specific analysis is necessary to determine whether these regulatory peptides function similarly or undergo specific adaptations to accommodate the distinctive physiology of cattle.

Nesfatin-1, a product of the precursor protein nucleobindin-2 (NUCB2), exerts notable anorexigenic effects by inhibiting food intake and influencing energy expenditure (Oh-I et al. 2006). Initially discovered in hypothalamic regions associated with appetite suppression, nesfatin-1 appears to have broader functions, including stress response, insulin sensitivity and metabolic regulation (Ayada et al. 2015, Stengel 2015, Dore et al. 2017, Schalla and Stengel 2018, Kras et al. 2022). Its presence in the central nervous system, gastrointestinal tract (GIT) and adipose tissue, suggests that nesfatin-1 may play multiple roles in energy homeostasis (Stengel et al. 2009, Ramanjaneya et al. 2010, Zhang et al. 2010, Goebel-Stengel et al. 2011, Kim et al. 2014). By contrast, ghrelin, another key regulatory peptide, is primarily produced in the stomach and operates as a potent hunger signal (Kojima et al. 1999, Kojima and Kangawa 2005, Müller et al. 2015). Its orexigenic properties involve stimulating appetite, promoting gastric motility and enhancing growth hormone secretion via the growth hormone secretagogue receptor (GHSR) (Kojima and Kangawa 2005, Akalu et al. 2020, Jiao and Luo 2022). This contrasting relationship - nesfatin-1 as anorexigenic factor versus ghrelin as an orexigenic factor – highlights their complex interplay within the same regulatory network.

Despite these opposing effects, nesfatin-1 and ghrelin share overlapping regulatory roles in energy balance, particularly within the hypothalamic pathways. This functional antagonism, sometimes described "sibling peptides", owing to their GIT origin and similar post-translational modifications (Chen et al. 2022), is reminiscent of other peptide pairs, such as glucagon and glucagon-like peptide-1 (GLP-1), which are both derived from the same proglucagon precursor but exert opposite influences on glucose metabolism (Sandoval and D'Alessio 2015). While ghrelin's gastrointestinal distribution has been characterized in several species (Mehdar 2021), including cattle (Jonova et al. 2022), corresponding data for nesfatin-1 are less abundant (Kras et al. 2022), creating a crucial knowledge gap in bovine physiology. Both peptides are also thought to interact with the same receptor, GHSR. GHSR, traditionally associated with ghrelin, has recently been proposed as a potential receptor for nesfatin-1, although this nesfatin-1's potential use of this receptor remains uncertain (Ozturk et al. 2015, Fan et al. 2018, Chen et al. 2022). Such a receptor-sharing mechanism introduces a fascinating element of regulatory complexity, as the receptor's activation could lead to divergent physiological outcomes, depending on the ligand involved. This possibility is especially compelling in the context of cattle, where efficient energy management is critical at different growth stages, ranging from the rapid development seen in calves to the maintenance-focused energy balance in adult animals (Diao et al. 2019).

This study focuses on the immunolocalization and protein levels of nesfatin-1, ghrelin and GHSR in different GIT organs of cattle to better understand how these peptides contribute to metabolic regulation across age groups. We hypothesize that nesfatin-1, ghrelin and GHSR display distinct localization patterns in the GIT of both calves and adult cattle, reflecting specific metabolic demands and developmental needs. The primary aim of this study is to compare the immunolocalization and quantifiable levels of nesfatin-1, ghrelin and GHSR across all stomach compartments and intestinal regions in adult and calf cattle, using immunohistochemistry (IHC) and ELISA techniques. Given the central role of energy balance in livestock growth and productivity, mapping the localization of nesfatin-1, ghrelin and their receptor in the bovine GIT may provide essential baseline knowledge for veterinary science.

Table 1. Primary and secondary antibodies used in the study.

Antibody	Host	Catalog number	Dilution	Manufacturer
Primary antibody				
Anti – nesfatin-1	mouse	H00004925-M03	1:500	Bio-Techne, Minneapolis, MN, USA
Anti-ghrelin	rabbit	PA1-1070	1:500	Thermo Scientific, Menzel-Glaser, Braunschweig, Germany
Anti-GHSR	rabbit	720278	1:100	Thermo Scientific, Menzel-Glaser, Braunschweig, Germany
Secondary antibody				
Anti-mouse/Anti-rabbit	goat	DPVB-HRP	RTU[1]	ImmunoLogic, Duiven, Netherlands

[1] RTU = Ready To Use

Materials and Methods

Animals

This study was conducted on healthy male Polish Holstein–Friesian cattle, divided into two age groups: six adult individuals (20-24 months old, weighing 768 ± 46 kg) and six calves (7-8 months old, weighing 218 ± 23 kg). All animals were obtained from the same farm to maintain consistency in housing, diet and environmental conditions. Both groups were managed under a semi-intensive feeding system, beginning with pasture grazing and transitioning to a total mixed ration approach, following the methodology outlined by Włodarczyk and Budvytis (2011) (Włodarczyk and Budvytis 2011).

The animals were slaughtered in a licensed local slaughterhouse under standard commercial conditions, following a fasting period of approximately 12 hours in accordance with routine pre-slaughter practices. The slaughter process and all subsequent procedures complied with the Council Regulation (EC) No. 1099/2009 of 24 September 2009.

Post-mortem examinations by the official veterinary inspector confirmed the animals' good health and the absence of gastrointestinal pathologies, validating the collected samples. Ethical approval by the Ethics Committee was not required under Polish law, as all procedures involved post-mortem examination of carcasses from animals that were intended for commercial use and human consumption.

Tissue Processing

Sections of the GIT (rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, colon) were collected immediately post-mortem, gently cleaned and subsequently preserved in liquid nitrogen before storage at −80 °C for ELISA, or fixed in 4% buffered formaldehyde for IHC. The tissue processing, IHC and ELISA methodologies were described in detail in a previous study (Kras et al. 2025). Briefly, formaldehyde-fixed samples were dehydrated, embedded in paraffin, sectioned (5 µm) and placed on SuperFrost® Plus slides (Thermo Scientific, Menzel-Glaser, Braunschweig, Germany). Frozen samples were homogenized in PBS, centrifuged and protein content was determined using a Pierce BCA kit (Thermo Scientific, Waltham, MA, USA).

