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Original article

Temporal characterization of conditions that promote functional capacitation of stallion sperm

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ABSTRACT

Equine in vitro fertilization (IVF) has historically been challenged due to lack of conditions that promote functional capacitation of stallion sperm. Capacitation success is often attributed to prolonged sperm incubation requirements. Advances to equine IVF are hindered by incomplete characterization of conditions that promote the functional capacitation of stallion sperm over time. Herein, we describe conditions that promote the functional capacitation of fresh, cooled, and frozen-thawed stallion sperm isolated by Percoll, Swim-Up, or Microfluidics (VetMotl). Fresh (diluted sperm) and previously cooled stallion sperm held at 5°C for 24–48 hr were maintained in capacitating conditions at 38°C or ambient temperature for up to 22 hr to determine motility, tyrosine phosphorylation, and acrosome integrity at 4 hr intervals. Holding sperm at 38°C was detrimental to motility while fresh and previously cooled sperm maintained at ambient temperature displayed maximum capacitation at 4 hr compared to prolonged holding times, regardless of sperm isolation technique. Importantly, sperm evaluated at 4 hr displayed the highest acrosome integrity. The functional capacitation of fresh sperm maintained under these conditions was challenged in a heterologous bovine IVF system where oocyte activation was confirmed by pro-nuclear formation and polar body extrusion. Frozen-thawed stallion sperm was then applied to an equine IVF system where Microfluidic sperm selection proved superior to Swim-Up regarding sperm kinematics, capacitation and acrosome status. Fertilization potential was confirmed by pro-nuclear formation and embryo development. These findings provide valuable information to conditions that support functional capacitation of stallion sperm towards advancing equine in vitro embryo production.
Data availability: Data is provided within the manuscript or supplementary information files. All data are available upon reasonable request to the corresponding author.

1. Introduction

Commercial production of live offspring in the equine industry is accomplished through assisted reproductive technologies including artificial insemination (AI), embryo transfer (ET), intracytoplasmic sperm injection (ICSI) and to a limited extent, somatic cell nuclear transfer [39]. The successful application of these strategies requires specific considerations for stallion sperm that are dependent upon conditions that promote functional sperm including motility, viability, capacitation, and acrosome status [24]. Despite species differences in sperm biology, assisted reproductive technologies for *in vivo* production of offspring are relatively similar, likely due to inherent characteristics of the female reproductive tract that promote and facilitate fertilization in a species-specific manner. However, important differences in sperm biology limit the translation of *in vitro* methods across large animal species for techniques such as ICSI and *in vitro fertilization* (IVF) for

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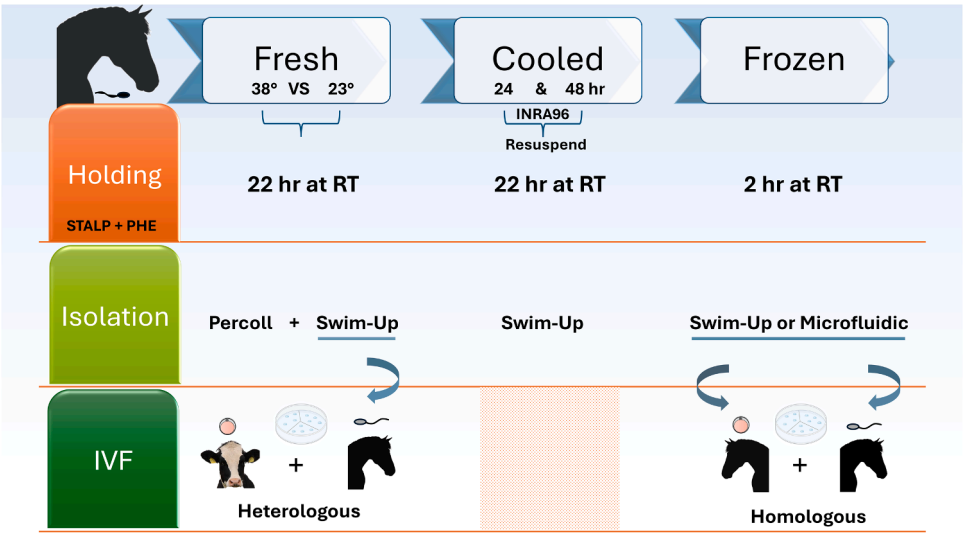


Fig. 1. Conditions and isolation procedure for challenging the capacitation status of stallion sperm. Temporal characterization of capacitation status was achieved for fresh, cooled and frozen-thawed stallion sperm with holding conditions consisting of a modified stallion-Tyrode's albumin lactate pyruvate medium containing glucose and the addition of penicillamine, hypotaurine and epinephrine (STALP + PHE). Fresh sperm was diluted in INRA 96 after collection for transportation within an Equitainer for further processing where ejaculates were split and maintained at either 38°C or 23°C (room temperature; RT) for evaluation up to 22 hr. Similarly, fresh semen was collected and cooled for 24 and 48 hr to replicate shipment, and then maintained at RT for 22 h in STALP. Frozen-thawed sperm was re-extended in STALP + PHE immediately after thawing. Sperm isolation procedures included a discontinuous Percoll gradient (90:45), Swim-Up, and Microfluidic device (Vetmotl) with all samples diluted to 20 x 10^6 total sperm/mL. Sperm from all conditions were analyzed at various time points up to 22 hr for tyrosine phosphorylation, acrosome integrity and kinematics. Fresh stallion semen selected by Swim-Up was applied to a heterologous bovine IVF system to determine pro-nuclear formation as an indication of functional capacitation. Frozen-thawed stallion sperm selected by both Swim-Up and Microfluidics were then challenged in a homologous equine IVF system to determine functional capacitation status by embryo development.

reasons that remain unknown. In bovine and human, IVF rates are quite successful, which permit application and implementation for clinical and commercial practices [14,17]. However, IVF in horses is not well-established, for reasons that have primarily been attributed to lack of ideal conditions that promote sperm capacitation-like changes required for fertilization [23].

Unlike horses, bull sperm capacitation can be induced with the addition of glycosaminoglycans such as heparin and the common incorporation of hypotaurine, penicillamine, and epinephrine during gamete co-incubation [35]. Stallion sperm do not seem to respond to these additives and attempts to induce capacitation have in general not been repeatable or highly effective [24]. Different inducers of capacitation such as calcium ionophore, caffeine, heparin, and procaine have not proven useful for equine IVF and embryo production [20,22,25,31]. Several techniques have been implemented to detect sperm capacitation in the equine, such as the assessment of acrosomal reaction, hyperactivation, intracellular calcium influx and primarily, the detection of tyrosine phosphorylation [8]. Nonetheless, these markers of capacitation do not necessarily reflect functional sperm for techniques such as IVF [24]. More recently, the successful induction of sperm capacitation was reported by pre-incubating sperm in capacitating conditions for 22 hr before gamete co-incubation, resulting in viable embryos and the birth of foals [12]. Blastocysts generated from stallion sperm held under capacitating conditions for 10 hr have also yielded comparable results to ICSI [27]. Similarly, the timing of pre-incubation with frozen-thawed sperm prior to fertilization has achieved varying results for blastocyst production [11]. Despite these recent reports of equine *in vitro* blastocyst production, limited information exists to define conditions that maximize functional sperm capacitation for fresh, cooled, and frozen-thawed sperm [13,21]. On the contrary, equine ICSI is effective, but tends to have limited success in cattle [3,15]. Unknown reasons for the discrepancy between IVF and ICSI success clearly highlight subtle differences in species-specific sperm biology that limit the application and translatability of these techniques.

In vitro embryo production (IVP) remains globally stable in humans and cattle, the latter of which represents the largest amount of embryos produced with the highest success rates [14,17,19]. In contrast, conventional IVP in horses has been relatively unsuccessful with few studies describing the birth of IVP foals [12,33]. Equine embryo production has faced many challenges for commercialization that have been minimized in other species but likely obfuscated in horses due to undetermined species differences and difficulties in obtaining oocytes from a species that are primarily mono-ovular, resistant to ovarian superstimulation, and managed with specific considerations as a non-traditional livestock species [39]. Historically, mares or stallions requiring assisted reproductive techniques beyond AI with cooled semen or frozen sperm were limited primarily to ICSI or oocyte-transfer, both of which required surgical transfer by standing flank laparotomy because culture of equine embryos beyond initial cleavage stages was unreliable and therefore not permissive to non-surgical transfer techniques. Despite recent advances to culture conditions that promote blastocyst development, little progress has been made to define reasons for IVF failure in horses [23]. Although multifactorial reasons hinder progress, lack of appropriate conditions and protocols to induce stallion sperm capacitation have been identified as a likely impediment to successful equine IVF [2,10,30]. Capacitation is the final maturation process that sperm undergo during their passage through the female reproductive tract and is required for unassisted fertilization [42]. Unlike sperm from other mammals, stallion sperm subjected to *in vitro* capacitating conditions often fail to carry out capacitation-related events such as hyperactivation, increased plasma membrane fluidity, and the acrosome reaction [24]. These important biochemical and structural changes enable sperm to bind the zona pellucida and penetrate the oocyte [41]. Conditions that successfully promote capacitation-like changes in stallion sperm while preserving acrosome integrity are essential for improving equine IVF.

