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Glutathione S-transferase Mu 3 is associated to *in vivo* fertility, but not sperm quality, in bovine

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ABSTRACT

In the dairy breeding industry, pregnancy of dairy cows is essential to initiate milk production, so that high fertility rates are required to increase their productivity. In this regard, sperm proteins that are indicative of sperm quality and/or fertility have become an important target of study. Glutathione S-transferase Mu 3 (**GSTM3**) has been established as a fertility and sperm quality parameter in humans and pigs and, consequently, it might be a potential biomarker in cattle. For this reason, the present work aimed to determine if GSTM3 could predict sperm quality and *in vivo* fertility in this species. Sperm quality was assessed with flow cytometry and computer-assisted sperm analysis. Immunoblotting and immunofluorescence analysis were performed to determine the presence and localisation pattern of sperm GSTM3. This enzyme was found to be present in bovine sperm and to be localised along the sperm tail and the equatorial segment of the head. No significant associations between sperm GSTM3 and sperm quality parameters were observed, except a negative association with morphologically abnormal sperm having a coiled tail. In addition, and more relevant, higher levels of GSTM3 in sperm were seen in bulls showing lower *in vivo* fertility rates. In conclusion, our data evidenced the presence of GSTM3 in bovine sperm. Moreover, we suggest that, despite not being associated with sperm quality, GSTM3 might be an *in vivo* subfertility biomarker in cattle sperm, and that high levels of this protein could be an indicative of defective spermatogenesis and/or epididymal maturation.

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Implications

In the dairy breeding industry, high fertility is required to increase productivity. The evaluation of fertility biomarkers is, therefore, of high relevance. Accordingly, the present work identifies and localises Glutathione S-transferase Mu 3 in bovine sperm and reveals that, despite not being associated to their quality, it could predict *in vivo* fertility in bulls. Although further research is required, sperm Glutathione S-transferase Mu 3 might be used as a molecular biomarker for *in vivo* fertility in cattle, which can maximise the efficiency and profitability of the dairy breeding industry.

Introduction

Over the years, the dairy breeding industry has selected males and females on the basis of their genetic traits for increasing milk production. Given that the lactation cycle is exclusively initiated by pregnancy, the prediction of bull sperm fertility is crucial to maximise the efficiency of the sector (Pryce et al., 2004; Miglior et al., 2017; Menezes et al., 2019). While the conventional spermiogram is the most commonly used method to evaluate male fertility, it is not always able to predict differences in fertility rates between males because it does not assess the physiological status of sperm (Krzyściak et al., 2020). Consequently, exploring novel sperm fertility biomarkers appears to be an interesting field of study.

Several molecular biomarkers have been reported as potential indicators of male fertility and subfertility (Krzyściak et al., 2020). Sperm proteins related to mitochondrial activity, such as Heat Shock Protein Family D Member 1, as well as antioxidant enzymes, such as glutathione peroxidases and glutathione S-transferases (**GSTs**), have been found to be relevant fertility

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biomarkers in sperm (Kwon et al., 2015). Specifically, GSTs have been reported to play a crucial role in cell detoxification, catalysing the conjugation of electrophilic substances into reduced glutathione (**GSH**) (Hayes et al., 2004).

Alpha, Mu, Omega and Pi GST classes have been described to be present in mammalian sperm, playing a triple role (Llavanera et al., 2019b) consisting of (i) cell detoxification (Fafula et al., 2019), (ii) cell signalling regulation (Cho et al., 2001) and (iii) fertilisation (Petit et al., 2013). In addition, previous research supports that GSTs are essential to maintain sperm quality by protecting the male gamete from oxidative stress (Fafula et al., 2019) through the activation of the JNK signalling pathway (Llavanera et al., 2021a). Furthermore, GSTM3, a specific GST, has been shown to be specifically relevant in sperm cells. This protein belongs to the Mu class of canonical soluble GSTs and is active as a dimer (Armstrong, 1997). It is expressed both in the testis, during spermatogenesis, and throughout the male reproductive tract (Li et al., 2010), and appears to be essential for proper sperm-oocyte binding, interacting with the zona pellucida (Petit et al., 2013). In men, high amounts of sperm GSTM3 are related to low sperm quality in patients suffering from oligozoospermia and varicocele (Botta et al., 2009; Agarwal et al., 2015). In boars, high levels of sperm GSTM3 are joined with small litter sizes (Kwon et al., 2015). GSTM3 has also been established as a sperm quality biomarker in pigs, since its abundance negatively correlates to sperm motility and mitochondrial activity, although its presence is necessary to maintain sperm quality (Llavanera et al., 2020b). Moreover, GSTM3 has been found to be a cryotolerance marker in porcine sperm (Llavanera et al., 2019a).

In spite of all the aforementioned, and to the best of the authors' knowledge, no previous study has investigated the presence and role of GSTM3 in bovine sperm. Considering the relevant function of GSTM3 as a potential quality and fertility biomarker in mammalian sperm, the aim of the present work was to determine the presence and localisation of GSTM3 in bovine sperm, as well as to address whether it could be a sperm quality and *in vivo* fertility biomarker in cattle.

Material and methods

Unless otherwise indicated, chemicals and reagents were purchased from Sigma-Aldrich (Saint Louis, MO, United States).

Animals and ejaculates

Seminal AI-doses used in this study were produced following the Spanish and European legislation for animal husbandry and welfare. Twelve healthy and sexually mature Holstein bulls from 1.5 to 2 years old were involved in this research. Animals were housed at Cenero AI centre in Gijón, Asturias (Spain), under standard feeding and housing conditions. Ejaculates were collected using an artificial vagina. A total of 2 087 heifers were inseminated, with an average number of 174 inseminated heifers per bull. Supplementary Fig. S1 shows the distribution of NRR among bulls. Ninety-day non-return rates (**NRRs**) to oestrus were used to assess *in vivo* fertility, through dividing the serviced heifers by the total number of inseminations.