IHC and ELISA

IHC involved antigen retrieval, blocking, incubation with primary antibodies (Table 1) and detection via a poly-HRP system with DAB staining (Kras et al. 2025). Control reactions validated antibody specificity. ELISA assays were conducted using commercial kits (nesfatin-1 – QY-E60251, Qayee Bio-Technology Co. Shanghai, China; ghrelin – QY-E60249, Qayee Bio-Technology Co. Shanghai, China; GHSR – QY-E60250, Qayee Bio-Technology Co. Shanghai, China) with absorbance measured spectrophotometrically. Assays were performed in duplicate, with intra- and inter-assay variations below 8% and 10%, respectively.

Semi-quantitative analysis

A semi-quantitative analysis of the IHC reaction was conducted following a previously described protocol (Crowe and Yue 2019) using ImageJ 1.52 software (Schneider et al. 2012). The measurements were calculated by dividing the mean gray value by the number of cell nuclei. To enhance accuracy, the protocol was adjusted to include manual counting of cell nuclei using the "multipoint" tool. Immunoreactivity (IR) results in the ENS were presented as the ratio of the mean gray value to the analyzed area, calculated using the "Polygon Selection" tool. IR in the ENS was assessed using a neuron-based sampling method. For each GIT segment and each animal, 100 neurons from the myenteric plexus and 100 neurons from the submucosal plexus

were randomly selected and analyzed. Images were captured under 400× magnification (objective 40×, ocular 10×), with each field covering an approximate area of 60 000 μm². Only clearly identifiable neuronal cell bodies were included in the analysis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 10.5.0 for Windows (GraphPad Software, San Diego, CA, USA). Prior to comparative analysis, all data were assessed for compliance with the assumptions of parametric testing. The Shapiro-Wilk test was used to evaluate the normality of distributions, and Levene's test was used to verify homogeneity of variances. When these assumptions were met, linear mixed-model was applied to determine the effects of two fixed factors: age group (young vs. adult cattle) and GIT segment. The presence of interaction between age and GIT segment was examined in all analyses. When a statistically significant interaction was observed ($p<0.05$), a full-factorial model was used, including pairwise comparisons between age groups within each GIT segment as well as comparisons between GIT segments within each age group. If no significant interaction was found, only the main effects were interpreted. In cases where data violated parametric assumptions, appropriate non-parametric alternatives (Aligned Rank Transform for full model, or Friedman's test with one factor) were used where applicable, post hoc tests were conducted using Tukey's multiple comparisons test for parametric data or Dunn's test for non-parametric data. Results are presented as mean ± standard error of the mean (SEM), and statistical significance was set at $p<0.05$.

Results

Nesfatin-1 immunolocalization

Nesfatin-1 was observed in all examined sections of the GIT, both in the mucosa and within the ENS (Fig. 1a-c). In the mucosa of the forestomachs, immunoreactive cells were visible in the flat epithelial layers beneath the keratinized surface (Fig. 2). In the abomasum, a high density of immunoreactive cells was clustered at the base of the glands, while in the duodenum the most intense IR was found in the duodenal glands and basal regions of intestinal crypts (Fig. 3). Single scattered cells with IR were also present throughout the mucosa. A similar pattern was noted in the jejunum, ileum and colon, with immunoreactive cells most abundant near the base of the glands. Statistical analysis of IR in the mucosal layer (Fig. 1a) revealed a significant interaction between age and GIT segment ($p<0.001$). In calves, mucosal IR was highest in the abomasum ($p<0.001$ vs. all other segments), followed by the jejunum and colon, which were significantly higher than the remaining segments ($p<0.001$). These remaining segments exhibited similarly lower levels. In adults, the abomasum also showed the highest IR ($p<0.001$), while all other segments had lower and statistically comparable immunoexpression. Comparison between age groups revealed that calves showed significantly higher mucosal IR in the abomasum ($p<0.001$), jejunum ($p<0.01$) and colon ($p<0.001$) compared to adults.

Within the ENS, nesfatin-1 was localized to neuronal cell bodies in both the submucosal and myenteric plexuses of all examined GIT regions (Fig. 4 and 5).

In the submucosal plexus (Fig. 1b), no interaction between age and segment was found, but the segment effect was significant ($p<0.001$). IR was significantly higher in the abomasum compared to the ileum ($p=0.017$), while no significant differences were observed between the other segments, nor between age groups.

In contrast, the myenteric plexus (Fig. 1c) showed a significant interaction between age and segment ($p<0.001$). In calves, IR was highest in the reticulum, which was comparable to the omasum and abomasum. These three segments (with the exception of the omasum) showed significantly higher IR than the colon ($p=0.006$), rumen ($p=0.010$) and duodenum ($p<0.001$). The jejunum and ileum had the lowest IR and were significantly different from all other segments ($p<0.001$). In adults, IR in the myenteric plexus was highest in the omasum, abomasum and colon, which were significantly greater than the other segments ($p<0.001$). When comparing age groups, significant differences were observed in the reticulum, where calves had higher IR ($p<0.01$) and in the colon, where adults showed higher IR ($p<0.05$).

Nesfatin-1 protein level

The concentration of nesfatin-1 did not exceed 1 000 000 pg/g of total protein (Fig. 6a). The protein level differed significantly between groups and was generally higher in calves compared to adults, with statistically significant differences observed in the rumen ($p<0.01$), reticulum ($p<0.05$), abomasum ($p<0.05$), jejunum ($p<0.001$) and ileum ($p<0.05$). Within groups, protein levels were relatively consistent across segments, except in calves, where a relatively higher nesfatin-1 protein level was noted in the rumen, ileum and colon compared to the omasum and duodenum.

