**Supplemental Materials and Methods**

(General methods)

*Oocyte recovery.* Immature oocytes were collected by transvaginal ultrasound-guided follicle aspiration from Quarter Horse-, Thoroughbred- and Warmblood-type research mares, as previously described by Jacobson et al. [34]. The recovered cumulus-oocyte complexes (COCs) were placed in commercial equine embryo holding medium (Vigro, Vetoquinol, Ft. Worth, TX, USA or EmCare, ICPbio, Henderson, New Zealand), and held overnight at room temperature (~22 ºC) or overnight to up to two days at 15 ºC, protected from light. Holding of equine oocytes under these conditions maintains meiotic arrest and does not have detrimental effects on meiotic or developmental competence [35-37]. Holding of oocytes allows their placement in maturation culture at a scheduled time.

Sporadically, ovaries became available from mares being euthanized for reasons unrelated to this study, and on these occasions, oocytes were recovered from the ovaries post-mortem by follicular scraping [38], held as described above, and used as defined for each study.

*Oocyte in vitro maturation.* After the holding period, COCs that were completely or partially denuded of cumulus, or showed signs of degeneration, were discarded. The remaining COCs were placed in 150-µL droplets of equilibrated maturation medium, with one to 15 COCs per droplet. The maturation medium consisted of M199 with Earle´s salts (Invitrogen, Carlsbad, CA, USA or Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 5 mU/mL FSH (Sioux Biochemicals, Sioux Center, IA, USA), and 25 µg/mL gentamicin (Invitrogen), under light mineral oil (Sage, Trumbull, CT, USA). The COCs were cultured at 38.2 ºC in a humidified atmosphere of 5% CO2 in air; onset of culture was coordinated so that oocytes had been in maturation culture for 28 to 35 h, as outlined in each experiment, at the time they were added to the sperm droplet for sperm-oocyte co-incubation. On removal from maturation culture, COCs were evaluated, and those that exhibited signs of degeneration were discarded. Except in Preliminary study 1, in which oocytes were denuded of cumulus before co-incubating with sperm, the COCs were used without knowledge of their maturation status, as the presence of the polar body could not be affirmed visually due to the surrounding cumulus.

*Semen collection.* Two mature stallions (Thoroughbred (stallion FS) and Arabian (stallion SM)) were used as sperm donors. Semen was collected using a Missouri-model artificial vagina (Missouri-model; Nasco, Ft. Atkinson, WI, USA). Sperm motility was evaluated visually or by CASA (IVOS II, Hamilton-Thorne, South Hamilton, MA, USA); only ejaculates with ≥ 70/45 (total/progressive) initial motility were used. Semen was either used immediately or extended with a milk protein-based extender (INRA96, IMV Technologies, Maple Grove, MN) as outlined for each experiment.

*Media.* All chemicals were purchased from Sigma unless otherwise indicated. Tyrode’s albumin lactate pyruvate medium [39] as modified by Alm et al [20],(H. Alm, personal communication 2003) was used for sperm preparation. Two TALP media were used: TALP-R, used for initial sperm resuspension and holding, and FERT-TALP, used for sperm pre-incubation and sperm-oocyte coincubation. TALP-R consisted of 112 mM NaCl, 2.7 mM KCl, 25 mM NaHCO3, 0.04 mM NaH2PO4, 0.05 mM MgCl2, 13.9 mM D-glucose, 5 mM HEPES, 10 mM sodium lactate, and 1 mM sodium pyruvate. Lyophilized bovine serum albumin (BSA; Sigma A3803, which was used for all BSA-containing media in the study) was weighed and added to a final concentration of 4 mg/mL. The medium was 290-305 mOsm and the pH was 7.4 after equilibration at 5% CO2 in air. FERT-TALP consisted of 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, and 6 mg/mL BSA, at 290-305 mOsm and pH 7.30 after equilibration at 5% CO2 in air. Media were prepared within 1 day of the study and dishes with droplets (for FERT-TALP, after addition of PHE as outlined below) were made immediately and placed in the incubator.