Ejaculates having 2–8 ml of volume, sperm concentration >10^9 spermatozoa per ml and total motility greater than 85% were cryopreserved. First, the concentration of ejaculates was adjusted using a commercial extender (Bioxcell; IMV Technologies L'Aigle, France) to 92 × 10^6 sperm per ml and then packaged into 0.25-ml straws. The cryopreservation procedure was performed by using a controlled-rate freezer (Digit-cool; IMV Technologies). Straws were then stored in a nitrogen tank. The thawing procedure consisted of warming sperm samples at 38 °C for 20 s in a water bath. A total of six straws per bull were pooled together (biological replicate) before the assessment of sperm quality, using two straws from three independent ejaculates per bull.

Sperm motility

Samples were diluted 1:3 (v:v) with Phosphate-Buffered Saline (**PBS**) and subsequently evaluated. A Computer-Assisted Sperm Analysis (**CASA**) system was used to determine sperm motility parameters (Integrated Sperm Analysis System V1.0; Proiser SL, Valencia, Spain) prewarmed at 38 °C. Sperm loaded into Leja chamber slides (Leja Products BV; Nieuw-Vennep, The Netherlands) were subsequently analysed by capturing 30 frames per second. The average path velocity at ≥10 μm/s was the threshold to consider a spermatozoon as motile, whereas the index of straightness at ≥70% was the threshold to consider a spermatozoon as progressively motile. A total of 1 000 sperm per replicate and two technical replicates per sample were assessed.

Sperm morphology

Sperm samples were diluted 1:3 (v:v) with PBS. Five μl of a diluted sample was used for each examination. Sperm samples were observed under an optical microscope (Olympus BX41) and evaluated using the SCA® Production software (Microptic S.L., Barcelona, Spain). Sperm were visually classified as morphologically normal or abnormal (abnormalities of the head size, shape and acrosome, isolated heads, folded and coiled tails, and proximal and distal droplets). Two hundred sperm per sample were examined.

Oxygen consumption rate

A SensorDish® Reader system (PreSens Gmbh; Regensburg, Germany) was used to evaluate oxygen consumption rate in sperm samples. A volume of 150 μl from each sperm sample was diluted in 850 μl of PBS and transferred onto Oxodish® OD24 plates before sealing them with Parafilm®. Plates were incubated at 38 °C for 3 h, and O_2 concentration was measured every 30 s. Oxygen consumption rate of each sample was subsequently calculated and normalised by the total number of viable sperm per well.

Flow cytometry analysis

Flow cytometry analysis was performed using a CytoFLEX cytometer (Beckman Coulter, California, USA). Sperm samples were diluted in PBS to a final concentration of 4 × 10^6 sperm per ml. Five sperm parameters were evaluated (sperm viability [**SYBR-14/PI**], intracellular calcium levels [**Fluo3-AM/PI**], intracellular Reactive Oxygen Species (**ROS**) levels [**H_2DCFDA/PI**], intracellular superoxide levels [**HE/Yo-Pro-1**], and chromatin (de)condensation [**CMA3/Yo-Pro-1**]). SYBR-14, Fluo3-AM, Yo-Pro-1 and H_2DCFDA were excited with the 488 nm laser, and their fluorescence was detected by the FITC channel (525/40). HE was excited with the 488 nm laser, and its fluorescence was collected through the PE channel (585/42). PI was excited with the 488 nm laser, and its fluorescence was detected by the PC5.5 channel (690/50). CMA3 was excited with the 405 nm laser, and its fluorescence was collected via the Violet610 channel (610/20).

Sperm viability was determined by double staining using SYBR-14 (32 nmol/l) and PI (7.5 μmol/l), based on the protocol of Garner and Johnson (1995). Intracellular calcium levels were evaluated through co-staining with Fluo3-AM (1.2 μmol/l) and PI (5.6 μmol/l), following the protocol described by Harrison et al. (1996). Overall ROS levels were determined after co-staining with H_2DCFDA (100 μmol/l) and PI (6 μmol/l), as described by Guthrie

and Welch (2006). The assessment of intracellular superoxide levels was performed by co-staining samples with Yo-Pro-1 (31.2 nmol/l) and HE (5 μmol/l), following a modification of the protocol of Guthrie and Welch (2006). Finally, chromatin (de)compaction was evaluated following double staining with CMA3 and Yo-Pro-1, as previously described by Llavanera et al. (2021b). Extended flow cytometry protocols are described in detail in Supplementary Material S1.

Immunoblotting analysis

Sperm samples were centrifuged twice at 3 000*g* for 5 min. Total protein content was extracted by mixing sperm samples with 400 μl of lysis buffer (RIPA buffer; Sigma), supplemented with 1% protease inhibitor cocktail (Sigma), followed by incubation on ice for 30 min. Samples were sonicated and subsequently centrifuged at 12 000*g* and 4 °C for 20 min. Finally, total protein of supernatants was assessed through a detergent-compatible method using a commercial kit (BioRad).

Ten μg of total protein was resuspended in Laemmli Reductor 4× buffer supplemented with 10% (v:v) β-mercaptoethanol and heated up to 95 °C for 5 min. Next, samples were loaded onto 12% Mini-PROTEAN TGX Stain-Free Precast Gels (BioRad) and electrophoresis was carried out at 200 V for 40 min at 4 °C. Total protein from the gel was quantified using the Stain-Free method and visualised using a G:BOX Chemi XL system (Syngene, Frederick, MD, United States). Following this, a Trans-Blot Turbo device (BioRad) was used to transfer proteins onto PVDF membranes. Membranes were blocked in blocking buffer (5% BSA in TBS) in agitation for 1 h. Thereafter, membranes were incubated with a primary rabbit anti-GSTM3 antibody (1:15 000; v:v) overnight at 4 °C and, subsequently, washed thrice with TBS-Tween-20 and incubated with an HRP-coupled secondary goat anti-rabbit antibody (1:30 000; v:v) for 1 h. Finally, membranes were washed five times, revealed with a chemiluminescent substrate and visualised with a G:BOX Chemi XL system (Syngene). To assess the specificity of the primary anti-GSTM3 antibody, a peptide competition assay was performed using an excess of the GSTM3 immunising peptide (20-fold regarding the primary antibody). Relative GSTM3 levels were quantified using Image Studio™ Lite v.3.1. (Licor) and normalised against the total protein from the blot. Two technical replicates per sample were analysed.