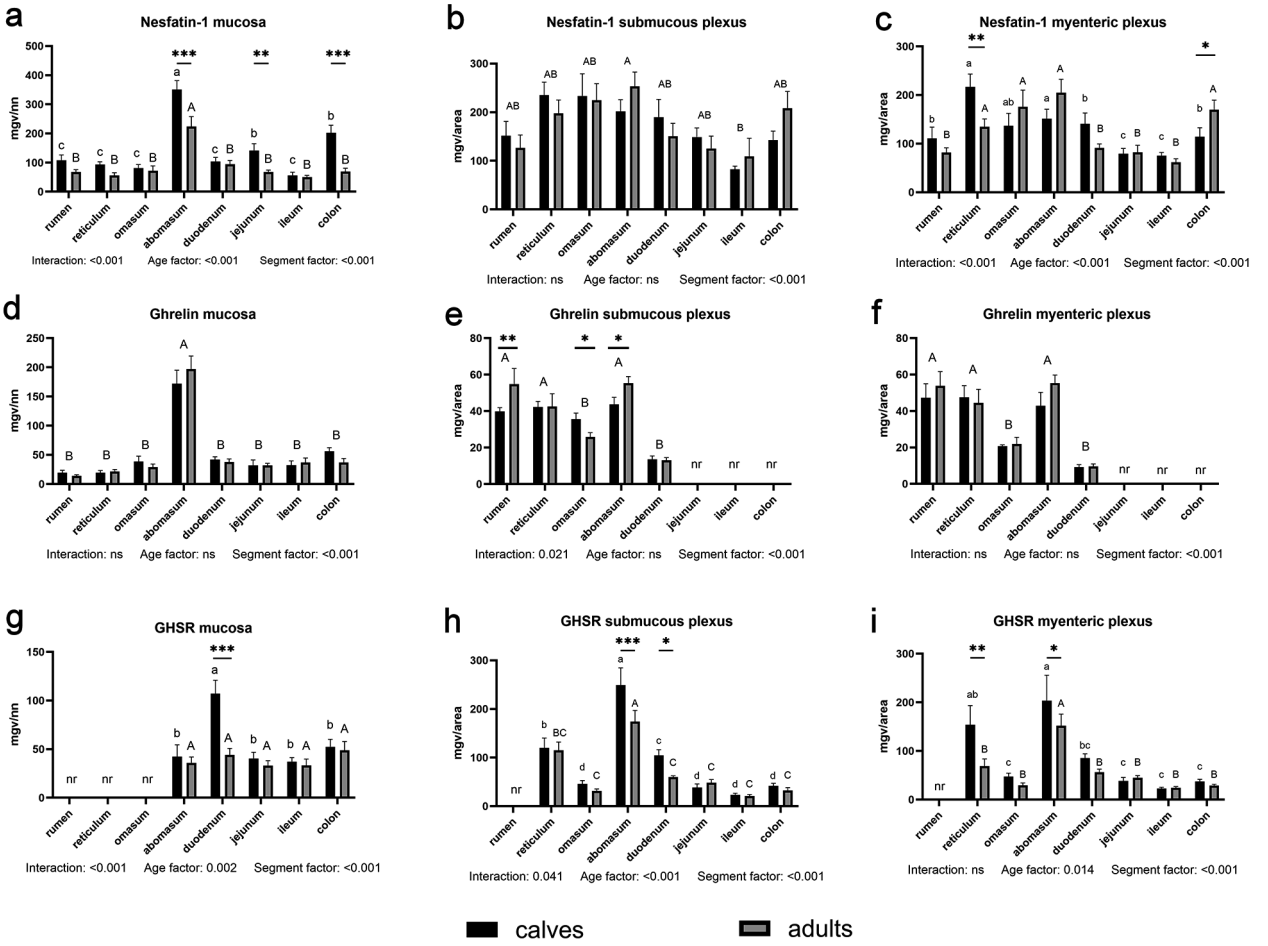

Fig. 1. Semi-quantitative determination of immunohistochemistry (IHC) reactions in the gastrointestinal tract (GIT) of calves and adult Holstein-Friesian bulls. Immunoexpression of nesfatin-1 (a, b, c), ghrelin (d, e, f) and GHSR (g, h, i) in the mucosa (a, d, g) was expressed as the ratio of mean gray value (mgv) to nuclei number (nn), whereas in the enteric nervous system (ENS) (b, c, e, f, h, i) it was expressed as the ratio of mgv to the surface area of the analyzed region. Different lowercase letters denote significant differences between GIT segments in calves, whereas different uppercase letters denote significant differences between GIT segments in adults ($p<0.05$), unless the interaction between the main factors or age as a main factor was not significant – in such cases, uppercase letters indicate differences between segments regardless of age group. The asterisks (*) highlight significant differences in the immunoreaction levels between calves and adults within a specific GIT segment (* for $p<0.05$; ** for $p<0.01$; *** for $p<0.001$).

Ghrelin immunolocalization

Ghrelin was primarily detected in the glandular cells of the abomasum, where its IR was most prominent. In the forestomachs, ghrelin-positive cells were localized in the outer epithelial layers, similarly to nesfatin-1 (Fig. 2). In the duodenum, single immunoreactive cells were present in the epithelium of the villi and in both duodenal and intestinal glands. In the jejunum and ileum, the distribution was similar to that of the duodenum, with the expected absence of duodenal glands. In the colon, immunoreactive cells were observed near the luminal surface (Fig. 3).

Statistical analysis of mucosal IR (Fig. 1d) revealed no interaction between segment and age, but the segment effect was significant ($p<0.001$). IR was highest in the abomasum, which showed significantly greater immunoexpression compared to all other GIT segments ($p<0.001$). There were no significant age-related differences in mucosal IR.

In the ENS, ghrelin was detected in both the submucosal and myenteric plexuses of the forestomachs, abomasum and duodenum (Fig. 4 and 5). In the duodenum, however, IR was sparse and limited to occasional ganglia. In both plexuses, ghrelin was located primarily in fibers within or surrounding the ganglia, rather than in neuronal cell bodies.

In the submucosal plexus (Fig. 1e), a significant interaction between age and segment was detected ($p=0.021$), although only the segment factor remained statistically significant overall ($p<0.001$). No IR was detected in the jejunum, ileum or colon. Among the remaining segments, the omasum showed significantly lower IR than all others ($p<0.001$). Age-related compa-

