Highlights

- Supplementation of antioxidant compounds to semen extender: taxifolin, glutathione.
- Sperm quality, functionality, and fertility of the native Bermeya goat breed.
- Taxifolin showed encouraging results for cryopreserving goat semen
- Use of frozen semen and artificial insemination in goats.

Title: Use of the flavonoid taxifolin for sperm cryopreservation from the threatened Bermeya goat breed.

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Abstract

Taxifolin is a plant flavonoid effective as an antioxidant. This study aimed to assess the effect of adding taxifolin to the semen extender during the cooling period before freezing on the overall post-thawing sperm variables of Bermeya goats. In the first experiment, a dose-response experiment was performed with four experimental groups: Control, 10, 50, and 100 μg/ml of taxifolin using semen from 8 Bermeya males collected by artificial vagina. In the second experiment, semen from 7 Bermeya bucks was collected and extended at 20 °C using a Tris–citric acid–glucose medium supplemented with different concentrations of taxifolin and glutathione (GSH): control (no antioxidant), 5 μM taxifolin, 1 mM GSH and both antioxidants. In both experiments, two straws per buck were thawed in a water bath (37 °C, 30 s), pooled, and assessed by CASA (motility) at 0, 2, and 5 h. and flow cytometry (viability, intact acrosome membrane, mitochondria membrane potential, capacitation, intracellular reactive oxygen species-ROS, mitochondrial superoxide, and chromatin status) at 0 and 5 h. of incubation at 38 °C. Data were analyzed with the R statistical environment using linear mixed-effects models. Treatment and incubation time were included as fixed factors. Taxifolin increased progressive motility at 10 μg/ml ($P<0.001$) but decreased total and progressive motility at higher concentrations ($P<0.001$). Viability decreased post-thawing in the three concentrations of taxifolin ($P<0.001$). Taxifolin at 10 μg/ml decreased ROS at 0 and 5 hours ($P=0.049$), while all doses decreased mitochondrial superoxide post-thawing ($P=0.024$) but not after the incubation. In experiment 2, 5 μM taxifolin or 1 mM GSH (alone or combined) increased total and progressive motility vs. the control ($P<0.01$). Taxifolin increased kinematic parameters such as VCL, ALH, and DNC ($P<0.05$). Moreover, viability was not affected by the concentration of taxifolin used in this study. Both antioxidants did not significantly affect ROS, mitochondrial superoxide parameters,

or any other sperm physiology parameters assessed. Incubation significantly affected all the parameters studied ($P<0.004$). Fertility after artificial insemination with doses supplemented with 5 μM taxifolin was 76.9% (10/13) and was not different from those inseminated with the control group (69.2%, 9/13). These results are promising for considering taxifolin as a supplement for goat semen cryopreservation.

Keywords: Taxifolin; Goat; Autochthonous breed; Sperm cryopreservation; Sperm quality.

1. Introduction

Semen cryopreservation is a crucial technique for conserving valuable or endangered breeds. Despite the significant and constant progress that has been made in sperm cryopreservation and artificial insemination (AI) in cattle, the use of frozen semen and AI in goats is strongly penalized by the poor quality of the thawed sperm [1]. The variability in fertility rates also supports this statement [2]. During cryopreservation, sperm cells are exposed to cold shock and atmospheric oxygen [3]. This leads to the overproduction of reactive oxygen species (ROS) and increases the susceptibility of the sperm cell membranes to lipid peroxidation [4], contributing to the lower sperm quality and fertility post-thawing [5-8]. Supplementation of antioxidant compounds to semen extender before to cryopreservation could be one of the strategies to prevent oxidative stress [1,9-10]. This procedure could reduce the detrimental impact of ROS, improving post-thawing sperm motility, viability, membrane, and DNA integrity in different species [11-14]. In goats, antioxidants have been added to the semen extender to improve frozen-thawed sperm quality [1,15-20]. Moreover, there is an interest in using natural compounds as antioxidant additives in semen extenders to reduce the harmful effects of ROS on sperm [10]. Taxifolin is a flavonoid found in olive oil, grapes, citrus fruits, and onions [21,22] and some pine trees [23]. It has a strong antioxidant effect [24], inhibits free radical formation at key stages of apoptosis, and was found to exhibit anticancer, neuroprotective, and anti-inflammatory effects [22-25]. As far as we know, there is only one report on animal reproduction supplementing the freezing extender with taxifolin for cryopreserving ram semen [26].

Breed and individual variability are also crucial in goat semen cryopreservation [27]. The goat breed Bermeya, native of Northern Spain and autochthonous of the mountainous region of Asturias, is considered at risk of extinction [28]. The efforts of the

breeders' association (Asociación de Criadores de Cabras Bermeyas, ACRIBER) together with the local government (Gobierno del Principado de Asturias, Spain) and the Regional Centre for Livestock Resources Preservation (SERIDA) have enabled its recovery. Moreover, mainly through ACRIBER's members, there is a renewed interest to rear this well-adapted traditional breed. Furthermore, the Bermeya, like many other autochthonous breeds, has not been characterized regarding sperm parameters both fresh and post-thawing [29].

Therefore, and as the main objective, this article provides information on taxifolin as a new antioxidant to include in the semen extender to improve post-thawed goat sperm quality and also provides essential information on sperm quality, functionality, and fertility of the Bermeya as an autochthonous breed.

2. Material and Methods

2.1. Reagents

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

2.2. Animals and facilities

Bermeya bucks (1.5 to 8 years old) were housed in a small ruminant facility at the Animal Biotechnology Center, SERIDA, Gijón, Spain, where the Genetic Resource Bank for Endangered Domestic Native Animal Species of Principado de Asturias is located and where the semen was collected and cryopreserved.