Immunofluorescence analysis

To determine the localisation of GSTM3 in bovine sperm, an immunofluorescence assay was performed. Sperm samples were adjusted to a final concentration of 5×10^6 sperm per ml, centrifuged at 600*g* for 5 min, resuspended in 2% paraformaldehyde and incubated for 30 min prior to being washed again. Then, 150 μl of diluted sperm was placed on a slide. Subsequently, slides were washed thrice and incubated with PBS supplemented with 1% Triton and 5% BSA for permeabilisation and blocking, respectively. Next, slides were incubated with the primary antibody, diluted at 1:250 (v:v) in blocking solution, at 4 °C overnight. For peptide competition assay, the GSTM3-specific immunising peptide was added 10 times in excess with regard to the primary antibody. Five rinses were performed before incubation with the secondary antibody, diluted 1:500 (v:v) in blocking solution, for 1 h in the dark. Finally, five rinses were performed, and samples were mounted with Vectashield mounting medium before coverage with a coverslip. Samples were observed under a confocal laser-scanning microscope (CLSM, Nikon A1R; Nikon, Tokyo, Japan) with predetermined acquisition settings and analysed using the Fiji ImageJ software (Schindelin et al., 2012). Brightness and contrast were homogenously adjusted in all images.

Statistical analysis

Data were analysed with IBM SPSS Statistics 27.0 (IBM, Armonk, NY, USA) and plotted with GraphPad Prism v.8 (GraphPad Software, La Jolla, CA, USA). First, normal distribution and homogeneity of variances were checked by running Shapiro-Wilk and Levene tests, respectively. Twelve biological replicates were used ($n = 12$), each one being considered a statistical case. The level of significance was set at $P \leq 0.05$.

Sperm samples were classified by their GSTM3 content in two groups on the basis of the median value. Sperm parameters were compared between the two GSTM3 content groups through a parametric *t*-test. Alternatively, a non-parametric Mann-Whitney U test was used when data did not meet normality and/or homoscedasticity assumptions. The Spearman's rank coefficient was used to determine the correlations of sperm GSTM3 levels with sperm quality and *in vivo* fertility parameters.

Results

Presence and localisation of Glutathione S-transferase Mu 3 in bovine sperm

Immunoblotting analysis was performed to determine the presence and relative content of GSTM3 in frozen-thawed bovine sperm. Immunoblotting analysis using an anti-GSTM3 antibody evidenced a single band of ∼48 kDa in every assessed sample

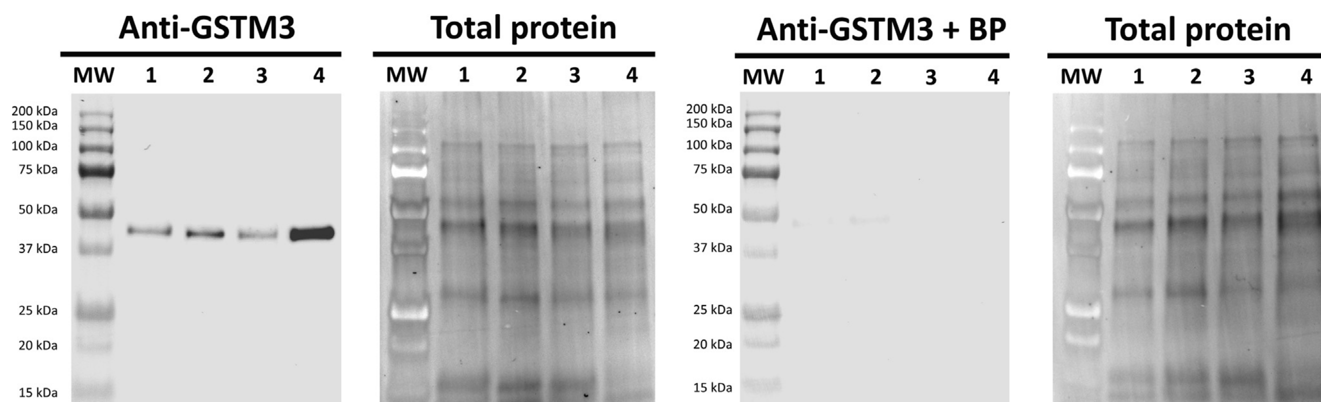

Fig. 1. Representative Western blots of GSTM3 in bovine sperm. (**A**) Incubation with the GSTM3 antibody (Anti-GSTM3) and its loading control (Total protein); (**B**) Incubation with the GSTM3 antibody and the corresponding immunising peptide (peptide competition assay), and its loading control (Total protein). MW: Molecular weight; 1–4 correspond to four independent sperm samples from different bulls. Abbreviations: GSTM3, Glutathione S-transferase Mu 3; BP, blocking peptide.

(Fig. 1A). This ~48 kDa band was absent from the blot incubated with the blocking peptide (Fig. 1B).

The localisation pattern of GSTM3 in bovine sperm was assessed by immunofluorescence. Sperm GSTM3 was found to be localised along the principal, mid and end pieces of the tail, as shown in Fig. 2. Moreover, a weaker GSTM3-specific signal was also found in the equatorial segment of the head. The peptide competition assay did not show green fluorescence.