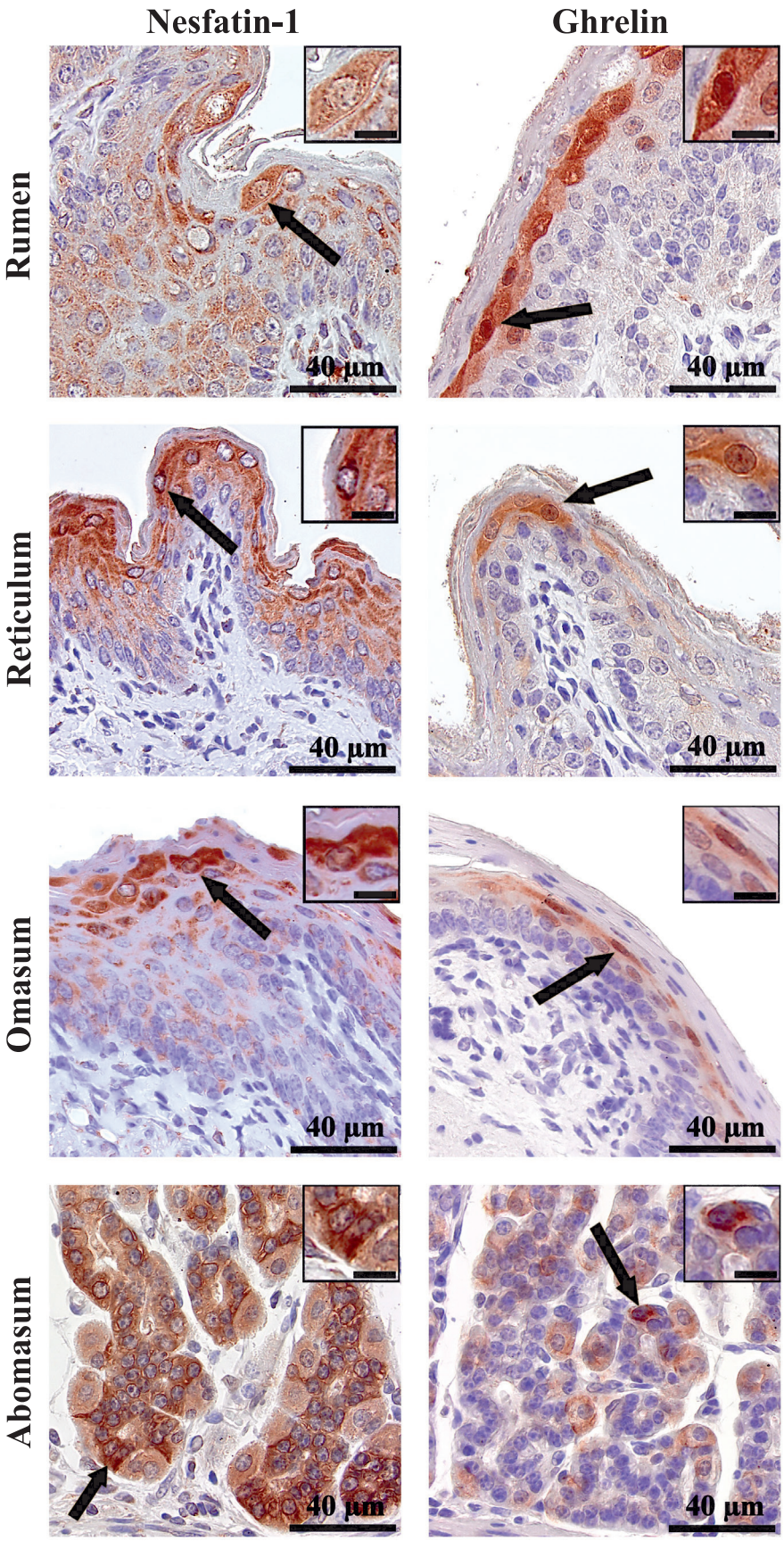

Fig. 2. Immunolocalization of nesfatin-1 and ghrelin in the mucosa of the rumen, reticulum, omasum and abomasum of Holstein-Friesian male calves. Black arrows indicate an example immunoreaction. In the top right corner, a magnified view of the reaction. Scale bar: 40 µm for the main image and 15 µm for the magnified reaction inset.