Bermeya does (3–6 years old) were kept on a farm near Gijón with facilities to perform laparoscopic AI.

All animals were managed according to procedures approved by the SERIDA Ethics Committee, and techniques were performed following the Spanish Policy for Animal Protection (RD53/2013), which conforms to the European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

2.3. Semen collection and cryopreservation

The semen doses were obtained from 15 Bermeya bucks using an artificial vagina (Cassou; IMV Technologies, L'Aigle, France). Ejaculates were routinely obtained during the breeding season (fall), as part of the Animal Biotechnology Center's objectives (germplasm cryobanking), twice per week, with part of the ejaculates being donated for the experiments. Two consecutive ejaculates were collected with a 30-minute recovery interval on each collection day and pooled. Fresh semen was assessed after collection, and only ejaculates with more than 1000×10^6 sperm/ mL, 75% of sperm motility, and 90% of sperm with normal morphology were accepted for freezing. Samples were frozen following the procedure described by Santiago Moreno et al. [3] with minor modifications. Briefly, semen was diluted 1/10 with TGC medium (Tris 313.7 mM, citric acid 104.7 mM, and glucose 30.3 mM; 345 mOsm/kg, pH=6.8) at room temperature (20 °C, RT) and centrifuged (900g, 15 min). The supernatant was then removed, and the pellet was resuspended at RT in TCG medium plus 12% egg yolk (v/v) and glycerol 4% (v/v) (freezing medium). The final sperm concentration was 400×10^6 sperm/mL. After 5 min RT, the diluted sperm samples were transferred to a programmable temperature controller-water bath (PolySience, Temperature Control Solutions, USA) at 20 °C, and the temperature was set to descend to reach 5 °C in 30 min (0.5 °C/min). Samples were maintained at this temperature for 2 h. At this point, aliquots of samples were loaded into 0.25 mL French straws (IMV; L'Aigle, France). Straws were frozen using a

programmable cell freezer (MiniDigitcool 1400, IMV): From 5 °C to −100 °C at a rate of 20 °C/min, from −100 °C to −140 °C at a rate of 10 °C/min. Then, the straws were plunged into liquid nitrogen and stored in a liquid nitrogen container. Semen doses were stored in the GRB-PA at SERIDA until used in this study. Thawing was performed in a water bath at 37 °C for 30 s. Two straws per buck were pooled and assessed.

2.4. Experimental design

Two experiments were performed to study the effect of taxifolin in buck semen. In both cases, samples were cryopreserved and assessed 10 min after thawing and after 5-h incubation at 38 °C (thermoresistance test). Motility and kinematic parameters were also analyzed at 2 h of incubation.

Experiment 1

As described previously, caprine semen from 8 Bermeya males was collected by an artificial vagina and extended at 20 °C. A dose-response experiment was performed with four experimental groups: Control (CTL), 10 (T10), 50 (T50), and 100 μg/ml (T100) of taxifolin (equivalent to 0, 33, 164, and 329 μM of taxifolin). Taxifolin was added just before the cooling process at 20 °C. Then, semen was cooled and frozen, following the previous standard procedure. The experiment was repeated four times (weekly collections from the same males).

Experiment 2

Semen from 7 Bermeya bucks was collected by artificial vagina and extended at 20 °C as described previously. Taking into account the results of Experiment 1, a lower taxifolin concentration was chosen for this experiment. The samples were split into four groups, one processed without supplementation (control, CTL) and the other three supplemented with 5 μM (1.5 μg/ml) taxifolin (T5), 1 mM GSH (GSH), and taxifolin +

GSH simultaneously (TG). The samples were cooled and frozen, as described previously. This experiment was replicated three times.

An artificial insemination trial for assessing the taxifolin supplement on fertility was performed using 29 Bermeya does. The experimental group that offered the best *in vitro* results (taxifolin group) was tested against the control group. Due to the limited number of females, only semen from three males was tested.

2.5. Evaluation of sperm motility and kinematic parameters by CASA (Computer Assisted Sperm Analysis)

Samples were diluted in BioxCell (IMV, L'Aigle, France) at 37 °C to a final concentration of 30×10^6 sperm/mL and loaded in a pre-warmed (38 °C) ISAS D4C20 counting chamber (ISAS; Proiser R+D, Paterna, Spain). At least 1000 spermatozoa in 4-6 fields were analyzed at 10x using a BX40 Olympus microscope equipped with a Basler scA780 video camera (Basler Vision Components, Ahrensburg, Germany) at 25 frames/s. The images were processed with the ISAS software v. 1.19 (ISAS; Proiser R+D, Paterna, Spain). Sperm total motility was recorded as the percentage of total motile spermatozoa (curvilinear path velocity > 10 μm/s) and sperm progressive motility as the percentage showing rapid and progressive movement (straightness of the average path ≥80%). Average kinetic parameters for each sample were recorded as curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity of sperm movement (LIN, %), straightness of the average path (STR, %), wobble coefficient (WOB, %), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), sperm dance (DNC, ALH×VCL), and sperm mean dance (DNCm, ALH×VCL/VSL).