Correlations of sperm Glutathione S-transferase Mu 3 with sperm quality and fertility

Fig. 3 shows Spearman's rank correlation coefficient between relative GSTM3 content, sperm quality and *in vivo* fertility parameters. No correlation between sperm GSTM3 content and sperm quality parameters was found ($P > 0.05$), excepting a negative correlation between GSTM3 levels and the percentage of morpholog-

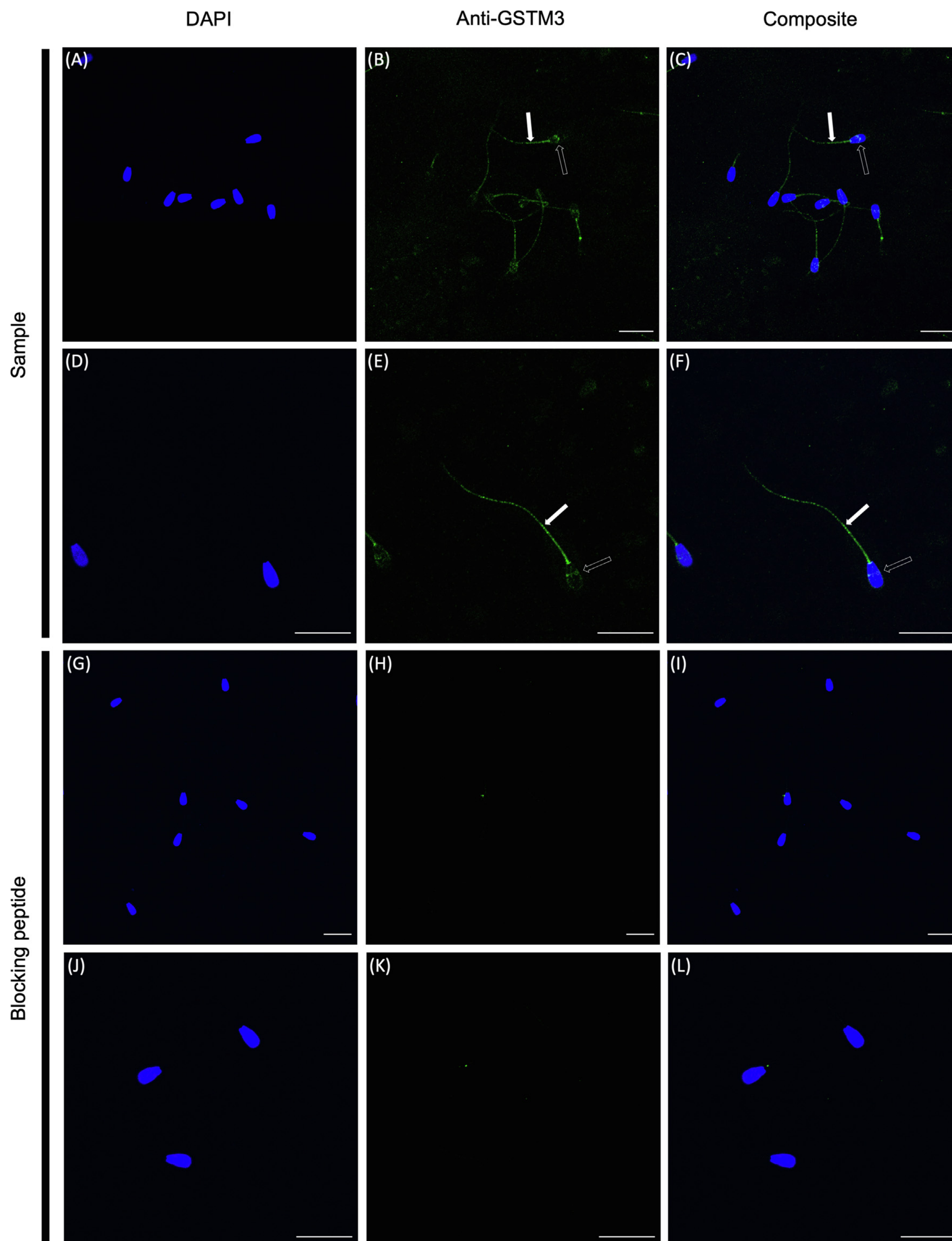

Fig. 2. Representative immunofluorescence analysis of GSTM3 in bovine sperm. (**A-F**) Representative bovine sperm sample (sample). (**G-L**) Peptide competition assay of the sample (blocking peptide). The nucleus is shown in blue (DAPI), whereas GSTM3 is shown in green. White arrows indicate GSTM3 in the sperm tail, whereas black arrows indicate GSTM3 within the equatorial subdomain of the head. Scale bars: 20 μm. Abbreviations: GSTM3, Glutathione S-transferase Mu 3.