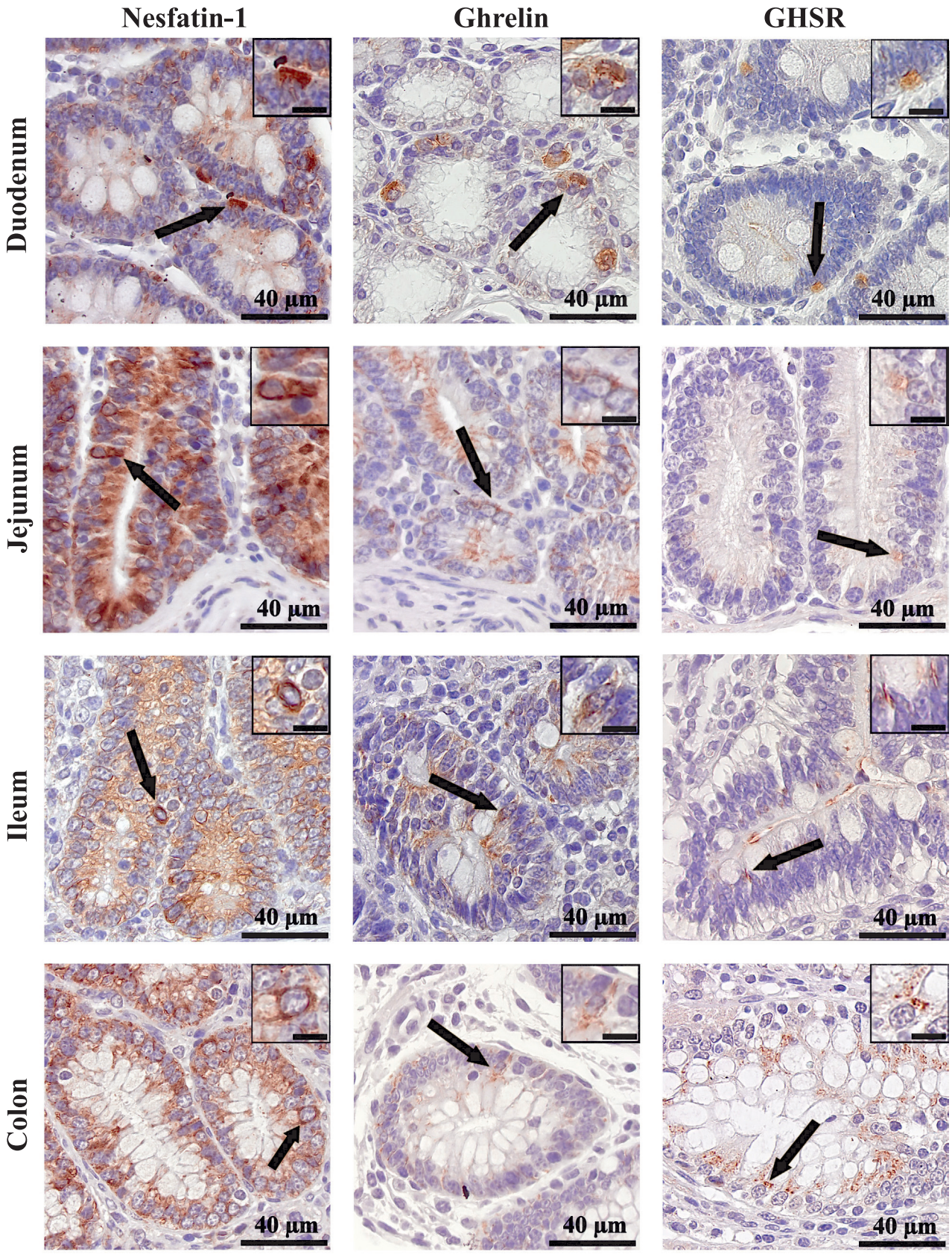

Fig. 3. Immunolocalization of nesfatin-1, ghrelin and GHSR in the mucosa of the duodenum, jejunum, ileum and colon of Holstein-Friesian male calves. Black arrows indicate an example immunoreaction. In the top right corner, a magnified view of the reaction. Scale bar: 40 µm for the main image and 15 µm for the magnified reaction inset.

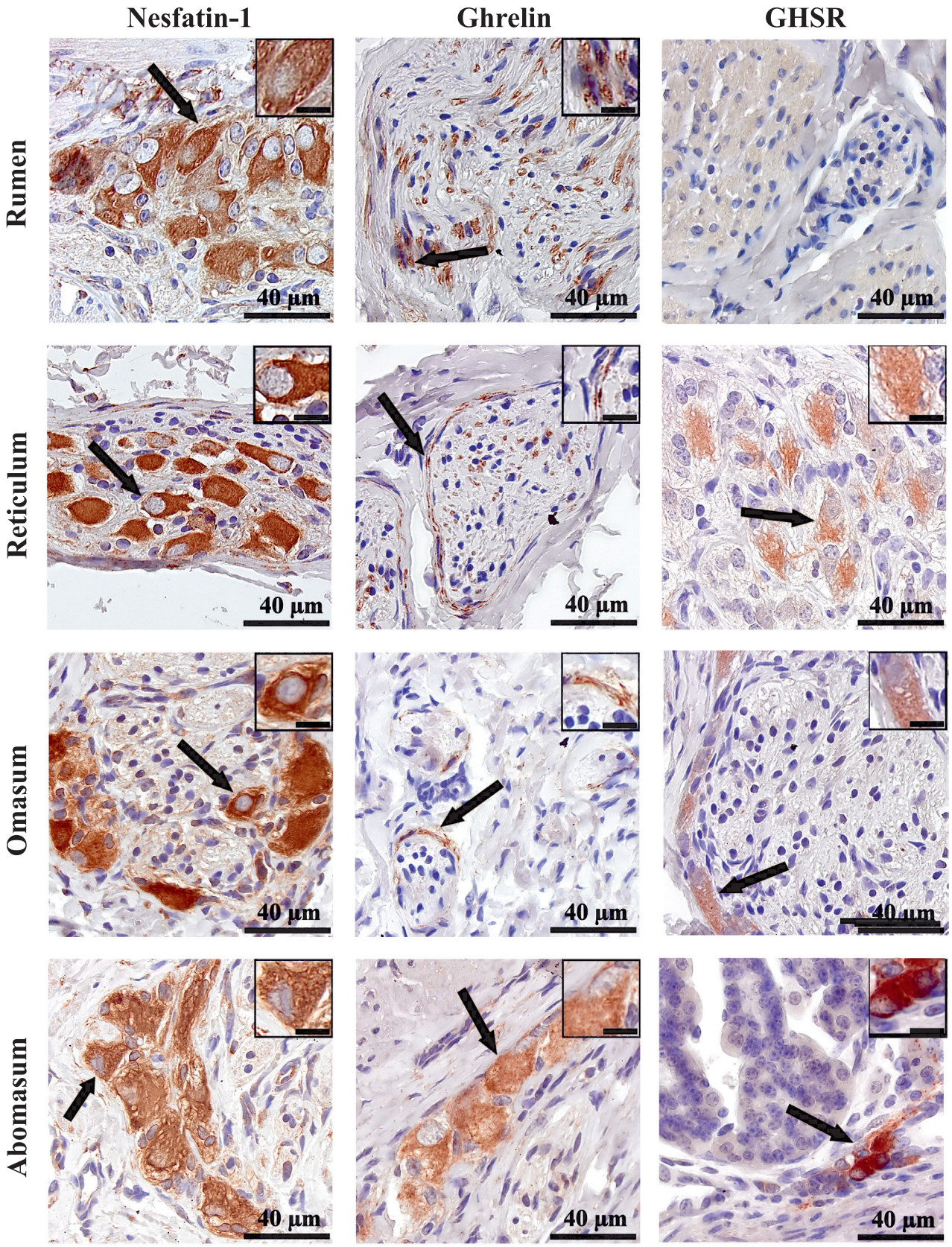

Fig. 4. Immunolocalization of nesfatin-1, ghrelin and GHSR in the enteric nervous system (ENS) of the rumen, reticulum, omasum and abomasum of Holstein-Friesian male calves. Black arrows indicate an example immunoreaction. In the top right corner, a magnified view of the reaction. Scale bar: 40 µm for the main image and 15 µm for the magnified reaction inset.