2.6. Flow cytometry assessment of sperm physiology

Flow cytometry was used to assess viability, apoptosis, acrosome, mitochondrial status, capacitation, cytoplasmic ROS (reactive oxygen species), and mitochondrial superoxide production. A CyAn ADP flow cytometer (Beckman Coulter, Inc., Brea, USA) fitted with three diode lasers (violet at 405 nm, blue at 488 nm, and red at 635 nm) was used for analyzing the samples after staining with two fluorophore combinations [10]. The first combination was: 4.5 μM Hoechst 33342 (H342, debris discrimination), 100 nM YO-PRO-1 (YP, apoptotic features), 2 μM Merocyanine 540 (M540, membrane disorder), 3 μM propidium iodide (PI, viability), and 100 nM MitoTracker deep red (MT, mitochondrial status). The second combination contained: 4.5 μM Hoechst 33258 (H258, viability), 5 μM CM-H_2DCFDA (CFDA, cytoplasmic ROS detection), 1 μM MitoSOX (MSX, mitochondrial superoxide), and 1 μg/ml peanut agglutinin conjugated with Alexa 647 (PNA, acrosomal status). The samples were adjusted to 1.7×10^6 sperm/ml in PBS 0.5% BSA containing the fluorochrome combinations and incubated at 38 °C for 15 min in the dark. The excitation/emission arrangement was: Violet laser line (405 nm) with a 450/50 filter for H258 and H342; blue laser line (488 nm) with a 530/40 filter for YO-PRO-1 and CFDA, a 575/25 filter for M540, and a 613/20 filter for PI and MSX; red laser line (633 nm) with a 665/20 filter for MT and PNA Alexa 647. The acquisition was controlled with the Summit software v. 4.3.02 (Beckman Coulter), acquiring at least 10,000 spermatozoa. Debris was gated out through a combination of FSC/SSC (forward/side scatter) and H342 regions ($H342^{neg/dim}$ as debris) or H258 ($H258^{neg}$ as debris). Data were saved as FCS v.3 files and analyzed with the Weasel v. 3.5 software (Frank Battye, Melbourne, Australia) [10].

2.7. Sperm chromatin assessment (Sperm Chromatin Structure Assay, SCSA)

Chromatin status was assessed by SCSA [30]. Briefly, 200 µl of samples at 2×10^6 ml^{-1} in TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, and 1 mM disodium EDTA, pH 7.4) were mixed with 0.4 mL acid-detergent solution (0.08 M HCl, 0.15 M NaCl, and 0.1% Triton X-100, pH 1.2). After 30 s, 1.2 mL of staining solution (6 µg/mL AO (Polysciences, Inc, Warrington, PA, USA) in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM disodium EDTA and 0.15 M NaCl, pH 6.0) was added to the tube. The tube was kept on ice for 3 min before analysis. A FACScalibur flow cytometer was used for analysis with the acquisition software CellQuest version 3.1 (Becton Dickinson). At least 5000 spermatozoa were analyzed per sample, exciting the AO with an Ar-ion laser at 488 nm and using a 530/30 filter for the green fluorescence of dsDNA-bound AO and a 650 long-pass filter for the red fluorescence of ssDNA-bound AO. Data were saved in flow cytometry standard (FCS) version 2 files. The DNA fragmentation index (DFI) was calculated for each spermatozoon as the red fluorescence ratio to total (red+green) fluorescence. From the %DFI, the standard deviation of DFI (SD-DFI) and the percentage of spermatozoa with a high fragmentation index (DFI >250; %DFI) were determined. Chromatin immaturity was estimated as %HDS, defined as the proportion of events with high green fluorescence.

2.8. Laparoscopic Artificial Insemination

Twenty-nine Bermeya does with proven fertility were randomly assigned to be inseminated with control or T5 (5 µM taxifolin) frozen-thawed doses. Females were estrus synchronized, followed by laparoscopic artificial insemination as described by Santiago-Moreno et al. [31]. Briefly, progestagen sponges were inserted (20 mg fluorogestone acetate, FGA, Chronogest1, Intervet International BV, Holland) at unknown stages of their estrous cycles (day 0) for estrus synchronization. The goats were

treated with equine chorionic gonadotropin (eCG, Foligon, Intervet International BV; 300 IU i.m.) and cloprostenol (Estrumate, Schering-Plough, S.A., Madrid, Spain; 120 mg dose i.m.) on day 9, and the sponges were removed on day 11. The goats were inseminated by laparoscopy at approximately 50 h after the withdrawal of the vaginal sponge. At the time of artificial insemination, human chorionic gonadotrophin at 400 IU (hCG, Chorulon, Intervet International BV) was administered. On day 35 after intrauterine insemination, the pregnancy status was assessed using an ultrasound scanner (ProScan CVM, Navarra, Spain) equipped with a 7.5-MHz transducer.

2.9. Statistical analysis

Data were analyzed in the R statistical environment [32] using linear mixed-effects models (lmerTest package) [33]. Treatment and incubation time were included as fixed factors, with the buck as the grouping factor in the random part of the models. The level of significance was set at $P \leq 0.05$. Pregnancies were assessed using the Chi-squared test. Results are described as mean±SEM unless otherwise stated.

3. Results

In experiment 1, taxifolin showed similar values to the control at 10 µg/ml for motility parameters (Fig. 1), with the higher 50 and 100 µg/ml concentrations showing reduced total and progressive motility (Fig. 1a and b; $P < 0.05$ for T100 at all incubation times), increased linearity and beat frequency, and lower ALH and dance parameters after incubation (Fig. 1f-l). Overall (main effects, compared with CTL), progressive motility increased in T10 ($P = 0.010$); however, total motility decreased in T50 and T100 ($P < 0.001$), and progressive motility decreased in T100 ($P < 0.001$). The flow cytometry analysis (Fig. 2) showed that taxifolin was detrimental to sperm physiology, more