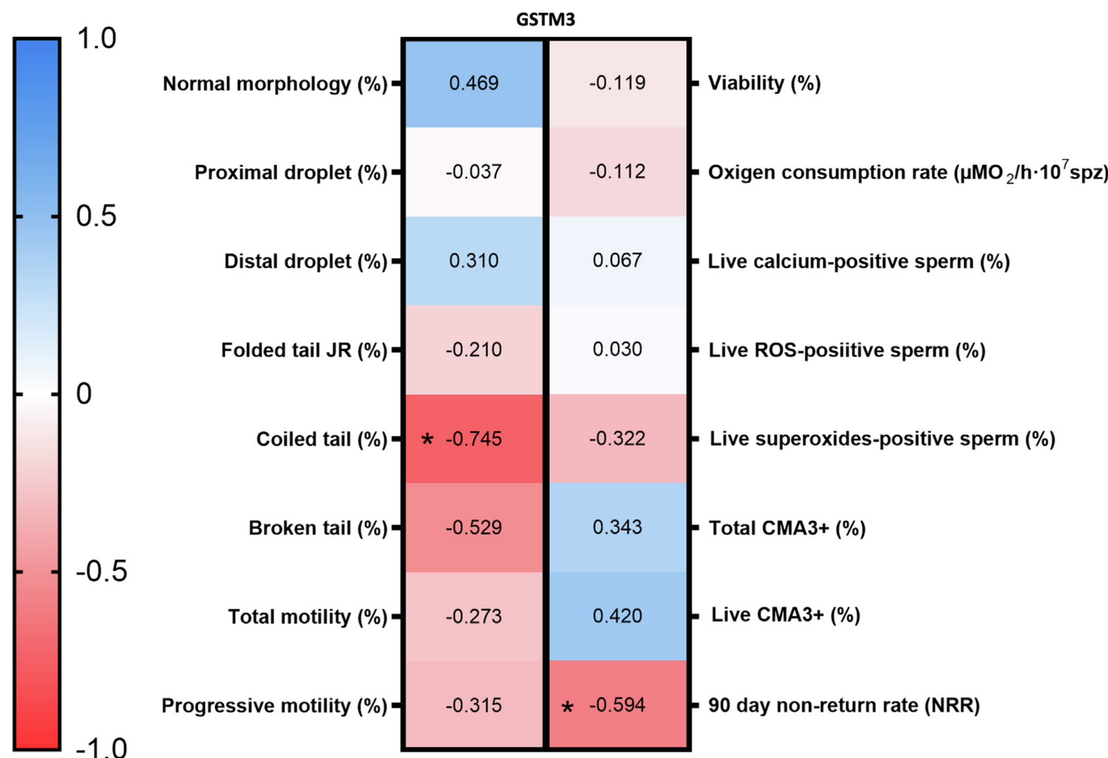

Fig. 3. Heatmap of the Spearman's rank correlation coefficients between relative GSTM3 content in bovine sperm and the different parameters evaluated to determine sperm parameters and *in vivo* fertility. (*) indicates significant correlations ($P < 0.05$). Abbreviations: GSTM3, Glutathione S-transferase Mu 3; JR, Jansen's ring; ROS, reactive oxygen species; $CMA3^+$, chromomycin A3-positive cells.

ically abnormal sperm with coiled tail ($R = -0.75$; $P < 0.05$). Moreover, a significant negative correlation was observed between sperm GSTM3 content and NRR ($R = -0.60$; $P < 0.05$).

Comparison of sperm quality parameters between Glutathione S-transferase Mu 3 groups

Sperm viability, total and progressive motility, and morphology were analysed as conventional sperm quality parameters. Each sperm parameter was compared between GSTM3 groups (low and high GSTM3 content). No significant differences**** ($P > 0.05$) between GSTM3 groups were found when assessing sperm viability (Fig. 4C), total motility (Fig. 4A), progressive motility (Fig. 4B) or morphology (Fig. 4D-I). An increased percentage of morphologically abnormal sperm with coiled tails, however, was found in the low GSTM3 group ($P < 0.05$).

Comparison of sperm metabolic parameters between Glutathione S-transferase Mu 3 groups

Sperm metabolism was evaluated through the analysis of the following sperm parameters: O_2 consumption rate (Fig. 5A), percentage of calcium-positive viable sperm (Fig. 5B), percentage of overall ROS-positive viable sperm (Fig. 5C), and percentage of superoxide-positive viable sperm (Fig. 5D). No significant differences ($P > 0.05$) between GSTM3 groups were observed in any of these metabolism-related parameters.

Comparison of sperm chromatin condensation status between Glutathione S-transferase Mu 3 groups

The putative relationship between sperm GSTM3 content and sperm chromatin (de)condensation status was evaluated through flow cytometry with a double CMA3/Yo-Pro-1 staining. Fig. 6 represents the distribution of the percentages of CMA3-positive cells in total and viable sperm populations between GSTM3 groups (i.e. low and high GTSM3 content). No significant differences ($P > 0.05$) in sperm chromatin condensation were found between GSTM3 groups.

Relationship between sperm Glutathione S-transferase Mu 3 content and in vivo fertility

To evaluate the relationship between sperm GSTM3 content and *in vivo* fertility, NRRs were compared between the two GSTM3 groups (i.e. low and high GSTM3 content). Furthermore, Spearman's rank correlation coefficient between the two parameters was analysed. A negative correlation between sperm GTSM3 content and NRR was observed ($R = -0.60$; $P < 0.05$) (Fig. 7B). Moreover, significant differences in NRR between low and high sperm GSTM3 groups were found ($P < 0.05$). Specifically, higher NRRs were observed in samples showing low levels of GSTM3 (Fig. 7A). Raw data of NRR and sperm GSTM3 levels of each bull are available in Supplementary Table S1.

Discussion

Molecular biomarkers have become relevant tools to predict the physiological status of sperm, which is unachievable with conventional semen analysis (Krzyściak et al., 2020). GSTM3 is an antioxidant enzyme that has been reported to be a useful biomarker for male infertility or subfertility in humans and pigs, as well as a predictor of sperm quality in these species (Botta et al., 2009; Kwon et al., 2015). In this regard, it is reasonable to suggest that sperm GSTM3 could be a potential biomarker for sperm quality and *in vivo* fertility in bovine species.