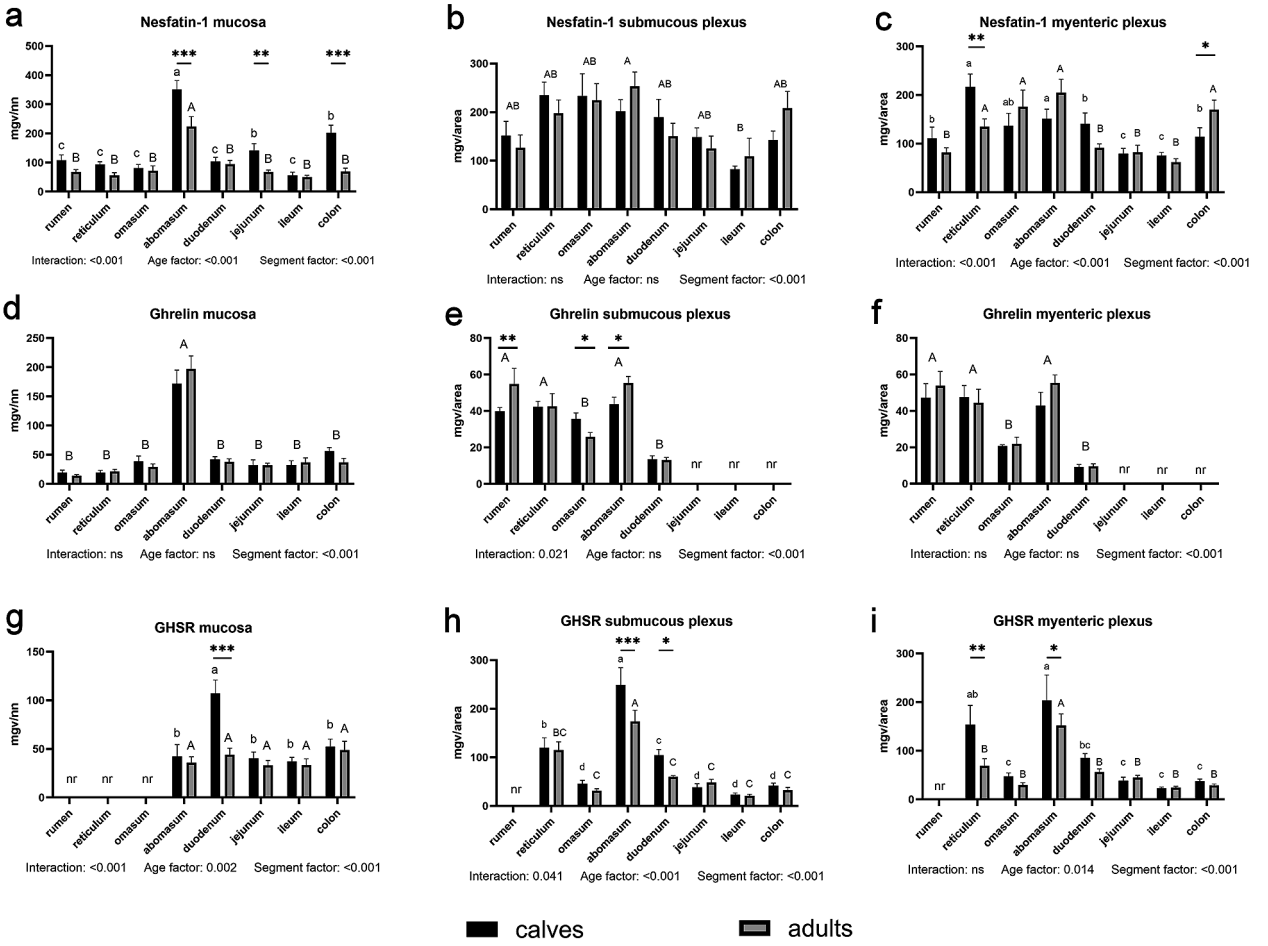

Fig. 5. Immunolocalization of nesfatin-1, ghrelin and GHSR in the enteric nervous system (ENS) of the duodenum, jejunum, ileum and colon of Holstein-Friesian male calves. Black arrows indicate an example immunoreaction. In the top right corner, a magnified view of the reaction. Scale bar: 40 μm for the main image and 15 μm for the magnified reaction inset.

risons revealed that IR was higher in adults in the rumen ($p<0.01$) and omasum ($p<0.05$), whereas in the abomasum, calves exhibited higher IR ($p<0.05$).

In the myenteric plexus (Fig. 1f), no interaction between segment and age was found, but the segment effect was again significant ($p<0.001$). As in the submucosal plexus, no IR was observed in the jejunum, ileum or colon and the omasum displayed significantly lower IR than all other segments ($p<0.001$). No significant age-related differences were observed in this plexus.

Ghrelin protein level

The highest concentration of ghrelin was found in the abomasum of both age groups, with a significant difference compared to the other segments. No other significant differences were observed either between groups or between segments (Fig. 6b).

GHSR immunolocalization

GHSR IR was not detected in the mucosa of the forestomachs. In the remaining GIT segments, sparse IR was observed in epithelial cells, with the most prominent signal found in the duodenum of calves (Fig. 1g and 3). Statistical analysis revealed a significant interaction between age and segment ($p<0.001$). In calves, the duodenum showed significantly higher IR than all other mucosal segments ($p<0.001$), while the remaining positive segments (abomasum, jejunum, ileum, colon) displayed significantly lower and comparable levels. In adults, there were no significant differences in mucosal IR between segments. When comparing age groups, IR in the duodenum was significantly higher in calves than in adults ($p<0.001$).

Within the ENS, GHSR was detected in neuronal cell bodies of both the submucosal and myenteric plexuses across all GIT segments except the rumen, where no IR was observed (Fig. 4 and 5).

In the submucosal plexus (Fig. 1h), statistical analysis showed a significant interaction between segment and age ($p=0.041$). In calves, IR was highest in the abomasum ($p<0.001$ vs. all other segments), followed by the reticulum and then the duodenum, both of which were significantly higher than the omasum, jejunum,