The objective herein was to identify and optimize functional capacitating conditions for stallion sperm permissive to *in vitro* fertilization. Sperm isolation procedures, holding conditions, and effects of cooling stallion sperm prior to induction of capacitation were determined with

an overall goal of maintaining motility and acrosome integrity while promoting capacitation as indicated by phosphorylation of tyrosine residues. Activation of bovine oocytes with stallion sperm was initially utilized to determine the functional capacitation status of sperm from conditions optimized herein. Frozen-thawed stallion sperm were then applied to a modified equine IVF system to determine the potential for embryo development. Results from these experimental conditions suggest that prolonged incubation of fresh stallion sperm at 38 °C is not required for functional capacitation and may be detrimental to motility. These data show that sperm maintenance at ambient temperature is sufficient and compatible with previously cooled and frozen-thawed stallion sperm for capacitation and in vitro embryo production.

2. Material and methods

2.1. Experimental design

Fresh and cooled stallion sperm were collected from commercial Quarter Horse stallions ($n = 2$) of proven fertility maintained at the University of Florida Horse Teaching Unit in months when stallions were exposed to a minimum of 14 hr of daylight. Cryopreserved sperm from 5 stallions was donated (Select Breeders Services, Chesapeake City, MD) and consisted of the following breeds: (Dutch Warmblood (2), Standardbred (1), Holsteiner (1), and Westphalian (1). Equine oocytes were sourced from a commercial enterprise and bovine oocytes collected from a local abattoir. All experiments were approved by the University of Florida Institutional Animal Care and Use Committee (202200000152) and performed in accordance with the relevant guidelines and regulations. Unless otherwise stated, all reagents were purchased from Sigma Aldrich® (St. Louis, MO, USA). The optimization of sperm capacitating conditions was performed under different conditions (Fig. 1):

2.1.1. Fresh diluted sperm maintained at 38 °C

Fresh sperm diluted in INRA 96 were isolated by two different techniques (Percoll vs. Swim-Up) and incubated at 38°C and 6% CO2 in capacitating media (STALP) for up to 22 hr. This experiment was designed in a 5×2 factorial arrangement, with the factors being sperm incubation times (0, 4, 8, 12 and 22 hr) and sperm selection technique (Percoll, Swim-Up), respectively.

2.1.2. Fresh diluted sperm maintained at 23°C

In the second condition, fresh sperm samples transported in INRA 96 were selected only by Swim-Up and incubated at 23°C (RT - Room Temperature) in capacitating media (STALP) for up to 22 hr. Sperm were isolated and selected as described below (Sperm processing and isolation), for extended maintenance under capacitating conditions.

2.1.3. Cooled sperm followed by 23°C incubation

The third condition challenged sperm by cooling for 24 and 48 hr at 5°C under conditions for semen transport using INRA 96. After the cooling period, sperm were then subjected to Swim-Up and resuspended at 23°C in capacitating medium (STALP). This experiment was designed in a 2×5 factorial arrangement, with the factors being sperm cooling times (24 and 48 hr) and incubation times at 23°C (0, 4, 8, 12 and 22 hr).

2.1.4. Cryopreserved sperm

For the last condition, frozen-thawed sperm from 5 different stallions were isolated by Microfluidic (VetMotl) and Swim-Up techniques. After isolation, sperm were incubated for up to 2 hr at room temperature (23°C) in STALP for further experiment.

2.2. Sperm processing and isolation

Ejaculates from two different stallions were collected using a Missouri style artificial vagina according to standard laboratory procedures. A minimum of 4–6 replicates was used for each experiment. Following collection, ejaculates were diluted 2:1 in INRA 96 extender (IMV Technologies) and placed in an Equitainer (Hamilton Thorne, Beverly, MA) at 5°C for transport to the laboratory (~15 min) to prevent over heating in the subtropical Florida climate. Ejaculates were split and sperm were isolated by either Percoll or Swim-Up method depending upon experiment. A stallion sperm capacitating medium (STALP) containing lactate, pyruvate, and glucose as energy sources was used in all experimental conditions in the absence of PHE for sperm processing and isolation (114 mM NaCl, 3.2 mM KCl, 25 mM NaHCO3, 0.4 mM NaH2PO4, 0.05 mM MgCl2, 4.6 mM D-Glucose, 10 mM HEPES, 10 mM Sodium Lactate, 0.5 mM Sodium Pyruvate, 1.5 mM CaCl2, Gentamicin and 6 mg/mL BSA). After sperm selection and dilution in STALP to adjust sperm concentration, PHE was added for sperm incubation as described below (154 mM NaCl, 0.93 mM $Na_2S_2O_5$, 5.2 mM sodium lactate, 0.23 mM hypotaurine, 0.46 mM penicillamine, and 0.046 mM epinephrine). The pH of STALP was previously adjusted to 7.3–7.4 prior to sperm processing and incubation for room temperature conditions devoid of CO2.

For Swim-Up, 200 μL of extended semen was placed at the bottom of 15 mL conical tubes and a 1 mL layer of GMOPS solution (Vitrolife®) was applied on top of the extended semen. Samples were incubated at 38°C and 6% CO2 for 20 min at a 45° angle with open lids to maintain equilibration. After incubation, 750 μL of supernatant was recovered and centrifuged at 750 x g for 5 min. The pellet was then resuspended in STALP with PHE at 20 × 10^6/mL in a final volume of 800 μL. Four tubes were used for each sperm preparation and the 750 μL supernatants were combined prior to centrifugation to recover sufficient amount of viable cells for incubation.

For Percoll separation, a discontinuous gradient was prepared with the addition of 1 mL of extended semen placed in each 15 mL conical tube containing 1 mL of Percoll 45% and 1 mL of Percoll 90% (1350 μL of Percoll diluted in 150 μL of HEPES Buffered Saline 10x). Samples were centrifuged at 750 x g for 10 min and the pellets were resuspended in 4 mL of STALP+PHE as described above [7]. Samples were then centrifuged a second time at 120 x g for 10 min and resuspended to 20 x 10^6 sperm/mL to 800 μL volumes in STALP + PHE. Two tubes per sample were used and the pellets were combined after centrifugation to recover sufficient amounts of motile sperm for incubation.

For Microfluidics, a VetMotl device designed for equine sperm was used for isolation. Sperm selection on this specific type of microfluidic device is based upon sperm movement from a bottom chamber towards an upper chamber through a filter with a defined pore size, allowing for an enriched motile sperm population. One straw (500 μL) of frozen sperm thawed at 37°C for 30 s was combined with 450 μL of STALP. A total of 850 μL of diluted sperm was applied to the inlet port of the device. Once injected, 750 μL of STALP (without PHE) primed the outlet port and concentration chamber. The device was placed in a petri dish and incubated at 38°C and 6% CO2 for 25 min allowing motile sperm to travel through the porous membrane of the device to the upper layer of STALP. Following incubation, 500–600 μL was aspirated from the outlet port and centrifuged at 300 x g for 5 min. The pellet was resuspended in STALP + PHE at 15 x 10^6 sperm/mL in a final volume of 200 μL and stored at room temperature (23°C) for 2 hr.

2.3. Sperm holding conditions

Following resuspension of isolated sperm, samples were maintained either at 38°C, or at 23°C (RT) depending upon experiment. In a separate cooling experiment, sperm were collected, extended in INRA 96 and then maintained for 24–48 hr within an Equitainer at ~5°C to replicate shipment of cooled stallion semen. After the cooling period, sperm were isolated by Swim-Up, centrifuged and then transiently warmed to 23°C (RT) for downstream analyses. For all sperm holding conditions, aliquots were warmed to 37°C for 10 min prior to analyses. Sperm for all fresh and cooled experiments were evaluated at 0, 4, 8, 12 and 22 hr after

their incubation period by removing an aliquot and returning the sample back to either incubation or to RT holding conditions in the dark. For frozen-thawed sperm, samples were limited to 2 hr of incubation at RT following isolation.

2.4. Sperm analysis

2.4.1. Kinematic assessment

Sperm kinematics were objectively quantified via Computer-Assisted Sperm Analysis (CASA-IVOS System, Hamilton Thorne, Beverly, MA). A minimum of 5 fields and 400 spermatozoa were observed on a 37°C heated stage by acquiring 30 frames at a rate of 60 Hz with minimum head brightness of 170, minimum head size of 5 μm^2, progressive minimum VAP cutoff of 50 μm/S, progressive minimum STR cutoff of 80 μm/S, slow cell VAP cutoff of 20 μ/S, and slow cell VSL cutoff of 30 μm/S. Parameters measured included total motility, progressive motility, VAP (average path velocity; μm/S), VSL (straight-line velocity; μm/S), VCL (curvilinear velocity; μm/S), STR (straightness = VSL x 100/VAP; %), LIN (linearity = VSL X 100/VCL; %), BCF (beat cross frequency; Hz) and ALH (amplitude of lateral head displacement: μm). All CASA parameters were captured using Leja® 4 chamber 20 μm slides warmed at 37°C with the addition of 3 μL of sperm sample (IMV, 025107).