evidently after the 5-h post-thawing incubation. Viability (as membrane integrity and sperm free from apoptotic features, Fig. 2a and b) decreased in the three concentrations of taxifolin (main effects: $P < 0.01$ for T10 and $P < 0.001$ for T50 and T100). Concurrently, the apoptotic ratio (Fig 2c), acrosomal damage (total and as the ratio of viable sperm, Fig. 2d and 2e) increased in the taxifolin treatments, especially at T100 and after the incubation (main effects as $P < 0.05$ for T50, $P < 0.001$ for total acrosomal damage, and $P < 0.001$ for T100). However, taxifolin tended to reduce the capacitation ratio, which increased with the incubation, but T50 reduced this, so the difference was non-significant (Fig. 2f; overall effects of T50 and T100 were $P < 0.01$). Mitochondrial activity significantly decreased after the incubation, while cytoplasmic and mitochondrial ROS increased (Fig. 2g-i). Whereas average values were similar post-thawing after the incubation, T50 and T100 showed significantly lower mitochondrial activity with higher mitochondrial superoxide production. However, T10 allowed slightly lower cytoplasmic levels with no significant differences for the other variables. No differences were observed for treatment or incubation for the chromatin status parameters, except for a small but significant increase of SD-DFI after the incubation (Fig. 2j-l).

In experiment 2, taxifolin 5 μM (TXF) and GSH 1 mM alone or combined (TXG) increased total and progressive motility vs. the control (Fig. 3a and b; main effects, $P < 0.001$). The incubation affected some kinematic parameters (Fig. 3). Whereas, in general, the treatments by incubation time did not significantly affect the kinematic parameters, the main effects analysis showed a significant positive effect of TXF for VCL, VSL, VAP, ALH, DNC, and DNCm ($P < 0.01$ except for DNCm, $P < 0.05$), and of GSH ($P < 0.05$) for VCL, ALH, DNC, and DNCm. The main effects analysis of TXG showed a negative effect on WOB ($P = 0.016$) and a positive one on DNCm ($P=0.006$). TXF and GSH positively affected sperm viability after the incubation (Fig. 4a and b).

Their combination lacked this effect, and it slightly increased the acrosomal damage as the ratio of viable spermatozoa (Fig. 4e). Furthermore, the incubation increased the acrosomal damage (Fig. 4d and e) capacitation-like features (Fig. 4f), and the mitochondrial superoxide (Fig. 4i) while decreasing mitochondrial activity (Fig. 4g) and cytoplasmic ROS (Fig. 4h). Contrarily, the treatments did not significantly affect these parameters. However, the main effects analysis showed a significant ($P < 0.05$) positive effect of TXF and GSH (but not TXG) on mitochondrial activity. After the incubation, chromatin status was slightly affected by GSH presence, with GSH and TXG significantly increasing %DFI and %HDS (Fig. 4k and l).

During the fertility trial, three does die for reasons unrelated to the study, with 26 does diagnosed by echography. Pregnancy diagnostic was 9/13 (69.2%) in the control group and 10/13 (76.9%) in the TXF group ($P = 1$ for χ^2, Monte Carlo test with 10^5 draws).

4. Discussion

This is the first report on using the flavonoid taxifolin for the cryopreservation of goat semen. Additionally, we present a practical case to improve sperm cryopreservation to preserve a local endangered breed, the Bermeya goat. Indeed, as a scientific article, our study shows, for the first time, the overall characterization of the post-thawing sperm quality of this breed. The control group in the two experiments displayed valuable information on post-thawed sperm quality for this autochthonous breed. Moreover, the fertility trial using frozen-thawed semen yielded excellent results in pregnancy rates, showing the potential of reproductive biotechnologies to recover and preserve this native breed.

Reports on the effects of taxifolin on animal reproduction are scarce, and studies on domestic animals are lacking. While quercetin, a close compound to taxifolin, has shown some beneficial effects in semen cryopreservation in different animal species [34-39], as far as we know, there is only one report on taxifolin, for freezing ram semen [26]. Therefore, the concentration of taxifolin adequate for freezing goat semen is unknown. Thus, Experiment 1 aimed to define the toxicity range of taxifolin. Taken together, these results suggest that both 50 and 100 μg/ml (by >100 μM range) could be toxic and, therefore, detrimental to sperm physiology during freezing/thawing, while the yield of 10 μg/ml (33 μM), whereas seemingly not optimal, indicated that lower concentrations could be adequate. These results motivated the second experiment, which supported this hypothesis. The much lower taxifolin concentration of 5 μM improved total and progressive motility with no effect on viability. In addition, taxifolin showed some antioxidant effects, decreasing cytoplasmic ROS in Experiment 1, but only after the incubation and at 10 μg/ml. However, these effects were non-significant in the second experiment with a lower dose of taxifolin. Paradoxically, 50 and 100 μg/ml caused an increase in spermatozoa producing mitochondrial ROS after incubating the samples. These contradictory results are in line with previous studies applying antioxidants to spermatozoa. Many studies have found low ROS in ruminant spermatozoa post-thawing, similar to our research [10,40-43]. Therefore, the supplementation with antioxidants shows detrimental or paradoxical effects that are attributed, at least in part of the cases, to altering physiological ROS levels. Several antioxidants have demonstrated toxic effects on ruminant spermatozoa if added before or after the freezing/thawing [41,42,44]. In fact, in some cases, antioxidants induce an increase in cellular ROS or associated parameters but do not necessarily cause a detrimental effect [43,44]. In this regard, the study of Bucak et al. [26] with taxifolin in sheep showed results similar to ours in sperm

motility and physiology. These authors found that taxifolin at the highest concentrations negatively affects sperm motility and viability. These results suggest related processes, but still, it is essential to assess the effect of taxifolin in sperm cryopreservation in each species of interest to apply specific solutions. Whereas a similar concentration, 10 μM, yielded acceptable results in ram, it might be practical to test lower concentrations in ram to confirm if further improvement occurs.