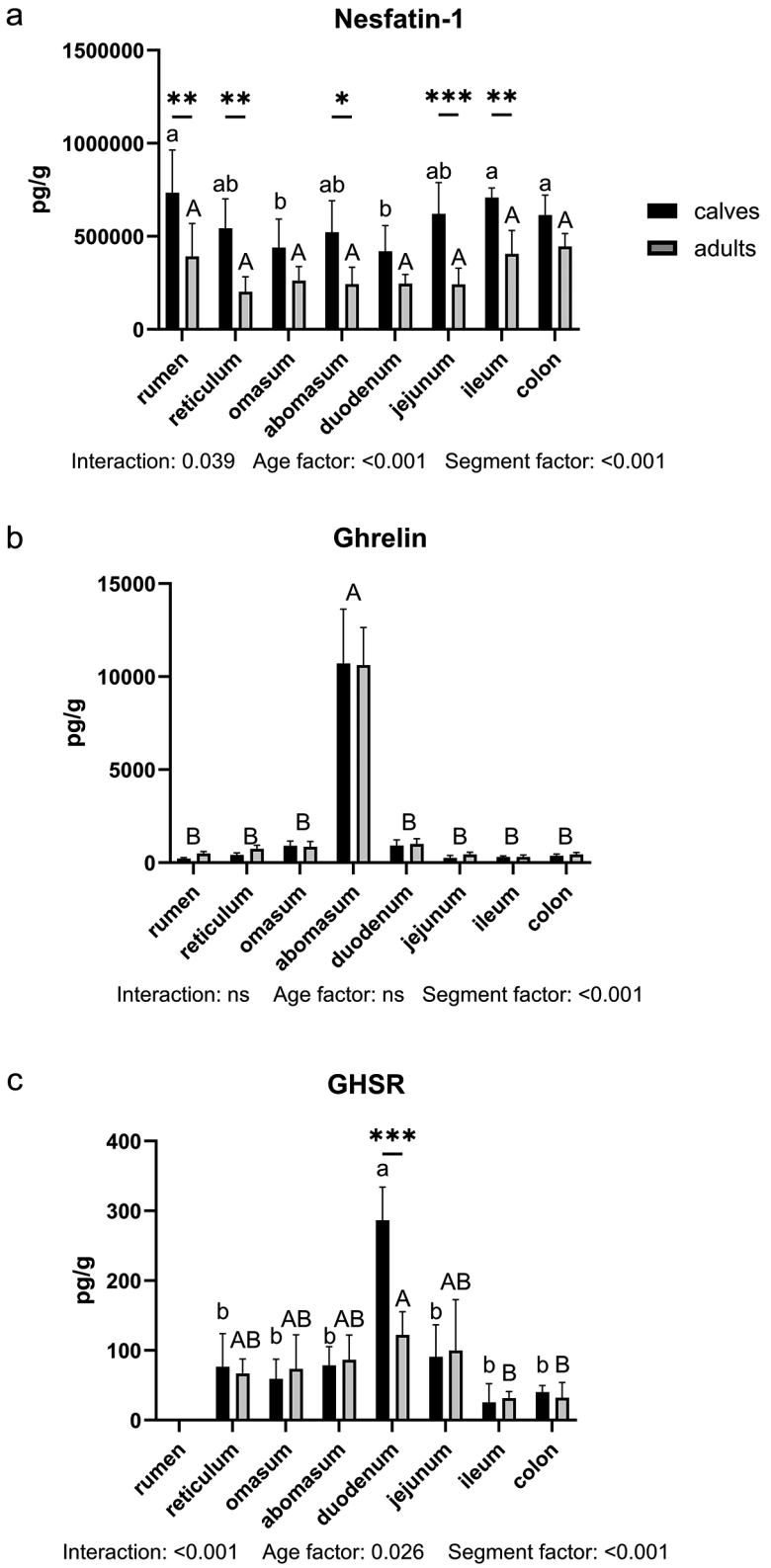

Fig. 6. Nesfatin-1 (a), ghrelin (b) and GHSR (c) concentration [pg/g total protein] in the gastrointestinal tract (GIT) of calves and adult Holstein-Friesian bulls. Different lowercase letters denote significant differences between GIT segments in calves, whereas different uppercase letters denote significant differences between GIT segments in adults ($p<0.05$), unless the interaction between the main factors or age as a main factor was not significant - in such cases, uppercase letters indicate differences between segments regardless of age group. The asterisks (*) highlight significant differences in the concentrations between calves and adults within a specific GIT segment (* for $p<0.05$; ** for $p<0.01$; *** for $p<0.001$).

ileum and colon ($p<0.001$). In adults, the abomasum also showed the highest IR ($p<0.001$ vs. all other segments), followed by the reticulum, while the remaining segments displayed lower and statistically similar immunoexpression. Between age groups, the abomasum ($p<0.001$) and duodenum ($p<0.05$) showed significantly higher IR in calves.

In the myenteric plexus (Fig. 1i), there was no interaction, but both segment ($p<0.001$) and age ($p=0.014$) had significant effects. In calves, IR was highest in the abomasum ($p<0.001$ vs. all other segments), followed by the reticulum, which was significantly higher than the omasum, jejunum, ileum and colon. The duodenum showed intermediate values, not significantly different from the lower-expressing segments. In adults, the abomasum again showed the highest IR ($p<0.001$), while all other segments were significantly lower and statistically comparable. In age comparisons, the reticulum ($p<0.01$) and abomasum ($p<0.05$) displayed higher IR in calves.

GHSR protein level

Protein level measurements show that GHSR is undetectable in the rumen. The highest level is recorded in the duodenum, where the only significant difference between groups is found ($p<0.001$), with higher receptor levels in calves compared to adults. In calves, aside from the significantly higher level in the duodenum compared to other segments, no other intersegmental differences are observed. In adults, however, a difference is noted between the duodenum and the ileum and colon (Fig. 6c).

Discussion

The results of this study provide significant insights into the immunolocalization and levels of nesfatin-1, ghrelin and GHSR in the GIT of Holstein-Friesian bulls, and supports the hypothesis that their levels and localization are age-dependent and region-specific within the bovine digestive system.