2.4.2. Capacitation status

Tyrosine phosphorylation (Tyrosine PO_3) detection and localization was performed as an indication of capacitation status by immunofluorescence [12]. Except for primary antibody addition, all washes were performed three times at ambient temperature with PBS. Briefly, a 50 μL drop of sperm at 20 x 10^6 total sperm/mL was placed on a slide and allowed to dry. Sperm were then fixed with 4% paraformaldehyde for 30 min and washed 3 times with PBS 1X. Permeabilization was accomplished with Triton-X (1%) for 15 min followed by 3 washes with PBS. Samples were then blocked for 2 hr at RT (10 mg/mL BSA) and washed 3 times with PBS. A 50 μL droplet of anti-phosphotyrosine primary antibody from mouse (1 mg / mL; Millipore Sigma #05–321, USA) diluted 1:100 in Ab buffer was incubated with sperm overnight (~15 hr) at 5ºC. Slides were then washed 3 times in PBS and then incubated for 1 h with secondary goat anti-mouse antibody conjugated to Alexa-Fluor 633, at a 1:100 dilution (ThermoFisher #A-21052, USA). Final washes were followed by addition of 15 μL of Vector mounting medium with DAPI (#H1200, Vector®) and coverslip. Fluorescence emission and detection were performed at 360/432 (DAPI) and 624/681 (Cy5), respectively. All images were captured with an AURAIII-UCGRnIR Light Engine (LED Lumencor 4-channel) compatible with Penta band filter cube on a Nikon TE2000U inverted microscope at 200X magnification and Nikon Elements software. Images were captured with an AMScope MA500 5MP color camera and compatible software. A minimum of 100 cells were counted per replicate and classified as positive when the flagellum was labeled red and negative for unlabeled flagellum.

2.4.3. Assessment of acrosomal status

Acrosome status was assessed through *Pisum sativum* agglutinin (PSA) staining associated with FITC fluorescence (Sigma, USA) and non-permeabilized sperm [32]. Briefly, 22.5 μL of Ident® solution (Hoechst 33342 stain; Hamilton Thorne, Beverly, MA) was added to 22.5 μL of sperm extended in STALP at 20 x 10^6/mL and 5 μL of FITC-PSA stock solution (100 μg/mL), achieving 10 μg/mL of FITC-PSA. Sperm samples were incubated for 10 min and 10 μL aliquots were placed onto slides previously heated to 37°C with coverslips. Fluorescence emission and detection were performed at 461/515 (FITC) and 360/432 (Hoechst 33342) respectively. A minimum of 100 cells per replicate were counted. Sperm were classified as acrosome-compromised when labeled in green and acrosome intact for those with unlabeled acrosomes. The same procedures were applied for frozen-thawed sperm but were accomplished concomitantly with tyrosine phosphorylation, which required permeabilization as previously described. Therefore, sperm were considered acrosome-compromised when no labelling was detected and acrosome intact when fully labeled.

2.5. Heterologous in vitro fertilization

To evaluate the activation capacity of equine sperm, bovine oocytes were co-incubated with stallion sperm according to embryo production procedures utilized in the laboratory with modifications for stallion sperm based on optimizations presented herein [28]. The use of bovine oocytes in a heterologous IVF system has been previously described and was therefore utilized as a tool to determine if optimization of holding conditions for stallion sperm were conducive to sperm penetration and fertilization [6,38,9]. Four replicates were conducted using fresh semen from two different stallions. Abattoir-derived bovine ovaries were obtained for isolation of immature oocytes originating from 3 to 8 mm follicles. Cumulus-oocyte-complexes (COCs) with a homogenous cytoplasm and a minimum of two layers of cumulus cells were selected, washed three times in holding medium and matured in groups of 10 within 50 μl drops of oocyte maturation media (OMM) (10% fetal bovine serum, 50 ng/mL Human Recombinant epidermal growth factor, 5 μg/mL Folltropin (Vetoquinol)) for 21 h at 38.5ºC and 5% CO_2 and atmospheric oxygen [28]. Matured bovine COCs were washed three times through HEPES embryo media and co-incubated with fresh stallion sperm isolated by Swim-Up [28]. Stallion sperm were isolated by Swim-Up procedure and independently pre-incubated at room temperature for 4 hr in capacitating conditions (STALP + PHE) prior to gamete co-incubation. The 4 hr time point was selected based on optimization results from previous experiments. Sperm were added to oocytes at a concentration of 1x10^6 total sperm/mL in IVF-TALP containing 6.0 mg/mL fatty acid free BSA, 0.2 mM sodium pyruvate, 5 μg/mL Gentamicin, and 20 μg/mL Heparin in a 35 X 10 mm dish. Penicillamine (0.5 mM), hypotaurine, (0.25 mM) and epinephrine (25 μM) were added to fertilization medium followed by gamete coincubation for 16 hr at 38.5ºC, 5% CO_2, 20% O2, and relative humidity [28]. Presumptive zygotes were vortexed for 5 min with hyaluronidase to remove cumulus cells and selected for culture in groups of ~10 per 50 μL drop in a defined culture medium containing 3.7 mg/mL fatty acid free BSA, 18.6 μl/mL essential amino acids, 9.3 μl/mL nonessential amino acids, 23.3 μg/mL under mineral oil in a 35 x 10 mm dish at 38.5ºC, 5.5% O_2, 5% CO_2 and humidity [28]. Embryo cleavage was assed 72 hr post-fertilization, followed by blastocyst development on day 7.5. Embryos with a distinct ICM and blastocele cavity were considered blastocysts.

2.6. Chromatin configuration

Presumptive zygotes obtained by exposing bovine oocytes to stallion sperm were evaluated for activation by DAPI staining to identify chromatin configuration, pro-nuclei formation, and polar body extrusion. Three IVF replicates were performed by using 3 independent ejaculates from two different stallions. One-cell presumptive zygotes were collected at 18, 24 and 42 hr post insemination (HPI) and fixed with 4% paraformaldehyde for 15 min (Fertilized group=163 oocytes total). Additionally, unfertilized oocytes were fixed at the same time points, respectively (Control group=145 oocytes total). Presumptive zygotes and unfertilized oocytes were mounted on a glass slide with a coverslip in groups of ~20 with Vectashield (Vector) antifade mounting medium containing DAPI for nuclear staining and imaged as described under 20X magnification (Ex 360–390, Em 432 and 40 ms exposure). Embryos with a minimum of two pro-nuclei and two polar bodies were considered fertilized while those containing two pro-nuclei but only one visible polar body were categorized as parthenotes. The absence of clearly distinguishable pro-nuclei were further categorized as immature or unfertilized oocytes.

Table 1
Kinematic and functional capacitation indices observed under different capacitating conditions for fresh and cooled stallion sperm. Total (TM) and progressive motility (PM), acrosomal status (% of compromised acrosome), and tyrosine phosphorylation detection (Tyrosine PO_3 positive) of fresh and cooled stallion sperm incubated for 22 hr in capacitating conditions at 38°C (fresh) or 23°C (fresh and cooled). Two to three ejaculates from two different stallions were used. Different superscripts ($^{a\text{-}d}$) indicate $P \leq 0.05$ between time points. Data presented as mean ± SEM.

Variables	0 hr	4 hr	8 hr	12 hr	22 hr	*P*-value Time
Fresh sperm at 38°C						
TM (%)	87.31 ± 3.44^{a}	43.14 ± 4.61^{b}	24.78 ± 4.41^{c}	21.99 ± 3.51 ^{cd}	15.68 ± 3.46^{d}	0.003
PM (%)	29.45 ± 4.49^{a}	7.40 ± 1.10^{b}	4.58 ± 0.73^{bc}	3.33 ± 1.07 ^{cd}	2.54 ± 0.57^{d}	0.003
Acrosome (%)	9.00 ± 2.07	18.95 ± 2.17	16.76 ± 2.33	18.44 ± 2.22	19.00 ± 2.28	0.1
Tyrosine PO_3 (%)	17.68 ± 4.13^{a}	45.14 ± 5.06^{b}	37.44 ± 3.62^{b}	21.83 ± 4.74^{a}	11.71 ± 1.94^{c}	0.02
Fresh sperm at 23°C						
TM (%)	80.43 ± 4.13^{a}	69.57 ± 3.80^{ab}	65.63 ± 4.00^{ab}	57.96 ± 5.09^{bc}	48.17 ± 6.86^{c}	0.003
PM (%)	24.13 ± 1.84^{a}	10.27 ± 0.94^{b}	10.96 ± 1.40^{b}	7.57 ± 1.4^{bc}	6.40 ± 1.78^{c}	0.0001
Acrosome (%)	16.42 ± 0.85^{a}	22.35 ± 2.05^{b}	25.80 ± 1.53^{b}	24.67 ± 1.21^{b}	22.05 ± 2.25^{b}	0.005
Tyrosine PO_3 (%)	23.38 ± 4.46^{a}	63.43 ± 3.17^{b}	66.28 ± 3.43^{b}	51.68 ± 4.42^{c}	45.17 ± 4.25^{c}	0.0001
Cooled sperm (24–48 hr) followed by 23°C						
TM (%)	70.25 ± 3.30^{a}	55.14 ± 4.97^{b}	51.13 ± 4.76^{bc}	47.71 ± 4.25^{bc}	39.20 ± 5.63^{c}	< 0.0001
PM (%)	31.99 ± 3.70^{a}	8.88 ± 0.96^{b}	7.75 ± 1.11^{b}	7.09 ± 0.98^{b}	5.68 ± 1.14^{b}	< 0.0001
Acrosome (%)	30.50 ± 1.84^{a}	38.75 ± 3.21^{bc}	43.25 ± 3.16^{c}	36.38 ± 2.95^{abc}	34.88 ± 2.35^{ab}	0.0317
Tyrosine PO_3 (%)	41.50 ± 2.74^{a}	73.00 ± 5.97^{bc}	79.13 ± 4.70^{c}	65.38 ± 6.99^{bc}	58.88 ± 5.40^{b}	< 0.0001