A PHE solution was prepared according to Leibfried and Bavister [40], as modified by Alm et al. [20] (H. Alm, personal communication, 2003). The following solutions were made in a base of embryo-quality distilled water containing 154 mM NaCl: **Solution A** (200 mL), 5.26 mM Na2S2O5 and 29.27 mM sodium lactate. **Solution B** (100 mL),1.00 mM hypotaurine. **Solution C** (100 mL), 2.02 mM penicillamine. **Solution D**: To 10 mL of Solution A, epinephrine was added at 10.09 mM. **Solution E**: 39 mL of Solution A combined with 1 mL of Solution D. The final solution (PHE stock solution) was prepared by adding 20 mL of Solution B, 20 mL of Solution C and 16 mL of Solution E to 32 mL of saline (154 mM NaCl). The final solution contained 154 mM NaCl, 0.93 mM Na2S2O5, 5.2 mM sodium lactate, 0.23 mM hypotaurine, 0.46 mM penicillamine, and 0.046 mM epinephrine. This stock solution was filtered and 200-µL aliquots were placed into 1.5 mL Eppendorf tubes, which were wrapped in aluminum foil and stored at -20 ºC.

For preparation of fertilization droplets, an aliquot of PHE was thawed at room temperature, protected from light, and 120 µL of PHE solution was added to 3 mL of FERT-TALP, resulting in final concentrations of 9 µM hypotaurine, 18 µM penicillamine, and 1.8 µM epinephrine. Then, 45 µL droplets of this medium (FT-PHE) were prepared in a 35-mm Petri dish under light mineral oil. The dishes were equilibrated at 38.2 ºC in a humidified atmosphere of 5% CO2 in air for at least 4 h and usually overnight before addition of sperm.

*Sperm preparation.* Raw or extended semen (200 µl), as outlined for each experiment, was layered under 1 mL GMOPS medium (Vitrolife, Englewood, CO, USA) without BSA in a 5-mL polypropylene tube, which was incubated at a 45o angle at 38 oC in air for 20 min. After incubation, the uppermost 750 µL of the medium was collected and was placed in a 1.5-mL Eppendorf tube and centrifuged. This and all subsequent centrifugations were performed at 700 x g for 5 min at room temperature (~ 22 oC).

In some experiments, sperm mitochondria were labeled by addition of MitoTracker Red CMXRos (Invitrogen, final concentration 100 nM) to the swim-up medium, as previously described for visualization of sperm tails after equine ICSI [42]. All other aspects of the sperm preparation procedure remained unchanged. After the first two replicates using this label, the concentration of MitoTracker was reduced to 10 nM.

In preliminary studies, A23187 was utilized. After centrifugation, the sperm pellet was resuspended in 495 µL Hanks Balanced Salt Solution with calcium and magnesium (HBSS; ThermoFisher Scientific, Waltham, MA) without additions. Then, 5 µL of an A23187 stock solution (100 µM A23187 in 5.2% DMSO) was added to the HBSS-sperm suspension to a final concentration of ~1 µM A23187. One min after A23187 addition, 25 μL of a concentrated BSA solution (lyophilized BSA dissolved in HBSS at a concentration of 100 mg/mL BSA) was added to quench A23187 action [43, 44], providing a final concentration of ~4.7 mg/mL. The suspension was centrifuged, the supernatant was removed, and the sperm pellet (~25 µL) was combined with TALP-R previously equilibrated at 5% CO2 in air, to achieve a final volume of 60 µL. In later studies in which A23187 was not used, all the above steps were similarly conducted with the exception that the BSA was added to the HBSS before it was used to resuspend the sperm and the A23187 stock solution was not added. Thus, in later studies without A23187, the steps were: 1) swimup; 2) centrifugation of the uppermost 750 µL; 3) resuspension of the resulting pellet (~25 µL) with 500 µL of HBSS containing 4.7 mg/mL BSA; 4) centrifugation; 5) resuspension of the resulting pellet with TALP-R to a volume of 60 µL.After resuspension with TALP-R, the sample was held in an incubator at 38 ºC in 5% CO2 in air while the concentration of the sperm suspension was determined via hemocytometer on a 2-µL aliquot. To standardize procedures, this holding period was fixed at 15 min. After this, a volume of the sperm suspension providing 50,000 sperm was added to the 45-µL FT-PHE droplet. If the volume of sperm suspension added was less than 5 µL, TALP-R medium was added to the droplet so that the total volume added was 5 µL. The final sperm concentration in the 50-µL droplet was 1 x 106/mL. The droplets containing sperm were incubated for various times (this period was designated “sperm pre-incubation”), as outlined for each experiment, at 38.2 ºC in a humidified atmosphere of 5% CO2 in air, before addition of oocytes or COCs.