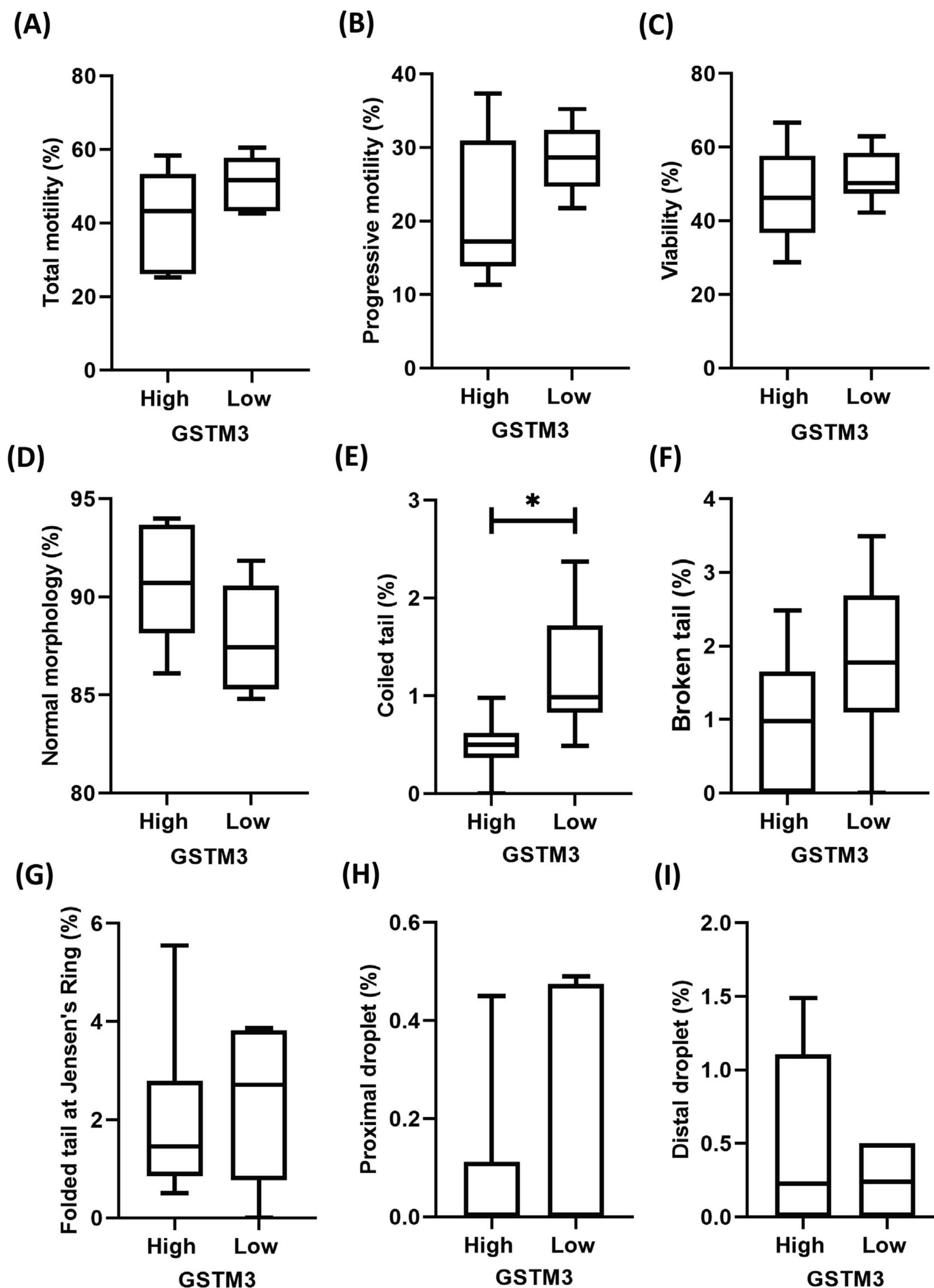

Fig. 4. Box plots representing the distribution of the percentages of (**A**) total motile sperm; (**B**) progressively motile sperm; (**C**) viable sperm; (**D**) morphologically normal sperm; (**E**) sperm with coiled tails; (**F**) sperm with broken tails; (**G**) sperm with folded tails at Jensen's ring; (**H**) sperm with proximal droplets; and (**I**) sperm with distal droplets, between bulls showing high/low sperm GSTM3 content (arbitrary units; AUs). (*) indicates significant differences between GSTM3 groups ($P < 0.05$). Abbreviations: GSTM3, Glutathione S-transferase Mu 3; spz, spermatozoa.

Immunoblotting and immunofluorescence analysis allowed us to confirm the presence and localisation of GSTM3 in bovine sperm, which, to the best of our knowledge, has not been previously described. A single band of ~48 kDa was observed in immunoblotting analysis, which was confirmed to be GSTM3-specific in the peptide competition assay. These results are consistent with the molecular weight of GSTM3 as, while this protein is known to be ~26 kDa in its monomeric form, it has been described to be stable as a homodimer only (Armstrong, 1997) which would explain the presence of the ~48 kDa band in blots. Yet, the unexpected increase in the molecular weight of GSTM3 could also be caused by post-translational modifications, which have been

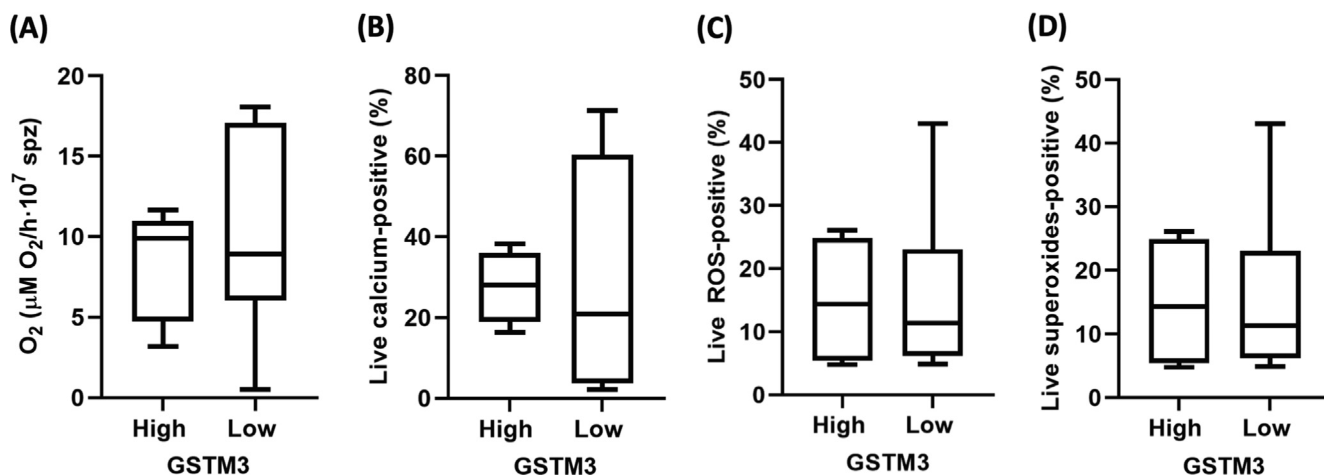

Fig. 5. Box plots representing the distribution of (**A**) O_2 consumption rate normalised against viable sperm (μM O_2/h × 10^7 sperm); (**B**) percentage of calcium-positive viable sperm; (**C**) percentage of ROS-positive viable sperm between bulls showing high/low sperm GSTM3 content (arbitrary units; AUs); and (**D**) percentage of superoxide-positive viable sperm. No significant differences between groups were observed. Abbreviations: GSTM3, Glutathione S-transferase Mu 3; spz, spermatozoa.