In the second experiment, we added glutathione (GSH) since it is a well-known antioxidant that has been used in the cryopreservation of semen from many species [44-52]. GSH has a critical role in protecting the cell, especially its membranes, from oxidative damage. We were interested in comparing its effectivity with taxifolin and investigating an interaction between both antioxidants. Whereas their effects differ, with GSH increasing total and progressive motility and viability, we did not detect statistical interactions. Therefore, their action mechanisms are likely to vary.

Whereas assessing sperm quality is a valuable estimator for cryopreservation success, the ultimate goal is to achieve good fertility post-thawing. This is important not only for economic reasons but also, as it is one of the objectives of the present study, to ensure the viability of germplasm banking for the preservation and dissemination of endangered breeds [29,53,54]. Laparoscopic artificial insemination (LAI) showed excellent results in pregnancy rates, both in control and in the taxifolin group. Goat fertility with frozen-thawed sperm, using LAI, usually ranges from 57% to 70% [55]. Taxifolin showed no detrimental effects and at least performed as well as the control group. In addition, the percentage of pregnancy rate was higher (not significantly, though). The low number of females used in this experiment was a limitation, affecting the power of the statistical test. Bermeya goat is a breed at risk of extinction, which constrained the number of available females. Nevertheless, these results are promising,

and taxifolin supplementation could be tested in commercial breeds with a larger number of females to collect evidence for an increase in post-thawing sperm fertility.

As a final comment, the post-thawing chromatin status of Bermeya goat spermatozoa was excellent, even in the absence of antioxidants, and both DNA fragmentation and chromatin compaction were little affected by post-thawing incubation. Interestingly, taxifolin (even at the higher concentrations of Experiment 1) did not affect the chromatin status, but GSH showed a small but significant effect on higher DNA fragmentation and lower chromatin compaction. This was observed in previous studies where GSH showed an overall protective effect during sperm cryopreservation, but these effects were evident, especially after a post-thawing incubation at physiological temperature [44,56]. Nevertheless, the experimental conditions and GSH concentration influence these effects [10,51]. These are likely caused by a reducing impact on disulfide bridges between protamines, causing a partial decondensation in some spermatozoa (with an apparent increase in DNA damage, but possibly due to lower compaction of the nucleus [57].

To sum up, taxifolin showed a lack of toxicity in the low micromolar range and some encouraging results for cryopreserving goat semen. Although taxifolin and GSH did not show evidence of synergic effects, each supplement showed benefits as supplements. In addition, the information obtained in this study could help the conservation of other autochthonous, non-commercial, or endangered breeds. Future experiments could confirm our findings on commercial breeds (with more extensive fertility analyses).

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Fig. 1. CASA results (mean±SEM) for Experiment 1, showing the effect of taxifolin at 10, 50, and 100 µg/ml (T10, T50, and T100; CTL: Control) on motility and kinematics variables directly post-thawing (PT), and after 2 and 5 h of incubation at 37 °C. VCL: Curvilinear velocity; VAP: Average-path velocity; VSL: Straight-path velocity; LIN: Linearity (VSL/VCL×100); STR Straightness (VSL/VAP); WOB: Wobble (VAP/VCL×100); ALH: Amplitude of the lateral displacement of the sperm head; BCF: Frequency of the flagellar beat; DNC: Dance (ALH×VCL); DNCm: Mean Dance (ALH×VSL/VCL). Treatments with different Latin letters differ by $P < 0.05$ within each incubation group, and those with different Greek letters differ by $P < 0.05$ among incubation groups and within the same treatment.

Fig. 2. Flow cytometry results (mean±SEM) for Experiment 1, showing the effect of taxifolin at 10, 50, and 100 µg/ml (T10, T50, and T100; CTL: Control) on physiology and chromatin structure variables directly post-thawing (PT), and after 2 and 5 h of incubation at 37 °C. PI: Propidium iodide; H258: Hoechst 33258; PNA: Peanut agglutinin (Alexa 647 conjugated); M540: Merocyanine 540; MT: MitoTracker deep red; MSX; MitoSOX; SCSA: Sperm chromatin structure assay; SD-DFI: Standard deviation of the DNA fragmentation index; %DFI: % spermatozoa with high DFI (fragmented DNA); %HDS: % spermatozoa with high DNA stainability (low compaction or chromatin immaturity). Treatments with different Latin letters differ by $P < 0.05$ within each incubation group, and with different Greek letters differ by $P < 0.05$ between incubation groups and within the same treatment.

Fig. 3. CASA results (mean±SEM) for Experiment 2, showing the effect of taxifolin at 5 µM (1.5 µg/ml) (TXF), GSH at 1 mM (GSH), their combination (TXG), or no

supplement (CTL: Control) on motility and kinematics variables directly post-thawing (PT), and after 2 and 5 h of incubation at 37 °C. VCL: Curvilinear velocity; VAP: Average-path velocity; VSL: Straight-path velocity; LIN: Linearity (VSL/VCL×100); STR Straightness (VSL/VAP); WOB: Wobble (VAP/VCL×100); ALH: Amplitude of the lateral displacement of the sperm head; BCF: Frequency of the flagellar beat; DNC: Dance (ALH×VCL); DNCm: Mean Dance (ALH×VSL/VCL). Treatments with different Latin letters differ by $P < 0.05$ within each incubation group, and with different Greek letters differ by $P < 0.05$ among incubation groups and within the same treatment. Whereas the treatments showed few significant effects within each incubation time (except for total and progressive motility), main effects analysis indicated significant effects on CTL for TXF ($P < 0.01$ for VCL, VSL, VAP, ALH, and DNC, and $P < 0.05$ for DNCm), for GSH ($P < 0.05$ for VCL, ALH, DNC, and DNCm), and for TXG ($P < 0.05$ for WOB and $P < 0.01$ for DNCm).