*Co-incubation of sperm and oocytes.* After sperm had been pre-incubated in the FT-PHE droplets for the designated period, COCs were removed from maturation culture and washed through four 100-µL droplets of FERT-TALP without PHE. One to three cumulus-denuded oocytes (Preliminary study 1) or COCs, in 1 µL of FERT-TALP medium, were added to each 50-µL FT-PHE droplet containing the pre-incubated sperm suspension. The dishes holding the droplets were placed back in the incubator under the same conditions (38.2 ºC in a humidified atmosphere of 5% CO2 in air). The sperm and oocytes were co-incubated for the duration designated in each experiment.

*Zygote culture.* At or before the time that oocytes (Preliminary study 1) or COCs were placed in the fertilization droplets, culture dishes holding 15-µL droplets of GL-FBS (commercial human embryo culture medium (Global medium, LGGG-050, LifeGlobal, Guilford, CT, USA) supplemented with 10% FBS), under oil were prepared and the dishes placed in 5% CO2 in air at 38.2 oC for equilibration. After the period of sperm-oocyte co-incubation, the oocytes or COCs were removed from the fertilization droplets, washed gently in GL-FBS and transferred to the droplets of GL-FBS. One oocyte or COC was placed per 15-µL droplet, to enable development to be followed over time. The time of transfer of the oocyte from sperm-oocyte co-incubation to the embryo culture droplet was designated Time 0 for reference to embryo age. The culture dishes were incubated in an atmosphere of 6% CO2, 5% O2 and the reminder N2 (Brom-de-Luna et al., 2019) for 36 h or as described for each experiment. This culture period immediately after removal of the oocyte/COC from the fertilization droplet was designated the *Zygote culture period*.

*Embryo culture.* In some experiments, to determine competence for blastocyst development, after the Zygote culture period, presumptive embryos were denuded by pipetting in the culture droplet. Oocytes found to be uncleaved at this time were stained and evaluated as described above to determine their chromatin status. Cleaved oocytes were transferred individually (one per droplet) to new droplets of GL-FBS (5 µL medium per embryo) unless otherwise noted in the Experimental Design. Culture was continued for 3.5 additional days, until Day 5 of culture. On Day 5, cleaved embryos were transferred to droplets of DMEM/F-12 with 10% FBS at a ratio of 5 µL medium per embryo, and cultured at 6% CO2, 5% O2 and the reminder N2 until blastocyst formation was recognized or until Day 10 of culture. Identification as a blastocyst was based on organization of an outer presumptive trophoblast layer with decreasing density of inner cells, as determined on evaluation on brightfield at 200 x [45]. Except where indicated, blastocysts were then vitrified as described by Canesin et al. [46].