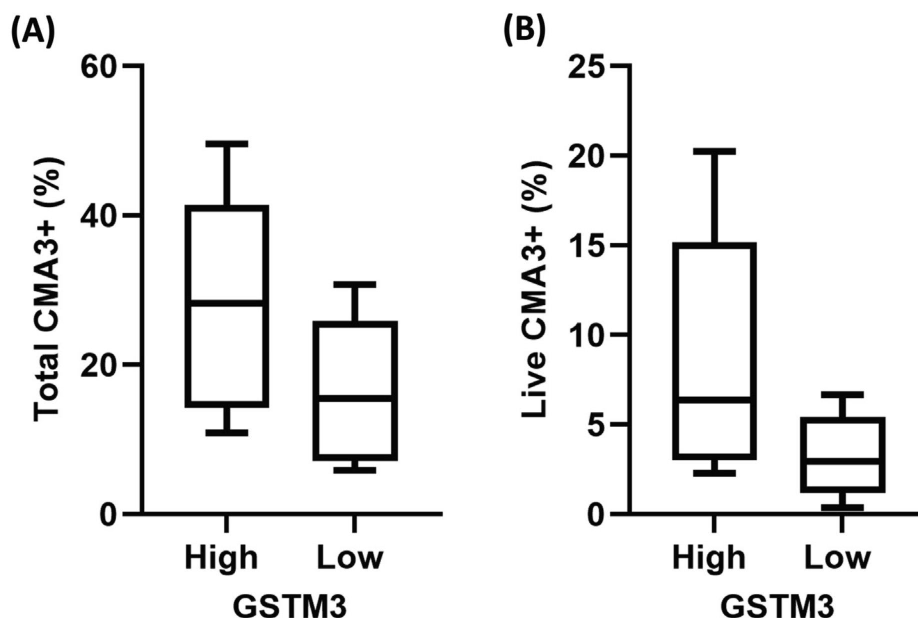

Fig. 6. Box plots representing the distribution of the percentage of (**A**) total and (**B**) viable CMA3-positive sperm between bulls showing high/low sperm GSTM3 content (arbitrary units; AUs). No significant differences between groups were observed. Abbreviations: GSTM3, Glutathione S-transferase Mu 3; $CMA3^+$, chromomycin A3-positive cells.

extensively reported in sperm (Samanta et al., 2016), or covalent protein-protein interactions. In spite of this, while a ~25 kDa band corresponding to monomeric GSTM3 was observed in the sperm of other species (Kwon et al., 2015; Llavanera et al., 2019a), it was not detected herein. Thus, although the presence of GSTM3 was confirmed in the present study, whether its homodimerisation, post-translational modifications and/or covalent protein-protein interactions occur in bovine sperm remains unknown.

Immunofluorescence analysis allowed determining the specific localisation of GSTM3 in bovine sperm. Sperm GSTM3 was found to be present along the sperm tail, comprising mid, principal and end pieces. Interestingly, this pattern partially differs from that observed in frozen-thawed sperm from other species. In pigs, sperm GSTM3 is localised only in the midpiece of the tail (Llavanera et al., 2019a) after cryopreservation, whereas in humans and goats, it is comprised in the acrosome and the postequatorial region (Gopalakrishnan et al., 1998; Petit et al., 2013). Interestingly, the localisation of GSTM3 in frozen-thawed cattle sperm seen in this study was similar to that reported for fresh but not for frozen-thawed pig sperm, where it is found in the sperm tail and the equatorial segment of the head (Llavanera et al., 2020b). Remarkably, the literature, together with our results, suggests a highly variable localisation pattern of sperm GSTM3 among mammalian species.

The association between sperm GSTM3 levels and their quality parameters was also evaluated in the present study. GSTM3 in porcine and caprine sperm has been reported to be essential to maintain sperm motility and mitochondrial activity (Gopalakrishnan and Shaha, 1998; Llavanera et al., 2020b). Interestingly, in this study, no significant correlation between GSTM3 levels and sperm

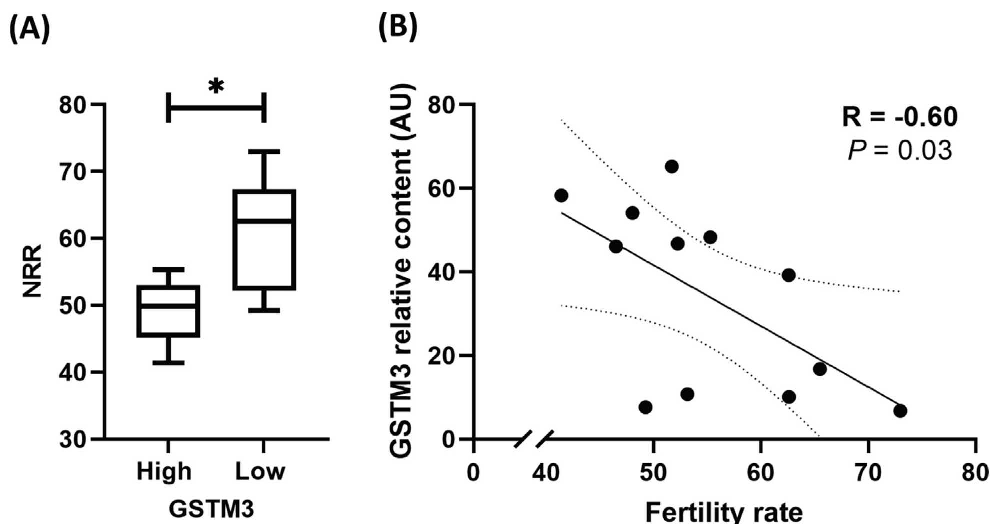

Fig. 7. (**A**) Box plot representing the distribution of non-return rates (NRRs) between bulls showing high/low sperm GSTM3 content (arbitrary units; AUs). (**B**) Scatter plot of Spearman's rank correlation coefficients between GSTM3 relative content and NRR. (*) indicates significant differences between GSTM3 groups ($P < 0.05$). Abbreviations: GSTM3, Glutathione S-transferase Mu 3.