Fig. 4. Flow cytometry results (mean±SEM) for Experiment 2, showing the effect of taxifolin at 5 µM (1.5 µg/ml) (TXF), GSH at 1 mM (GSH), their combination (TXG), or no supplement (CTL: Control) on physiology and chromatin structure variables directly post-thawing (PT), and after 2 and 5 h of incubation at 37 °C. PI: Propidium iodide; H258: Hoechst 33258; PNA: Peanut agglutinin (Alexa 647 conjugated); M540: Merocyanine 540; MT: MitoTracker deep red; MSX; MitoSOX; SCSA: Sperm chromatin structure assay; SD-DFI: Standard deviation of the DNA fragmentation index; %DFI: % spermatozoa with high DFI (fragmented DNA); %HDS: % spermatozoa with high DNA stainability (low compaction or chromatin immaturity). Treatments with different Latin letters differ by $P < 0.05$ within each incubation group, and with different Greek letters differ by $P < 0.05$ between incubation groups and within the same treatment.

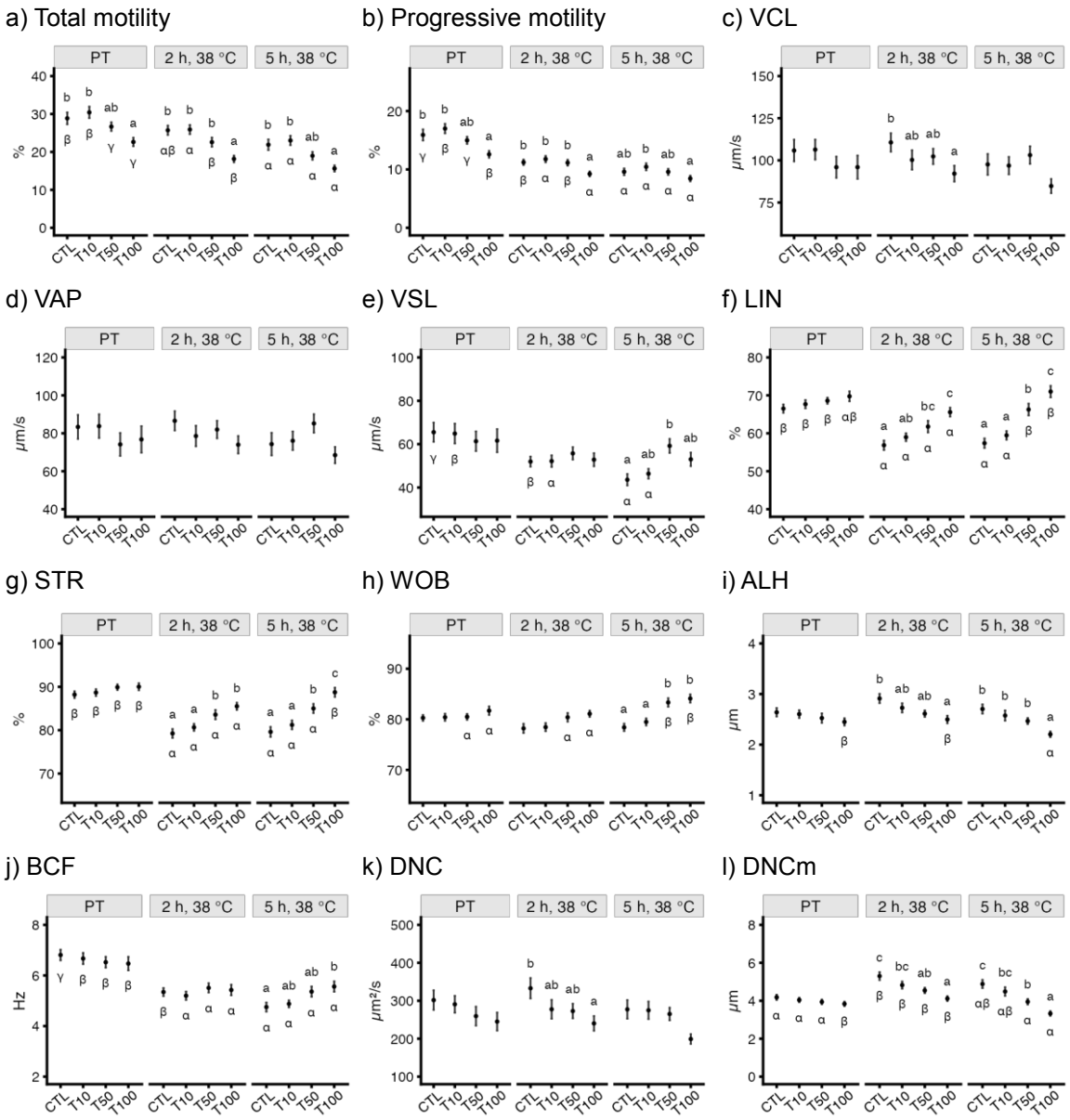

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Figure 2

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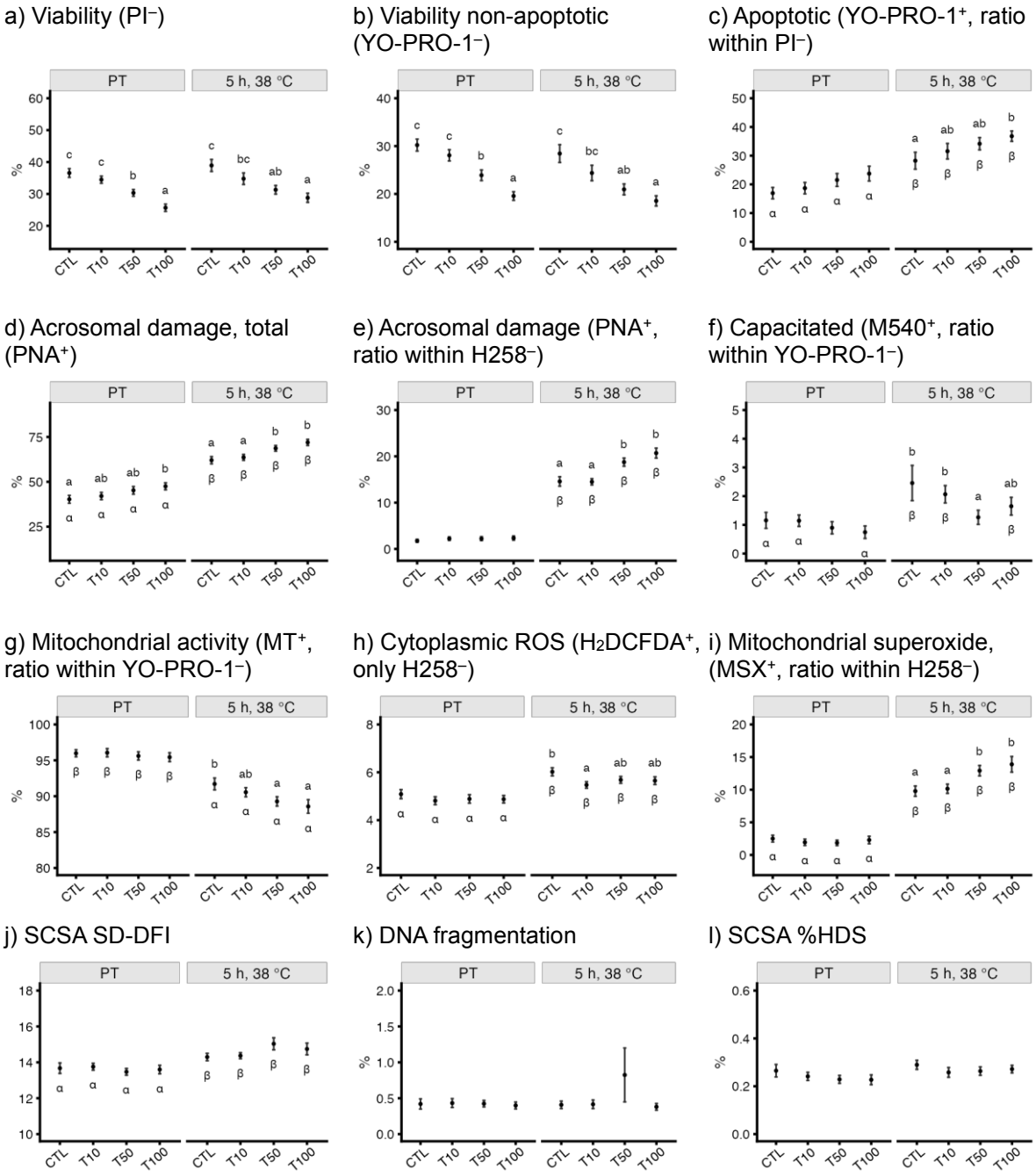

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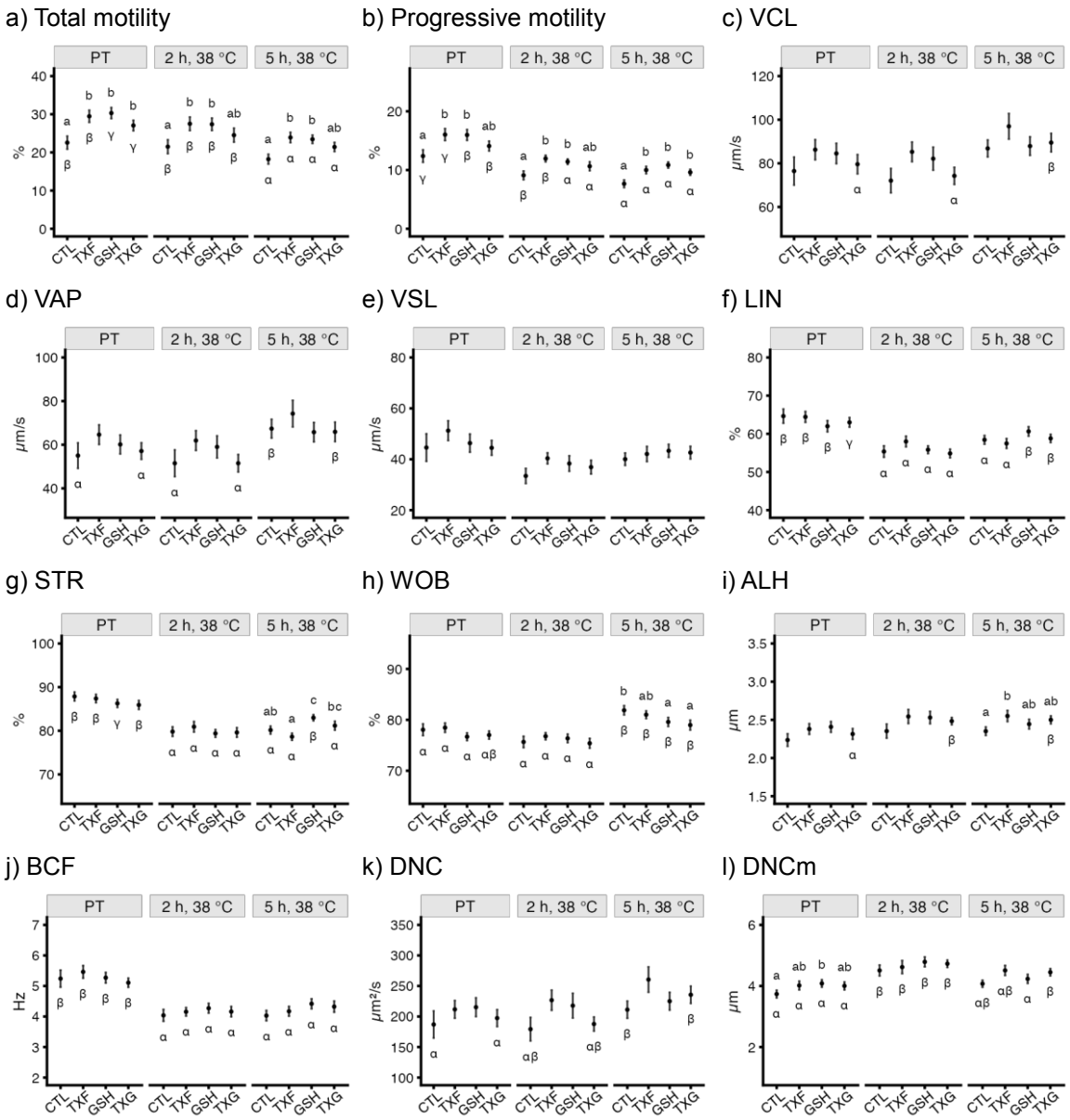