*Staining and evaluation of oocytes.* For simplicity in this report, one-cell oocytes exposed to sperm are referred to as “oocytes” regardless of their developmental stage. If oocytes were to be fixed and stained for evidence of fertilization, the cumulus, if present, was removed by gentle pipetting without use of hyaluronidase, and the oocytes were fixed in buffered formol saline (Animal Reproduction Systems, Chino, CA, USA). The fixed oocytes were mounted on slides and stained using a commercial mounting medium containing DAPI (SlowFade Diamond Antifade Mountant with DAPI; Invitrogen, Carlsbad, CA, USA) and the chromatin configuration was assessed using a fluorescence microscope (Zeiss AxioObserver) with a 365-nm excitation filter. Fertilization status was classified as described by Hinrichs et al., (2002) with modifications. Briefly, oocyte chromatin configurations were classified as metaphase (regardless of presence of a polar body, as the polar body may degenerate with prolonged culture; [47]); anaphase/telophase; pronuclear; cleaved, (having two or more cells with normal-appearing nuclei present); and abnormal (immature or degenerating chromatin). Sperm chromatin was classified as condensed, decondensing (larger area or irregular shape, but still recognizable as a sperm head) and pronuclear. Oocytes exhibiting metaphase, anaphase/telophase or pronucleus formation, with sperm chromatin present, were classified as fertilized. Cleaved embryos were classified as having 2, 3-4, 5-6, or 7-8 nuclei. Fertilization rates were expressed as (fertilized + embryos) / (metaphase oocytes + fertilized + embryos), to omit oocytes that may have been immature or degenerating at the time they were placed in co-culture with sperm.

In experiments in which sperm had been stained with MitoTracker during swimup, oocytes were counter-stained with DAPI as described above and evaluated under fluorescence microscopy with both 365-nm and 545-nm excitation filters. Photomicrographs were taken at different focal planes and the images were reconstructed using the “Auto-blend layers” function in Photoshop.

Confocal microscopy was performed on a subset of intact COCs after co-incubation with MitoTracker-labeled sperm, for assessment of chromatin, sperm tail and acrosome status. For this, the COCs were removed from the fertilization droplet, washed gently in Dulbecco’s phosphate buffered saline (DPBS; Sigma) with 2 mg/ml BSA, and placed in 4% paraformaldehyde (1 part Electron Microscopy Sciences 16% paraformaldehyde aqueous solution, EM grade, Thermo Fisher Scientific: 3 parts DPBS) for a minimum of 15 min. The COCs were rinsed in DPBS with 2 mg/ml BSA, then transferred to 0.1% Triton-X 100 in DPBS and held at room temperature for 20 minutes, washed in DPBS with BSA, then placed in a solution of 20 µg/ml Alexa Fluor 488-conjugated lectin from Arachis hypogaea (peanut agglutinin, PNA; Thermo Fisher) in DPBS and incubated for 30 minutes in a humidified atmosphere at 37 °C.

The COCs were then washed and placed in a solution of 10 µg/mL Hoechst 33342 (Invitrogen) in DPBS for 5 minutes, then rinsed and placed on a slide (one COC per slide) with the minimum medium possible. A 10-µl droplet of non DAPI-containing mounting medium (SlowFade Diamond Antifade Mountant, Invitrogen) was placed over the COC, and the preparation was carefully covered with a glass coverslip, suspended from the slide by placing paraffin at the corners, and the coverslip compressed until it touched the medium. After 5 min, the coverslip was secured by brushing nail polish around the edges. The slides were stored at -20 oC until they were analyzed (maximum 5 days storage).

Confocal images were acquired on a Leica (Mannheim, Germany) SP5 II confocal microscope with a 63x (1.2 NA) water immersion objective lens. Hoechst, Alexa Fluor 488-PNA and MitoTracker Red CMXRos were sequentially excited with 405, 488, and 543 nm lasers respectively and fluorescence signals were detected with a Hybrid detector. Emission widows were set to 415-472 nm, 498-551 nm, and 558-736 nm for Hoechst, FITC-PNA, and MitoTracker Red CMXRos, respectively, and Z-stacks were acquired at a step size of 0.5 µm.