2.7. Equine in vitro fertilization

Immature cumulus-oocyte complexes (COCs) were collected by transvaginal ultrasound-guided follicle aspiration from two mares on consecutive days and were transported directly to the lab at 22ºC in a holding medium supplemented with 10.0 mM HEPES [28]. Recovered COCs were washed through three drops of HEPES medium and placed in 50 μL drops of preequilibrated oocyte maturation medium (OMM) containing 1 mg/mL estradiol, 10% fetal bovine serum, 50 ng/mL human recombinant epidermal growth factor, and 5 μg/mL Folltropin (Vetoquinol). Oocyte maturation duration included 26–28 hr at 38.5ºC with 5% CO2, atmospheric oxygen and humidified air. Matured COCs were evaluated for cumulus expansion and then placed in 43 μL drops of TALP medium supplemented with 6.0 mg/mL fatty acid-free BSA, 0.2 mM sodium pyruvate, 5 μg/mL gentamicin, and 20 μg/mL heparin (IVF-TALP). Pooled, frozen-thawed stallion sperm from 2 stallions were isolated by either Swim-Up or microfluidic selection within independent replicates and were adjusted to a concentration of $20x10^6$ total motile sperm/mL. A total of 5–7.5 μL of sperm was added to the drops for a final concentration of ~2 – 2.5 $\times 10^6$ total sperm/mL. Penicillamine (0.5 mM), hypotaurine (0.25 mM), and epinephrine (25 μM) were added to fertilization drops followed by gamete coincubation for 10–15 h at 38.5ºC, 5% CO2, atmospheric O2, and humidified air. Zygote co-culture was considered day 0 (D0). Presumptive zygotes were vortexed for 3–5 min with hyaluronidase to remove cumulus cells, washed through 5 drops of HEPES, and cultured in pre-equilibrated 25 μL drops of commercial embryo culture medium (Global, LGGG-050, LifeGlobal, Guilford, CT, USA) supplemented with 10% FBS. All presumptive zygotes from each replicate were cocultured together in a single, 25 μL drop at 38.5ºC, 5% CO_2, 10% O_2, and humidified air. Oocytes from each mare were cultured in single drops/mare for the entirety of the culture period and un-cleaved embryos were not discarded. On day 3 of culture, all embryos were washed through 1–3 drops of culture medium to remove any cumulus cells remaining after initial vortexing and were subsequently placed in fresh, pre-equilibrated drops of the same media. On day 5 of culture, all embryos were moved to 50 μL drops of pre-equilibrated DMEM/F-12 (Sigma, D0697) supplemented with 10% FBS at 38.5ºC, 5% CO_2, 10% O_2, and humidified air.

2.8. Statistical analysis

Data were analyzed using the SAS System for Windows software (SAS Institute Inc. 9.3; Cary, NC, USA). Guided data analysis was considered to evaluate the normality of the residuals (Gauss distribution) and homogeneity of the variances (Shapiro-Wilk test) and when significant ($P < 0.05$), were transformed. The MIXED procedure (repeated measures) was used to analyze interactions between time*treatment factors. For the variables that interacted ($P < 0.05$), the effect of treatment (sperm selection technique or cooling time) at each time point was considered and vice versa. For variables that did not interact ($P > 0.05$), time and treatment factors were analyzed separately. Stallion and replicate were considered as random effects. Differences between treatments were evaluated using parametric methods (Student's *t*-test or ANOVA [LSD test]) by PROC GLM procedure. The significance level used to reject H0 was 5%. Data are presented as the untransformed mean and standard error of the mean.

3. Results

3.1. Sperm incubation at 38 °C is detrimental to motility and capacitation over time

Initial conditions utilized fresh sperm from two different stallions to determine effects of maintaining sperm at 38°C on motility and capacitation status ($n = 4$ reps total, 2 per stallion). Ejaculates were split and sperm were isolated by Swim-Up or Percoll and maintained for up to 22 hr to determine if differences in functional assessment parameters were due to isolation procedures. No interaction between time points (0, 4, 8, 12 and 22 hr) and sperm selection techniques was observed (Percoll and Swim-Up; $P < 0.05$; Suppl. Table 1). Accordingly, time points were compared regardless of sperm selection techniques (Table 1) and the latter were compared regardless of time, resulting in no difference in any parameters between isolation techniques (Suppl. Table 2). Total motility decreased by over 50% in 4 hr, accompanied by a decline in additional kinematic parameters (Table 1 and Fig. 2A). Similarly, maximum sperm capacitation was reached at 4 hr of sperm incubation (~40%), which remained constant until 8 hr and then reduced significantly after 12 hr (Table 1 and Fig. 2B). The percentage of acrosome compromised sperm (AC) remained constant over time, indicating no premature acrosome reaction during the incubation period under capacitating conditions (Table 1 and Fig. 2C).

3.2. Stallion sperm maintained at 23 °C supports motility and capacitation

Because sperm motility was compromised over time when incubated

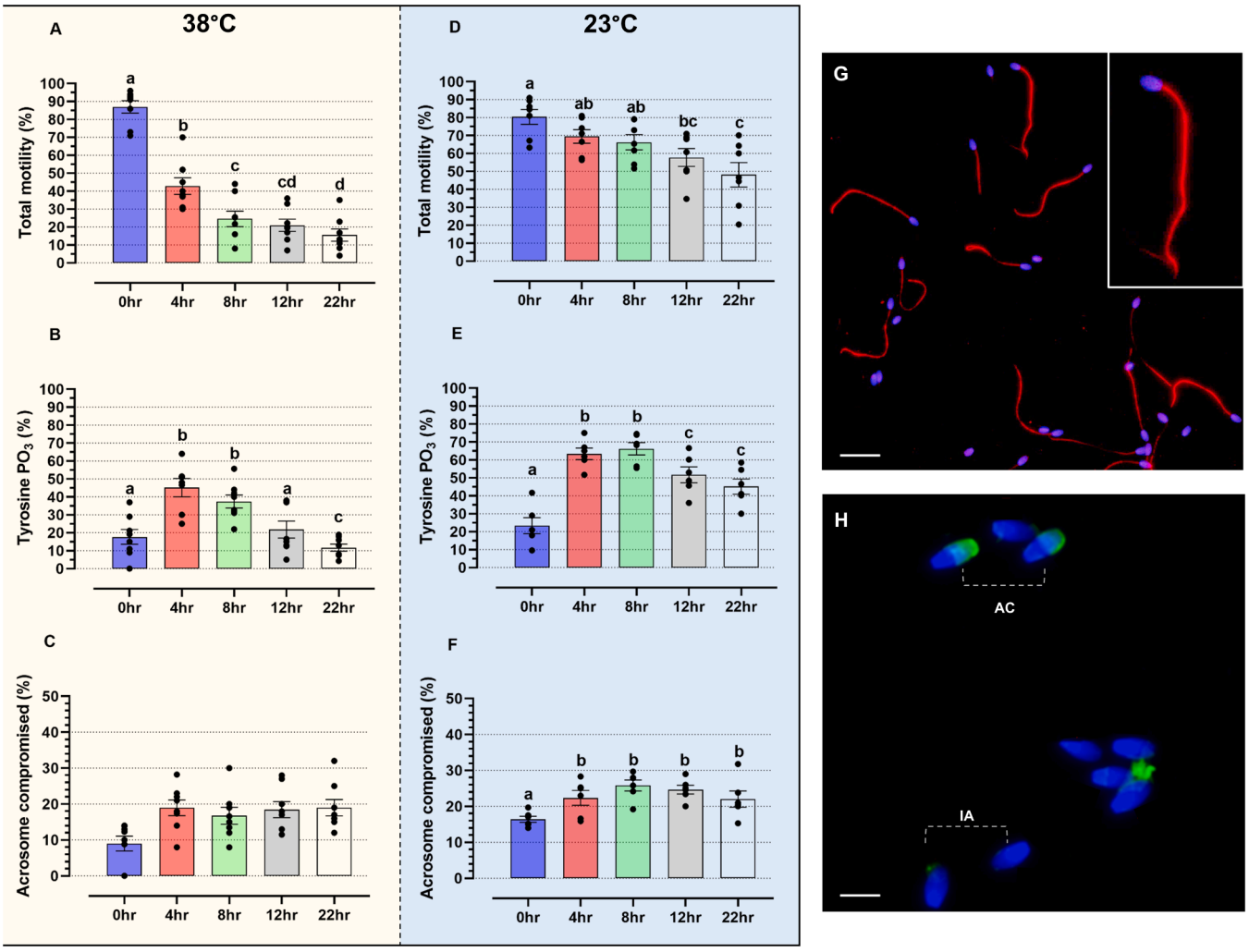


Fig. 2. Stallion sperm maintained at 23°C supports motility and capacitation while holding sperm at 38°C is detrimental. Fresh stallion sperm was collected, the ejaculates split and then isolated by Percoll and Swim-Up followed by incubation for 22 hr in capacitating conditions at 38°C **(A-C)** or 23°C **(D-F).** Samples were analyzed for total motility **(A,D)**, tyrosine phosphorylation detection **(B,E)** and non-intact acrosome status **(C,F)**. For sperm samples incubated at 38°C, the comparison between time points was performed regardless of the sperm selection technique (Percoll or Swim-Up) due to no differences detected in any parameters (Suppl. Table 1). Two ejaculates from two different stallions ($n = 4$) were used for each sperm selection technique, totaling 8 observations per time point. For sperm incubated at 23°C, three ejaculates from two different stallions ($n = 6$) were isolated by Swim-Up. Different letters (a-d) indicate $P \leq 0.05$ between time points. Data presented as mean ± SEM. Immunofluorescence image **(G)** representing capacitated sperm with nuclei identified by DAPI (blue) and anti-tyrosine phosphorylation detection localized to flagella (red). **(H)** Non-permeabilized sperm with acrosomes labelled (green) as detected by PSA-FITC counterstained with DAPI indicate acrosome-compromised sperm (AC) while unlabeled sperm contain intact acrosomes (IA). Scale bars= 20 μm (G) and 5 μm (H).