quality and metabolic parameters was observed. Moreover, no differences between high and low GSTM3 groups were seen. These results suggest that GSTM3 is not related to sperm quality and metabolism in cattle. Specifically, given the role of GSTM3 as an antioxidant enzyme, the lack of correlation between this enzyme and ROS levels in sperm is interesting. The fact that GSTM3 shows an association with sperm motility and mitochondrial activity in porcine and ovine, but not in bovine, indicates that the involvement of this protein on sperm physiology is variable among mammalian species. Moreover, sperm GSTM3 was not found to be associated to ROS levels in cattle, which concurs with previous research in humans and pigs and leads one to posit that this enzyme is not involved in cell detoxification in these species.

The results obtained in the present work suggest a putative relationship between sperm GSTM3 levels and sperm morphology, specifically in tail malformations. The percentage of coiled tail sperm was negatively associated with relative GSTM3 content, suggesting that low levels of this enzyme are associated to an increased percentage of sperm with a coiled tail. Accordingly, the percentage of sperm showing this abnormality was also found to be higher in the group with lower GSTM3 content. Sperm malformations can be originated during spermatogenesis (primary malformations) or sperm maturation along the epididymis (secondary malformations) (Briz and Fàbrega, 2013), and the coiled tail is known to be a secondary malformation (Nisa et al., 2018). The association between GSTM3 and secondary malformations has already been discussed in a previous study assessing the function of pig GTSM3 in seminal plasma (Llavanera et al., 2020a). In that study, a role of GSTM3 during epididymal maturation, but not in ejaculated sperm, was suggested (Llavanera et al., 2020a). These results agree with those reported herein, evidencing that GSTM3 might play a key role during epididymal maturation rather than in sperm antioxidant capacity.

Regarding the putative relationship between GSTM3 levels and sperm chromatin status, our work did not find any significant relationship between both parameters, suggesting that GSTM3 is not involved in sperm chromatin (de)condensation. In contrast, Tarozzi et al. (2009) reported a negative relationship between the antioxidant capacity of seminal plasma and the protamination status of sperm chromatin in humans. These differences could be explained because sperm GSTM3 might not play an antioxidant role on sperm, which agrees with the fact that no relationship between its levels and the parameters concerning oxidative stress was observed. Due to the low number of samples used in both studies, nevertheless, further research to elucidate the relationship between antioxidant enzymes and sperm chromatin status is required.

As previously described in other mammalian species, sperm GSTM3 could be related to male fertility, since it is a membrane-bound protein that interacts with the zona pellucida during fertilisation (Botta et al., 2009; Agarwal et al., 2015; Llavanera et al., 2019b). Our results evidenced a negative correlation between sperm GSTM3 content and *in vivo* fertility in bovine sperm. In effect, low sperm GSTM3 levels were found in highly fertile males when compared to subfertile individuals. These results agree with those reported by other authors assessing the relationship between sperm GSTM3 and *in vivo* fertility in humans and pigs, with lower levels of GSTM3 in highly fertile ejaculates (Botta et al., 2009; Kwon et al., 2015). The increased levels of sperm GSTM3 in males showing reduced fertility might be associated to an increased rate of defective spermatogenesis and/or epididymal maturation (Sabeti et al., 2016). Because the main role of GSTM3 is known to be cell detoxification, spermatogonia with high oxidative stress and/or inadequate spermatogenesis may enhance the expression of GSTM3 (Llavanera et al., 2019b). Consequently, sperm with defective spermatogenesis and subsequent impaired fertility might show higher levels of GSTM3, indicating increased oxidative stress during spermatogenesis (Gharagozloo and Aitken, 2011). Moreover, given the previously mentioned association between GSTM3 content and secondary malformations during epididymal maturation (Llavanera et al., 2020a), this antioxidant enzyme could also be implied in this process. Our results are consistent with those reported in men and boars showing sperm GSTM3 as a potential fertility biomarker in these species. In this regard, although further research is required, sperm GSTM3 may be used as a biomarker of *in vivo* fertility rates in bovine, which is of great interest for the dairy breeding industry. Furthermore, these results pave the way for future research regarding the role of sperm GTSM3 in other mammalian species.

In conclusion, immunoblotting analysis evidenced the presence of GSTM3 in bovine sperm for the first time. Immunofluorescence results confirmed the species-specific expression pattern of GSTM3 in bovine sperm, reported along the sperm tail and the equatorial segment, and differing from those observed in other mammalian

species. When analysing its potential role as a sperm quality biomarker, no significant association between GSTM3 and sperm quality and metabolism parameters, such as ROS levels and sperm chromatin (de)condensation, was observed. Interestingly, high GSTM3 levels were related to a lower percentage of sperm showing tail morphologic abnormalities, suggesting a putative function of this enzyme during epididymal maturation. Finally, we also assessed if GSTM3 could be a male *in vivo* fertility biomarker in cattle. As previously described in humans and pigs, high GSTM3 levels in bovine sperm were found to correlate with low fertility rates. Thus, although further studies involving a larger number of animals are required to confirm our results, this work suggests that sperm GSTM3 could be used as a fertility biomarker in bovine.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100609.

Ethical Approval

Not applicable.

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Authors' contributions

ML and **MY** conceived the study. **CT** handled animals and conducted sperm cryopreservation and **AI**. **FG**, **ML**, **EV-V**, **SR** and **AD-B** conducted laboratory analysis. **FG**, **ML** and **MY** participated in the discussion of the results. **FG** and **ML** wrote the Manuscript. **MY** revised and edited the Manuscript. All authors contributed to the finalised Manuscript, read and approved the final version.

Declaration of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be defined as a potential conflict of interest.

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Data and model availability statement

None of the data were deposited in an official repository. The datasets used and/or analyses during the current study are available from the corresponding author on reasonable request.

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