*Staining of sperm for protein tyrosine phosphorylation*. For assessment of protein tyrosine phosphorylation in sperm under conditions that support IVF, duplicate droplets of sperm in FT-PHE were prepared identically to and incubated side-by-side with droplets used for IVF, with the exception that sperm were not stained with MitoTracker. After the designated period of pre-incubation, as outlined for each experiment, the contents of the droplet, which contained 50,000 sperm, were carefully aspirated from beneath the oil overlay and transferred to a 12-mm poly-L lysine-coated coverslip (Corning BioCoat coverslips, ThermoFisher) in an individual well of a 5-well culture dish (Minitube USA, Verona, WI) and the sperm were allowed to settle onto the coverslip for 15 minutes.

Protein tyrosine phosphorylation was also assessed in control (extended) sperm and in sperm incubated in a high-pH environment, as outlined in the individual experiments. For extended sperm, an aliquot containing 1 x 106 cells was washed twice in DPBS, then centrifuged and the pellet resuspended to ~ 1 million sperm per ml in DPBS. This was then loaded onto coverslips as for the sperm from the pre-incubation droplets, above.

After the sperm had settled on the coverslip, the medium was aspirated and the cells were fixed by addition of 100 µl of 4% paraformaldehyde in DPBS. After 15 min, the coverslips were rinsed three times, and 0.1% Triton X100 in DPBS was added. After 2 minutes, the coverslips were again rinsed and were stored under DPBS at 4 oC until stained.

For staining, the coverslips were rinsed in DPBS then blocked with 10% normal goat serum (AbCam, Waltham, MA) in DPBS for 4 hours at room temperature, or overnight at 4 oC. The coverslips were then exposed to primary antibody (anti-phosphotyrosine clone 4G 10, EMD Millipore Sigma), 1:100 (v:v) in 10% normal goat serum, for 1 hour at room temperature. Coverslips were washed and exposed to the secondary antibody (Alexa Fluor 633 goat anti-mouse IgG (H+L), ThermoFisher) 1:100 (v:v) in 10% normal goat serum for 1 hour. After washing, the coverslips were exposed to 10 µg/ml Hoechst 33258 (Invitrogen) in DPBS for 2 min before being rinsed again and mounted onto slides with mounting medium (Fluoromount G, ThermoFisher).

Confocal images were acquired using the confocal system noted above, with Hoechst and anti-phosphotyrosine/Alexa Fluor 633 sequentially excited with 405 and 633 nm lasers respectively. Emission windows were set to 415-503 nm for Hoechst and 643-762 nm for Alexa Fluor 633, with a Z step size of 0.29 µm. All fluorescent images were obtained using one standardized exposure setting to allow semi-quantitative assessment of intensity of PY-related fluorescence. Duplicate sperm preparations processed simultaneously but without addition of the primary antibody served as a control for non-specific binding of the secondary antibody.

*Evaluation of sperm for acrosome status and viability via flow cytometry.* The contents of a pre-incubation droplet (~50,000 total sperm in 50 µL) were placed in an Eppendorf tube and diluted to 250-500 cells/µl in PBS. The samples were incubated with 5 ng/mL FITC-conjugated PNA (Sigma) and 37.5 nM propidium iodide (PI) at 37 oC in the dark for 10 minutes.

This suspension was placed in the feed of the flow cytometer (CytoFLEX, Beckman Coulter, Inc., Brea, CA, USA). The voltage settings used were FSC 103, SSC 295, with 488-nm laser excitation. FITC fluorescence was collected through a 525/40 nm bandpass filter, and PI fluorescence collected through a 690/50 nm bandpass filter. A total of 10,000 events were evaluated per sample and data were acquired using a log scale. The data was collected and analyzed using the Cytoflex S Benchtop Flow Cytometer and CytExpert Software, version 2.4 (Beckman Coulter).