at 38°C, a second condition was implemented to determine effects of sperm kinematics and capacitation when fresh stallion sperm was maintained at 23°C for 22 hr. Sperm isolation was conducted by Swim-Up based on previous results indicating no differences in kinematic and capacitation parameters, and also due to compatibility with equine assisted reproductive procedures (Suppl. Table 3). Three to four ejaculates from two stallions (6–7 replicates) were collected, diluted 2:1 in INRA 96 extender and placed in an Equitainer for further processing. Sperm were isolated as described and equilibrated to 23°C (RT) in capacitating conditions. Total motility was maintained for 8 hr and then marginally reduced to ~50% after 22 hr of incubation (Fig. 2D; Table 1). There was an increase in the amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) over time concomitant with a decrease in progressive motility, linearity, straightness, average velocity (VAP) and straight-line velocity (VSL), suggestive of sperm hyperactivation as an indication of capacitation (Suppl. Table 3). Similar to sperm held at 38°C, maximum sperm capacitation was achieved at 4 hr of sperm incubation (65%), which remained constant until 8 h and then reduced significantly after 12 hr (Table 1 and Fig. 2E). The percentage of acrosome compromised sperm (AC) increased significantly at 4 hr and thereafter remained constant until the end of incubation (Table 1; Fig. 2F).

3.3. *Cooling stallion sperm for up to 48 hr is compatible with sperm isolation and capacitation*

The objective of this experiment was to determine the effect of cooling semen at 5°C for up to 48 hr prior to incubation in a capacitating medium (STALP) at RT (23°C) to replicate conditions requiring collection and transport of cooled stallion semen for equine IVF (Fig. 3A). Regardless of cooling period (24 or 48 hr), no interaction was detected for any variable across time when challenged by subsequent RT incubation (Fig. 3 B,D; Table 1, Suppl. Tables 4,5). Accordingly, data collected following the cooling period were combined regardless of cooling time to determine sperm kinematics and capacitation status (Table 1; Fig. 3). Similar to previous experiments, total motility remained above 50% at 4 hr, while progressive motility remained highest immediately after the cooling period when equilibrated to 23°C

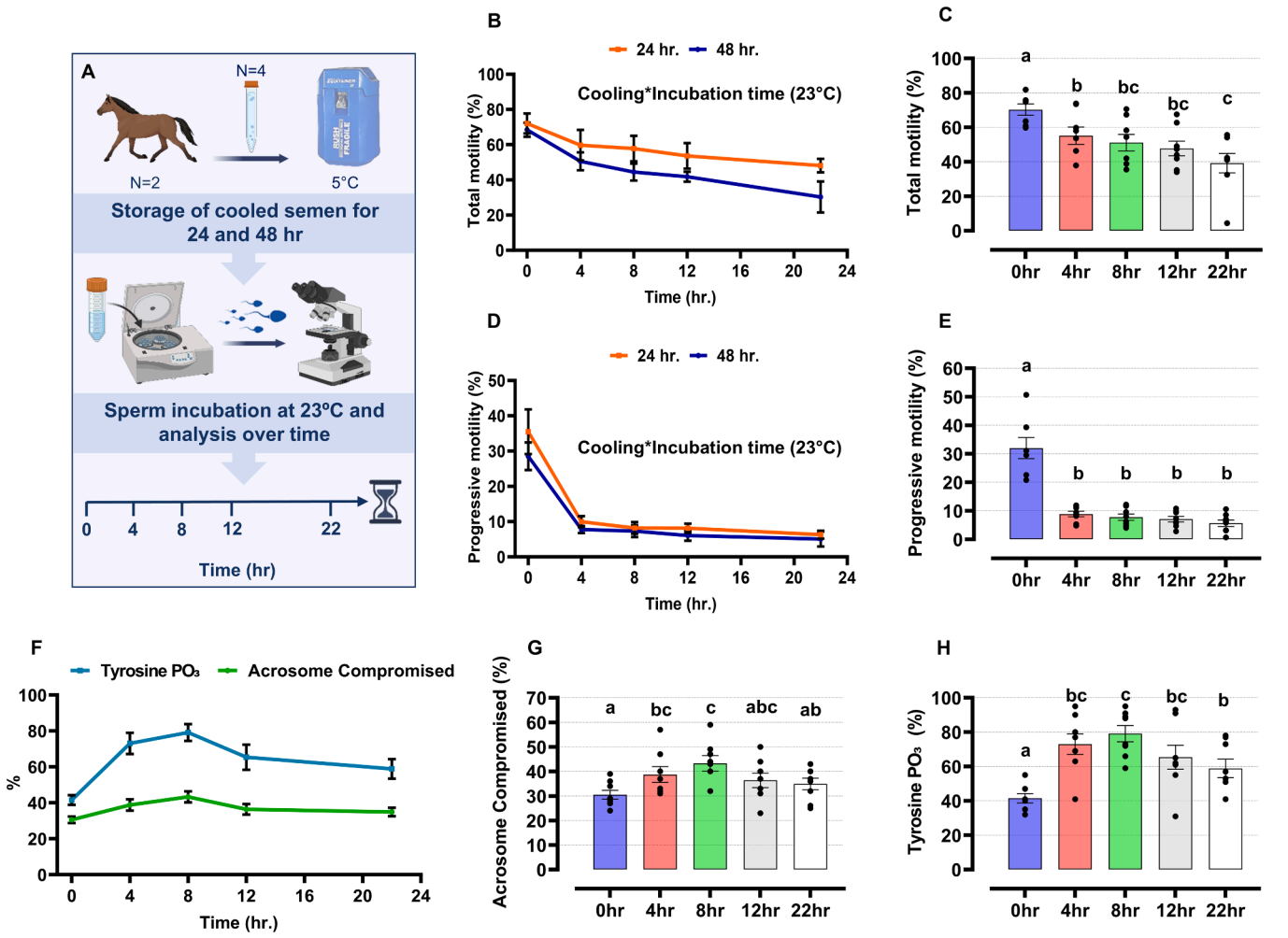


Fig. 3. Cooling stallion sperm for up to 48 hr is compatible with sperm isolation and capacitation. Two ejaculates from two stallions ($n = 4$ total) were collected, extended with INRA 96, and cooled to 5°C for 24 and 48 hr in replicate using commercial Equitainers. Following the cooling period, sperm were isolated by Swim-Up, resuspended in capacitating media, incubated for an additional 22 hr at 23°C and analyzed at 0, 4, 8, 12 and 22 hr **(A)**. Total motility **(B,C)**, progressive motility **(D,E)**, acrosome integrity **(F,G),** and tyrosine phosphorylation detection **(F,H)** were quantified at each time point by CASA and immunocytochemistry. No differences between cooling time were detected (24 vs 48 hr), so comparisons between time points were performed regardless of sperm cooling time, totaling 8 observations per time point. Different letters (a-d) indicate $P \leq 0.05$ between time points. Data presented as mean ± SEM.

and then warmed to 37°C for analyses (Fig. 3B - E). Regarding capacitation status, the percentage of cells positive for tyrosine phosphorylation increased over time, reaching maximum capacitation at 4 hr followed by a reduction at 22 hr of incubation (Fig. 3F,H; Table 1). In addition, Beat-cross frequency (BCF) increased over time while the other kinematic variables decreased from 4 hr onwards (Table 1). The percentage of acrosome-compromised sperm remained relatively constant over time, with a peak of 39% at 4 hr of incubation (Fig. 3F,G; Table 1).

3.4. *Frozen-thawed stallion sperm does not require prolonged holding for maximum capacitation*

In continuation of initial experiments with fresh and cooled stallion sperm, characterization of frozen-thawed samples for kinematic parameters and capacitation status over time was conducted to identity optimal conditions compatible for IVF. Sperm isolation by Swim-Up and Microfluidics (VetMotl) was compared to maximize conditions for fully functional sperm kinematics and capacitation. Straws of frozen sperm collected from a single ejaculate of 5 different stallions were thawed at 37°C for 30 s and evaluated prior to further isolation for kinematic assessment by CASA (pre-isolation). Following pre-isolation assessment, sperm were subjected to both Swim-Up and Microfluidics and maintained for two hours at room temperature (23°C) in capacitating conditions (STALP). At each hour (0, 1 and 2), samples were heated to 37°C and analyzed for sperm kinematics, acrosome integrity, and tyrosine phosphorylation. Isolation of sperm by Microfluidics compared to Swim-Up at 0 hr proved superior for selecting motile (72% vs. 30%) and progressively motile sperm, (19.9% vs 7.5%) and for each time point thereafter (Fig. 4A,C). Both isolation techniques resulted in a decrease of sperm motility over time (Fig. 4B,D). Maximum capacitation was achieved immediately after sperm isolation (0 hr) for both techniques and remained constant over time while maintaining tyrosine phosphorylation and acrosome staining (Fig. 4E). However, sperm isolated by Microfluidics presented greater capacitation rates at 0 and 1 hr when compared to Swim-Up (Fig. 4F). Acrosome integrity also remained constant over time for both sperm selection techniques whereas samples selected by Microfluidics had more intact acrosomes across all time points compared to Swim-Up isolation (Fig. 4G).