An interesting finding was the higher level of nesfatin-1 compared to ghrelin and GHSR, despite the animals being in a fasting state. Nesfatin-1 is recognized for its central anorexigenic activity, opposing the appetite-stimulating effects of ghrelin (Chen et al. 2022). However, despite its central role in suppressing food intake, peripheral nesfatin-1 secretion may fulfill alternative physiological functions. As a result, its level in the GIT during fasting would not necessarily be low, since it may play distinct local roles unrelated to appetite regulation, which has been confirmed in other species (Kras et al. 2022). The highest levels of nesfatin-1 were observed in the abomasum, consistent with the pattern seen for ghrelin, suggesting a functional relationship between these two peptides, as previously discussed in the context of their classification as "sibling peptides" despite their differences. Furthermore, the presence of nesfatin-1 in epithelial cells of the forestomachs, particularly in layers close to the lumen, indicates a local regulatory function within the GIT. Studies have shown that the rumen epithelium is metabolically active and additionally expresses numerous genes involved in immune responses (Fregulia et al. 2021). Therefore, IR in this area may suggest that nesfatin-1 could play either a metabolic or immune-related role. Supporting this, nesfatin-1 has been shown to reduce leukocyte infiltration and pro-inflammatory cytokines in a rat model of intestinal ulcers (Kolgazi et al. 2017). Similar anti-inflammatory effects were observed in other tissues, including the vascular endothelium and lungs, where nesfatin-1 inhibited NF-κB signaling and macrophage activation (Meng et al. 2021, Cheng et al. 2022). However, in certain pathological contexts such as rheumatoid arthritis, it may enhance inflammatory responses (Chang et al. 2023). These findings indicate that the immunoregulatory effects of nesfatin-1 may be tissue- and context-specific. Interestingly, the intense localization of nesfatin-1 within the ENS further supports the notion of its potential involvement in broader aspects of GIT physiology. Although the absence of a clearly identified receptor complicates the interpretation of its local actions, it remains plausible that nesfatin-1 signals via an as yet uncharacterized receptor – possibly involving GHSR—or that it exerts paracrine effects within this region. Nesfatin-1 in cattle has previously been identified solely in plasma (Aydin 2013, Morton et al. 2018); therefore, no available literature exists for comparison with our findings. However, studies conducted on animals such as rats and pigs demonstrated a similar pattern of nesfatin-1 immunolocalization within the GIT, aligning with the distribution observed in our findings (Kras et al. 2022). The increased peptide levels observed in calves may be attributed to age-related physiological factors, including differences in diet, the immature state of the GIT, or regulatory processes that are still undergoing transition and have not yet stabilized, in contrast to those observed in adult animals. Studies also suggest a link between nesfatin-1 and sexual maturation, which may be reflected in the increased nesfatin-1 levels in developing calves (Ranjan et al. 2019).

The IR and concentration of ghrelin followed the expected pattern, with the highest levels observed in the abomasum, consistent with findings in other species (Date et al. 2000, Hayashida et al. 2001). This consistency across species reinforces the evolutionary conser-

vation of ghrelin's role in various physiological processes, including appetite regulation and potentially local actions within the stomach or other functions in the GIT (Kojima and Kangawa 2005, Müller et al. 2015). However, the detection of ghrelin in the ENS of the stomach and duodenum was unexpected. Although the IR was not particularly strong, a clear pattern of ghrelin localization within fibers surrounding ENS neurons suggests that ghrelin may act via the vagus nerve or other neural pathways to mediate gastrointestinal signaling. This interpretation aligns with experimental data showing that ghrelin reduces neuronal activation in the nodose ganglion, a key hub of vagal sensory neurons, following gastric distension. This effect is abolished by vagotomy, confirming that intact vagal afferents are necessary for ghrelin's action on gastric sensitivity (Meleine et al. 2020). Moreover, ghrelin receptors in these neurons co-localize with TRPV1 and ASIC3, ion channels involved in mechanosensation, suggesting that ghrelin modulates gastric afferent excitability at a peripheral level. Complementary findings by Perelló et al. (2022) further support the presence of ghrelin receptor immunoexpression in vagal sensory pathways, and provide a broader framework for ghrelin's neuromodulatory role in gut-brain communication beyond its classic endocrine functions (Perelló et al. 2022). As mentioned previously, ghrelin was detected in the squamous epithelial cells of the forestomachs. However, the absence of ghrelin receptors in the mucosa of all forestomachs suggests that ghrelin may be secreted in this location for a paracrine or systemic effect. This supports the hypothesis that ghrelin could act as a signaling molecule to distant targets via neural or circulatory routes, rather than exerting direct effects within the forestomachs.

Studies on cattle have shown the highest ghrelin levels in the abomasum, consistent with the current results (Arne et al. 2021, Jonova et al. 2022). However, Karakoç et al. (2022) reported ghrelin presence in the smooth muscle of the abomasum, which contrasts with the current findings (Karakoç et al. 2022). This discrepancy could be attributed to methodological differences or variation in sample handling, but further investigation is warranted to clarify this point. According to available data (Sakata and Sakai 2010), ghrelin levels are low in fetuses and increase with age, indicating that ghrelin levels are age-dependent. The lack of differences between calves and adult bulls in our study may suggest that the final ghrelin levels are established before full maturity in cattle, which could reflect its important role in processes not directly linked to the complete development of the digestive tract.

Among the three molecules studied, GHSR exhibited the lowest levels of IR. Notably, GHSR was absent in the rumen, both in ELISA and IHC analysis. In the other forestomachs, GHSR was detected only within the ENS, suggesting that its primary role in these regions might be related to neural signaling rather than local action. This may suggest that ghrelin secreted in the forestomachs does not exhibit a local effect, but rather acts in a systemic manner. In the intestines, however, GHSR was present in both the mucosa and ENS, supporting a dual role in both local tissue response and neural signaling. In the available literature, there is only one study that examined the localization of GHSR in cattle, and it focused exclusively on the abomasum. Karakoç et al. (2022) identified the presence of GHSR in epithelial and parietal cells of the abomasum (Karakoç et al. 2022). However, their study does not provide data on the presence of IR in ENS ganglia, leaving this issue unresolved, especially since our study showed a more intense reaction in the ENS of the abomasum than in the mucosa.

A potential limitation of this study is that nesfatin-1 is derived from its precursor protein, NUCB2. Consequently, the antibodies used in the study may have bound not only to nesfatin-1 but also to NUCB2. This cross-reactivity could have influenced the specificity of the detected signals, potentially affecting the interpretation of the results. In particular, this factor may have contributed to the relatively high nesfatin-1 concentrations observed in comparison to ghrelin and GHSR. A similar consideration applies to ghrelin, which is produced from the precursor protein preproghrelin and may therefore be detected by the antibody alongside its mature form.

This study provides a detailed map of nesfatin-1, ghrelin and GHSR distribution in the bovine digestive tract, highlighting both organ- and age-dependent patterns. High nesfatin-1 levels during fasting and its presence in the ENS suggest broader functional roles beyond appetite regulation. The consistent pattern of ghrelin localization reinforces its conserved function in gastric signaling, while the restricted presence of GHSR in the ENS of the forestomachs underscores its potential role in neural regulation. These findings lay the groundwork for further veterinary research into the complex endocrine and neural interactions governing bovine digestion and metabolism.

Acknowledgements

We would like to express our deepest gratitude to Professor Siemowit Muszyński and Professor Ewa Tomaszewska for their invaluable support during our research and the development of this article. Their guidance, insightful feedback and expertise have been instrumental in shaping our work.

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