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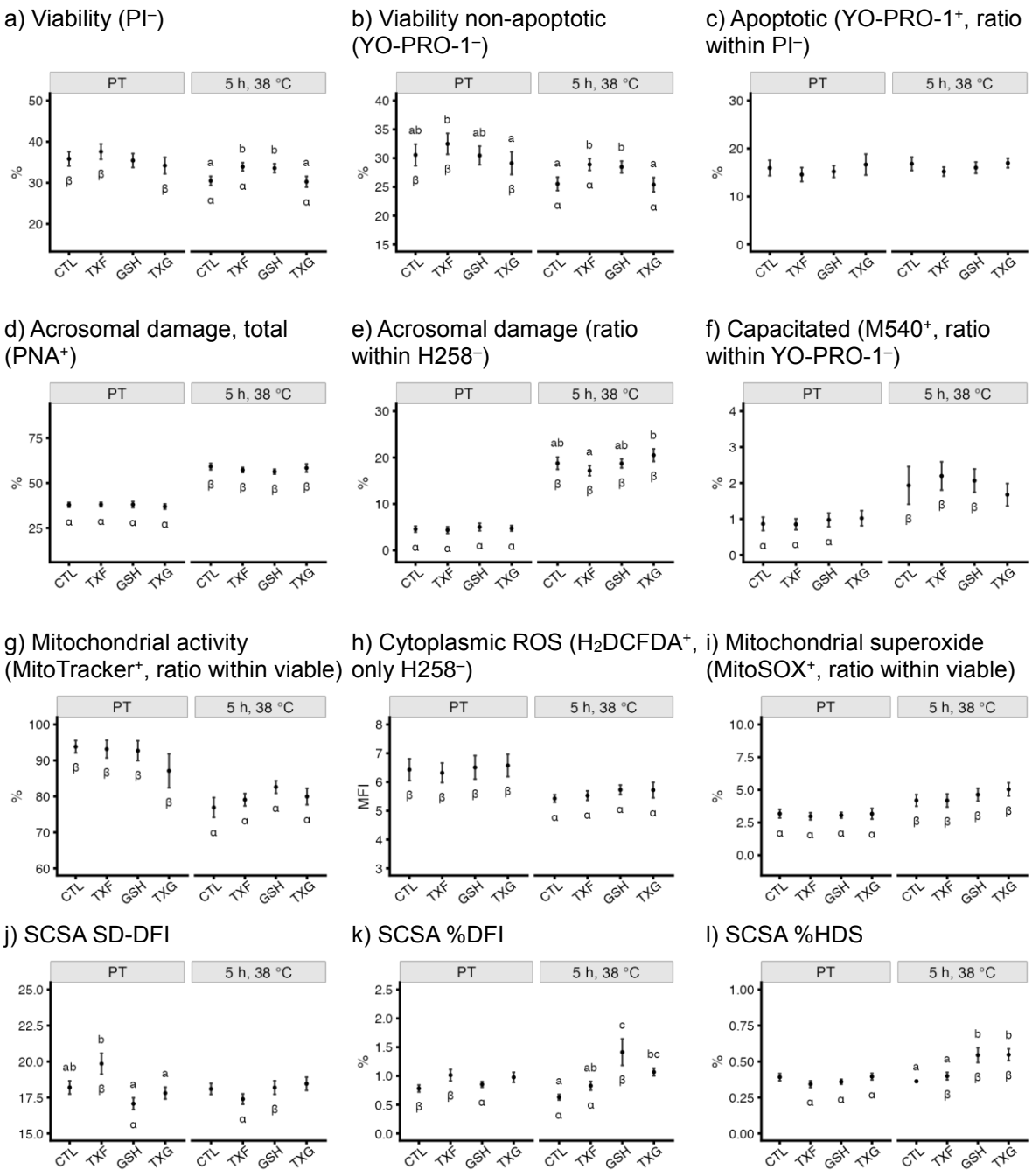

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