3.5. *Heterologous in vitro fertilization of bovine oocytes with stallion sperm*

The application of bovine oocytes in a heterologous IVF system has been previously described and was therefore utilized to determine the

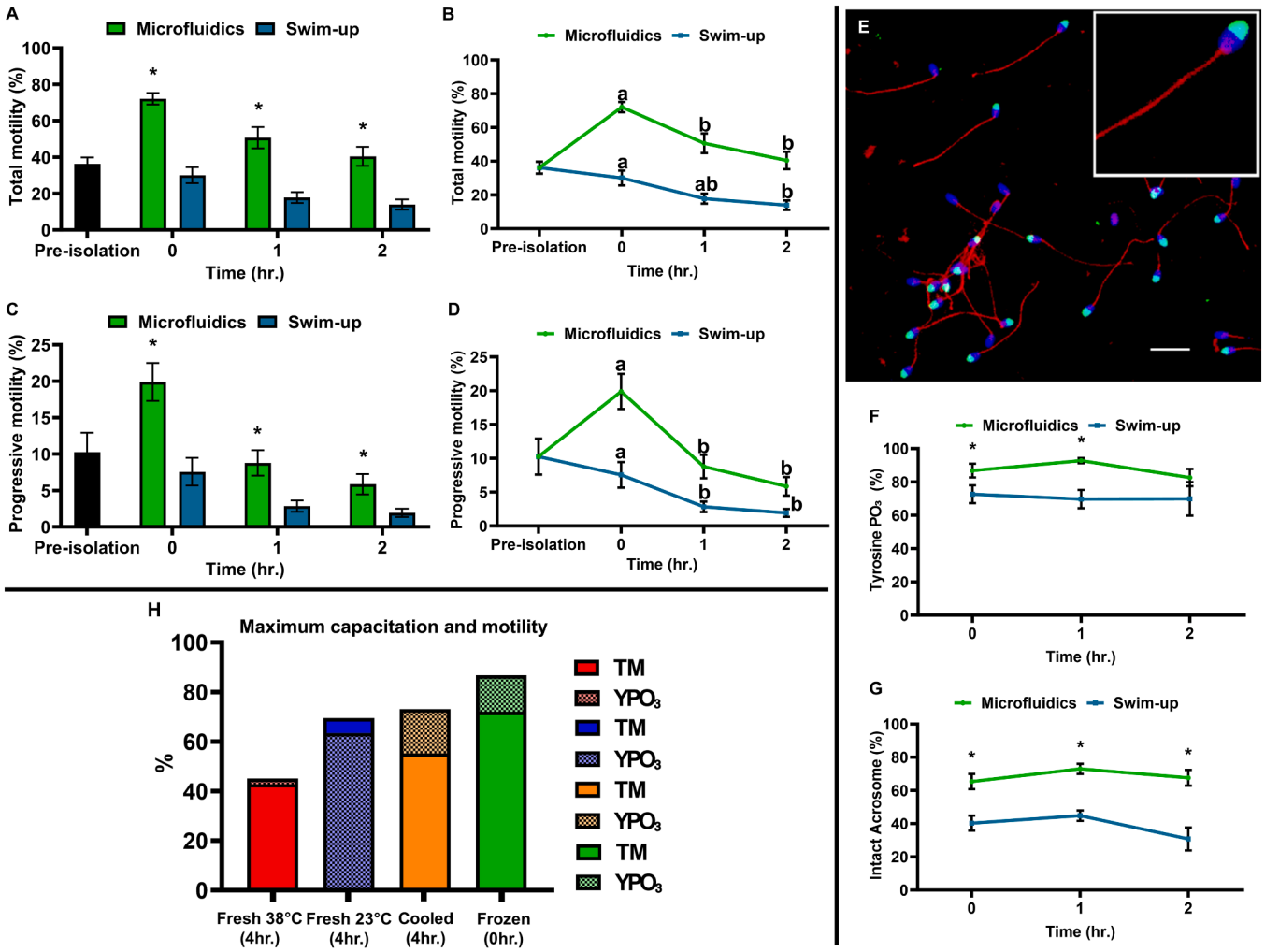


Fig. 4. Frozen-thawed stallion sperm achieve maximum motility and capacitation immediately after sperm selection. Frozen-thawed sperm from 5 stallions (2 reps each) were isolated by both Microfluidics and Swim-Up, incubated in capacitating conditions at 23°C and analyzed at 0, 1 and 2 hr. Total motility **(A,B),** progressive motility **(C,D)**, tyrosine phosphorylation detection **(E,F)** and acrosome integrity **(G)** were quantified at each time point. Different letters (a-d) indicate $P \leq 0.05$ within a single selection technique but between time points. Asterisks (*) indicate $P \leq 0.05$ between sperm selection techniques at each time point. Data presented as mean ± SEM. **(E)** Representative immunofluorescence image showing permeabilized sperm positive for tyrosine phosphorylation (red) and co-stained with FITC-PSA (green) indicating intact acrosome and DAPI (nuclei labeled in blue). **(H)** Summary of ideal time point and incubation conditions to achieve maximum motility and capacitation for fresh sperm held at 38°C or 23°C (room temperature – RT), cooled sperm followed by maintenance at RT, and frozen-thawed sperm also maintained at RT. Scale bar (E)= 20 μm.

efficacy of sperm isolation and holding conditions for promoting functional capacitation and thus activation potential of stallion sperm [6,38,9] (Fig. 5A). Bovine oocytes were fertilized with fresh stallion sperm pre-incubated for 4 hr in capacitating conditions at room temperature as described based on initial experiments that indicated maximum capacitation and maintenance of sperm motility and acrosome integrity. Four independent replicates of IVF resulted in an average cleavage rate of 42% and 7% blastocyst development (Fig. 5B). Oocytes not exposed to stallion sperm were included within replicate as an internal control to account for parthenogenesis. Control embryos yielded an average cleavage rate of 23% and a blastocyst rate of 0.9% (Fig. 5B). Nuclear staining (DAPI) of presumptive zygotes confirmed activation of bovine oocytes with stallion sperm as indicated by pro-nuclei (PN) and polar body extrusion (Fig. 6). Activation rates of 7, 31 and 23% were confirmed at 18, 24 and 42 hr post insemination, respectively (Fig. 6). Mature oocytes (MII) and immature oocytes with germinal vesicle and germinal vesicle breakdown (GV, GVBD) chromatin configuration were also identified in both fertilized and unfertilized (control) groups (Fig. 6).

3.6. Isolation of frozen-thawed stallion sperm by Microfluidics supports equine in vitro embryo development

The functional capacitation of frozen-thawed stallion sperm isolated by Microfluidics was further challenged by determining compatibility for equine IVF. Equine oocytes were aspirated from two mares and placed in maturation conditions for expansion to COCs within 25–28 hr (Fig. 7A,B). Gamete co-incubation was initiated immediately after sperm isolation based on functional characterization data indicating maximum motility and capacitation status of frozen-thawed sperm following isolation (Fig. 4H). Two independent replicates of equine IVF were performed in which 4 oocytes from the first replicate were co-incubated with pooled stallion sperm ($n = 2$ stallions) selected by Microfluidics and 2 oocytes from the second replicate were co-incubated with pooled sperm from the same two stallions selected by Swim-Up. The relatively small number of oocytes was designed to replicate typical availability of oocytes from mares challenged by small ovarian reserves or poor egg quality. Embryo cleavage (2-cell), morula development, and blastocyst expansion were evaluated (Fig. 7). Data from combined replicates resulted in 66.6% 2-cell cleavage and 33.3

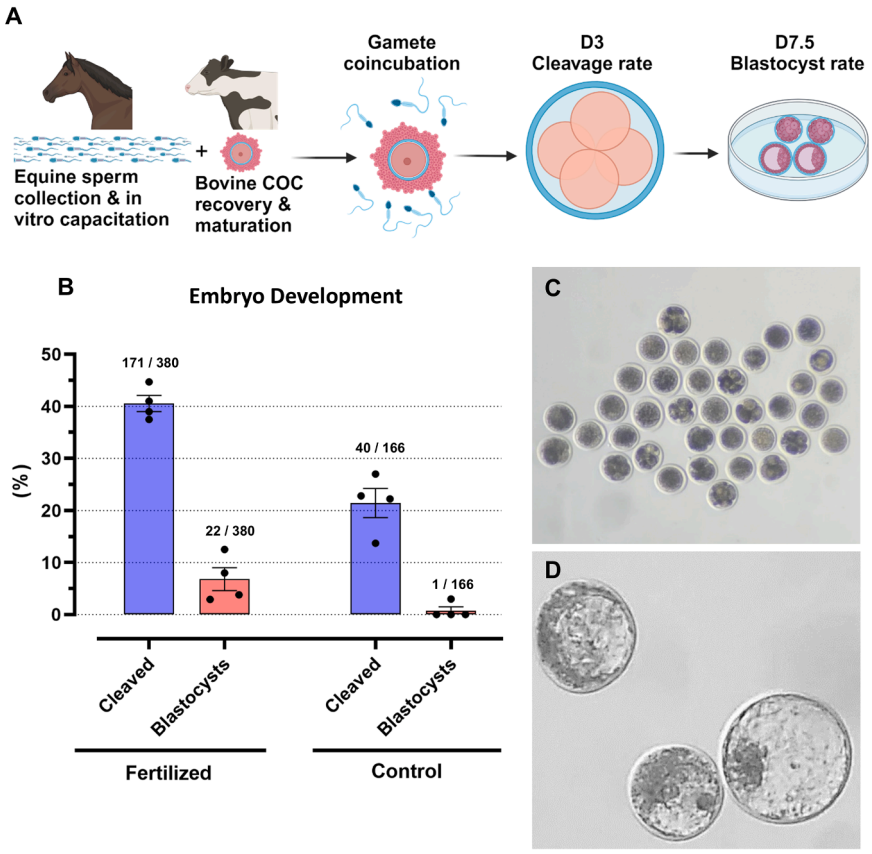


Fig. 5. Heterologous IVF to determine functional capacitation of fresh stallion sperm. Fres sperm from four ejaculates and two different stallions were isolated by Swim-Up, pre-incubated for 4 hr at room temperature in capacitating conditions and subjected to gamete co-incubation using bovine oocytes. **(A)** Graphical representation of workflow for exposing bovine oocytes and stallion sperm to generate embryos. **(B)** Embryo development success depicting cleavage and blastocyst rates for bovine oocytes fertilized with stallion sperm and oocytes not exposed to sperm (control). **(C)** Bright field images depicting day 3 (D3) embryos including 2-cell, early morulas, and (**D**) D7.5 blastocysts (Scale bar = ~100 μm).

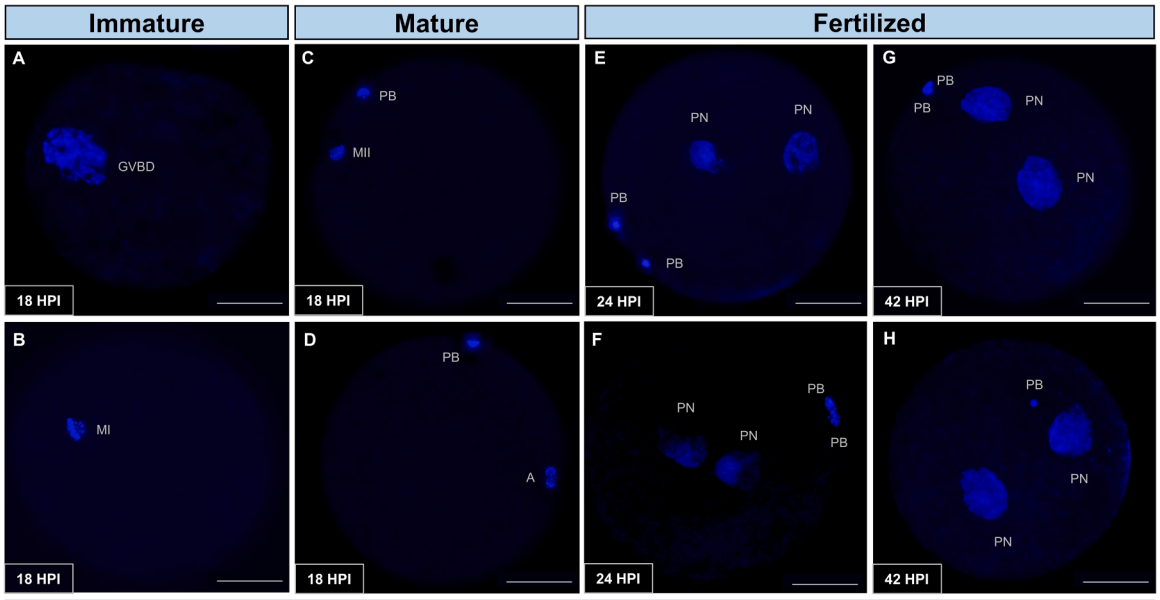
expanded blastocyst development (Fig. 7F), with blastocysts containing a clear blastocele cavity and noticeable thinning of the zona-pellucida.

4. Discussion

Unlike bovine and humans, stallion sperm seem to require unique and undefined responses to elicit capacitation-like changes and the acrosome reaction [23]. In addition, stallion sperm appear resistant to stimulation with capacitating agents used in other species [24]. Such capacitating agents are at least partially inefficient to trigger the acrosome reaction, changes in plasma membrane permeability, hyperactivation, and tyrosine phosphorylation concomitantly. Some events related to capacitation can occur independently, which may mean that complete and functional capacitation does not always occur despite phenotypical observations [18]. Type 10 sAC, cAMP analogues and caffeine can induce tyrosine phosphorylation without triggering stallion sperm hyperactivation [29,36]. Lysophospha-tidylcholine and progesterone are capable of inducing acrosomal reaction while PHE is capable of activating tyrosine phosphorylation in stallion sperm [5,12,16]. However, the complexity of capacitation events and incomplete knowledge to describe discrete measures of functional capacitation in stallion sperm continue to result in lack of established protocols and identification of agents that promote functional capacitation of equine sperm.

To address sperm-specific reasons for IVF failure in horses, the present study aimed to characterize temporal capacitation events to determine optimal *in vitro* conditions for holding sperm. The need to fully characterize temporal aspects of stallion sperm capacitation is supported by IVF reports indicating various success attributed in part to different sperm holding times prior to gamete co-incubation [11,12,27]. In the present study, stallion sperm were maintained at 38°C and 23°C in attempt to identify optimal conditions that maintained motility over time while promoting capacitation potential and maintaining acrosome integrity. Traditional sperm isolation procedures were also compared (Percoll vs. Swim-Up) and no interaction between incubation times and sperm selection technique was observed. Holding sperm for 22 hr at 38°C was detrimental to motility and therefore likely not conducive to IVF efficiency due to subpar motility standards observed in other species. To mitigate the negative effects of warmer temperatures, fresh stallion sperm was held at room temperature (23°C) and achieved ~60% capacitation status while maintaining acrosome integrity, which is important to prevent precocious acrosome reaction that may be detrimental to gamete interaction and zona penetration. Maintaining stallion sperm at RT in capacitating conditions aligned with maximum tyrosine phosphorylation at 4–8 hr and importantly, was supported by kinematic data to suggest that these sperm are compatible for IVF procedures. The observation of ~60% tyrosine phosphorylation suggests that stallion sperm are not necessarily resistant to capacitation, but instead must be maintained in conditions that support motility to prevent loss of viability and thus capacitation. Although plasma membrane integrity was not applied as an indicator of viability, a high correlation between motility and membrane integrity is well-established [1,37]. Therefore, kinematic characteristics were used as an indicator of sperm survival over time under capacitation conditions. A precedence for temperature reduction from 38°C to 30°C in capacitating medium with PHE has also proven beneficial for maintenance of motility, viability and acrosome integrity [26].

In a study conducted by Parker et al., it was not possible to induce



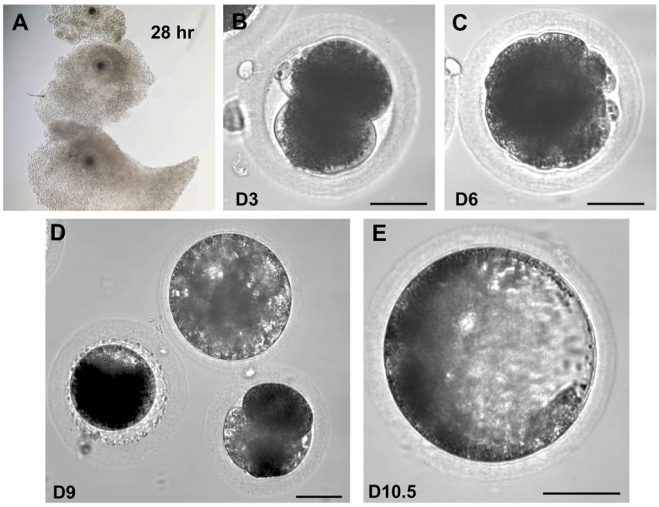
HPI	Fertilization Group	Total (N)	Immature	Mature	Fertilized/Total	Parthenote
18	Control	60	22 (37%)	38 (63%)	0 (0%)	0 (0%)
	Sperm	69	15 (22%)	48 (70%)	**5 (7%)**	1 (1%)
24	Control	46	17 (37%)	27 (59%)	0 (0%)	2 (4%)
	Sperm	42	13 (31%)	16 (38%)	**13 (31%)**	0 (0%)
42	Control	39	17 (44%)	20 (51%)	0 (0%)	2 (5%)
	Sperm	52	16 (31%)	21 (40%)	**12 (23%)**	3 (6%)

Fig. 6. Pro-nuclei and polar body formation in bovine zygotes exposed to stallion sperm. Representative fluorescent images depicting immature **(A,B)**, mature **(C,D)** and fertilized/activated **(E-H)** oocytes stained with DAPI at 18, 24 and 42 hr post insemination (HPI). Embryos with a minimum of two pro-nuclei and two polar bodies were considered activated or fertilized while those with two pro-nuclei but only one visible polar body were categorized as parthenotes. The absence of clearly distinguishable pro-nuclei were further categorized as immature or un-fertilized oocytes. Chromatin structures are indicated by the following: GVBD= germinal vesicle breakdown, MI/MII= metaphase I and II, A= anaphase, PB= polar body, PN= pro-nucleus. Scale bar= 50 μm.

capacitation of stallion sperm *in vitro* through the addition of heparin, a reagent commonly used in sperm from different species. However, equine sperm incubated *in vivo* within the reproductive tract of estrous ewes undergo capacitation, demonstrating that interactions between sperm and the female reproductive tract facilitate capacitation [34]. On the other hand, by performing sperm pre-incubation in capacitating media (STALP + PHE) in the present study, it was possible to achieve sperm capacitation while maintaining sperm motility and thus viability over time. These results demonstrate that stallion sperm resistance to capacitation may be overcome before gamete co-incubation by holding sperm in conditions that promote maintenance of motility over time.

Because many assisted reproductive techniques in horses rely upon cooled semen, experiments were conducted herein to replicate conditions of cooled and shipped stallion semen by maintaining samples at 5°C for 24–48 hr before holding at RT for up to 22 hr. Surprisingly, there was no interaction between cooling time (24 vs. 48 hr) and subsequent time of RT incubation under capacitating conditions, suggesting that cooled stallion semen are compatible with current protocols for promoting capacitation following shipment. Despite a prolonged cooling period, total motility remained ~70% after cooling and was stable for up to 12 hr under these capacitating conditions at RT before dropping below 50% total motility. Interestingly, stallion sperm cooled prior to Swim-Up and re-extension in STALP + PHE had a higher percent of tyrosine positive sperm as numerically compared to fresh sperm maintained over time. In support of these observations, cryopreservation and thawing of stallion sperm also induced capacitation-like changes, likely due to changes in the stability and permeability of the plasma membrane [13,40]. Similarly, frozen-thawed stallion sperm also presented a higher population of acrosome compromised sperm following sperm isolation prior to RT incubation, in alignment with recently reported observations [11]. The increased incidence of acrosome destabilization from fresh, cooled, and then frozen sperm are not likely associated with viability, as sperm motility remained comparable across all conditions when maximum motility was observed. Instead, acrosome destabilization is likely due to inherent stress associated with cooling and freezing processes. One limitation to these studies is the number of stallions used to determine the effect of an individual stallion on holding conditions for sperm. Although individual stallions are likely to respond differently to sperm holding conditions, these findings provide an alternative approach to holding stallion sperm that appears more compatible for reproductive procedures than traditional prolonged culture conditions.

To partially address historical dogma that deficiencies in equine IVF success are specific to stallion sperm rather than of maternal origin, sperm function was challenged by gamete co-incubation with an established bovine *in vitro* embryo production system [28]. Equine IVF has not only been challenged by lack of efficient sperm protocols, but also due to inconsistencies associated with protocols for oocyte maturation, gamete co-incubation, and embryo culture. Prior to IVF, fresh stallion sperm were maintained at 23°C for 4 hr in capacitating conditions to allow maximum capacitation in coordination with maintained motility. Pro-nuclei formation and blastocyst development in embryos demonstrate that functional capacitation of stallion sperm can be achieved *in vitro*. Importantly, the current study also suggests that *in vitro* fertilization with sperm from some stallions may be delayed, as supported by failure to observe pro-nuclei formation until 24–42 hr for oocytes fertilized with one stallion (N = 2 ejaculates) compared to traditional evaluation at ~15 hr for bovine, human, and porcine zygotes. The evidence for delayed fertilization is further substantiated by



F	Cleavage (%) N	Blastocyst (%)	Grade	Size (μM)	Zona Thickness (μM)
Rep 1	3/4 (75%)	1/4 (25%)	1	176	14 μM
Rep 2	1/2 (50%)	1/2 (50%)	3	202	6 μM
Combined	4/6 (66.6%)	2/6 (33.3%)	-	-	-

Fig. 7. Frozen-thawed stallion sperm selected by Microfluidics and Swim-Up promotes equine embryo formation. Six COCs were aspirated from a 19-year-old mare and matured for ~28 hr. After oocyte maturation and evaluation of cumulus cell expansion **(A)**, the oocytes were fertilized with frozen-thawed stallion sperm selected by Microfluidics or Swim-Up, generating cleaved embryos **(B)**, morulas **(C)**, and expanded blastocysts (**D,E**). Two replicates of equine IVF were performed in which 4 oocytes from replicate 1 were fertilized with pooled sperm selected by Microfluidics and 2 oocytes from replicate 2 were fertilized with pooled sperm selected by Swim-Up. Embryo cleavage yielded 75% (3/4) and 50% (1/2) followed by 25% and 50% blastocyst rates, respectively. The combined replicates presented a 66.6% cleavage rate and 33.3% blastocyst rate **(F)**.

equine IVF blastocyst formation obtained with frozen-thawed sperm on D9.5–10.5, several days later than most species. In agreement, Felix et al., demonstrated the need for prolonged sperm incubation to trigger capacitation-related events in equine sperm for fertilization to occur [12]. To address the possibility of parthenogenetic activation, a control group (oocytes not exposed to sperm) were maintained in replicate, resulting in a lower cleavage rate (23%) and production of a single blastocyst. Despite blastocyst development observed in these embryos, the likelihood of incomplete fertilization sufficient to activate development without paternal contribution to the embryo genome is acknowledged. Incorporation of the equine genome into bovine zygotes has not been reported, and thus blastocyst development from heterologous IVF is likely due to sufficient activation induced by stallion sperm, similar to chemical activation permissive for parthenogenetic production of blastocysts. However, the visualization of pro-nuclei and two polar bodies at 24 and 42 hr after gamete co-incubation demonstrate that equine sperm under capacitating conditions were capable of penetration and decondensing. Establishment of interspecies genome incorporation was beyond the scope of this study.

Although the optimization of fresh stallion sperm conditions herein was conducive to capacitation and embryo development in a heterologous system, prolonged pre-incubation of frozen-thawed stallion sperm does not seem beneficial nor conducive to equine IVF. When selecting frozen-thawed sperm by both Microfluidics and Swim-Up, sperm achieved maximum capacitation and motility immediately after sperm isolation, unlike previous experiments using fresh or cooled sperm. These observations are supported by cryocapacitation events described as freezing and thawing processes that promote structural and biochemical changes that are similar to physiological sperm capacitation [13,40]. In agreement with these findings, Felix et al. indicated that a shorter pre-incubation period is required to induce frozen-thawed sperm capacitation compared to fresh sperm utilized for equine IVF (9 vs. 22 hr) [11]. Additionally, recent studies conducted using frozen-thawed sperm report a 10-hour sperm incubation period to induce sperm capacitation [27]. Sperm kinematics were assessed after this period to ensure that motility was above 20% [27]. In the present study, after 2 hr of incubation, motility decreased to ~40%, indicating a drastic drop compared to the initial incubation period (0 hr = ~70%). Because maximum motility and sperm capacitation occurred immediately after sperm selection, this time point was considered optimal for fertilization under our experimental conditions. Differences in frozen-thawed sperm isolation procedures are likely affected by the individual stallions and nuances in handling protocols.

The addition of frozen-thawed stallion sperm to mature oocytes in two consecutive and independent replicates lead to the production of two *in vitro* derived expanded blastocysts. Noteworthy changes to the IVF procedure included shorter maturation times of oocytes (~26 hr) devoid of shipping and pre-maturation conditions despite current industry standard practices for equine ICSI procedures. In addition, the relatively low number of oocytes used for gamete-coincubation (2−5) is significantly lower than bovine embryo protocols while more closely representing clinical practice in challenged mares. Blastocyst production originated from oocytes collected from an aged 19-year old acyclic mare, thus demonstrating compatibility of sperm procedures despite sub-optimal maternal conditions. The observation of comparatively delayed blastocyst formation on D9.5 and 10.5 is supported by initial PN observation in embryos produced by heterologous IVF at 24–42 HPI. These observations suggest that sperm from some stallions may require additional time for complete fertilization unlike bovine, porcine, and human embryos. Such a requirement is likely specific to lack of adequate *in vitro* conditions compared to *in vivo*, as D7 blastocysts are routinely flushed from inseminated mares. Despite observed embryo development, the number of replicates and oocytes used for these experiments are insufficient to determine efficiency in the described procedures for equine IVF production as noted by recent success using frozen-thawed sperm for equine IVF [4,11,27]. Alternatively, the timing of capacitation and fertilization events described herein suggest that sperm from some stallions are likely unique in requirements for timing of

capacitation and successful fertilization. Given variability among stallions, breeds, and methods used in different laboratories, optimization is likely required within experimental conditions to achieve optimized performance.

5. Conclusion

Functional capacitation of fresh and previously cooled stallion sperm can be achieved under conditions at ambient temperature that maintain motility over time and are permissive to capacitation-like changes promoting tyrosine phosphorylation while maintaining acrosome integrity. Unlike fresh and cooled sperm, cryopreserved stallion sperm achieve maximum capacitation and motility immediately following isolation. Procedures for isolating and maintaining stallion sperm at room-temperature for 4 hr demonstrate functional capacitation through oocyte activation in a heterologous bovine IVF system. Similarly, frozen-thawed stallion sperm can support equine embryo development. Combined, these data provide a comprehensive characterization of temporal aspects and conditions that promote functional stallion sperm while suggesting that maternal factors including oocyte maturation and timing of gamete co-incubation play vital roles in successful production of *in vitro* fertilized equine embryos.

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CRediT authorship contribution statement

JDAL responsible for data collection, analyses and preparation of manuscript. JEG, LK, and MM made significant contributions to data collection, experimental design and graphical representation. VB contributed to intellectual support, experimental design and manuscript edits. JWC responsible for semen collection and stallion logistics. JHFP, PF, MM contributed intellectual support. CT provided oocytes and shipping logistics. BD responsible for experimental design, theory, data collection, analyses, manuscript preparation, and original concepts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.repbio.2026.101201.

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