PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

MESTRADO EM CIÊNCIA ANIMAL

TALITA RAQUEL CAVICHIOLI SEBASTIÃO

EFEITO DA α-L-FUCOSIDASE SOBRE A MODULAÇÃO DA ZONA PELUCIDA E DESENVOLVIMENTO DE EMBRIÕES BOVINOS PRODUZIDOS *IN VITRO*

Presidente Prudente – SP
2022

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO

MESTRADO EM CIÊNCIA ANIMAL

TALITA RAQUEL CAVICHIOLI SEBASTIÃO

EFEITO DA α-L-FUCOSIDASE SOBRE A MODULAÇÃO DA ZONA PELUCIDA E DESENVOLVIMENTO DE EMBRIÕES BOVINOS PRODUZIDOS *IN VITRO*

Dissertação apresentada a Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciência Animal.

Orientador: Prof. Dr. Anthony César de Souza Castilho

Presidente Prudente – SP
2022

636.089
S443e

Sebastião, Talita Raquel Cavichioli.
Efeito Da α-L-Fucosidase sobre a modulação da zona pelucida e desenvolvimento de embriões bovinos produzidos *in vitro* / Talita Raquel Cavichioli Sebastião. – Presidente Prudente, 2022.
55f.: il.

Dissertação (Mestrado em Ciência Animal) - Universidade do Oeste Paulista – Unoeste, Presidente Prudente, SP, 2022.
Bibliografia.
Orientador: Anthony César de Souza Castilho.

1. Produção *in vitro* de embriões. 2. Polispermia. 3. Expressão gênica. 4. Blastocisto. I. Título.

Catalogação na fonte: Michele Mologni – CRB 8-6204

TALITA RAQUEL CAVICHIOLI SEBASTIÃO

EFEITO DA α-L-FUCOSIDASE SOBRE A MODULAÇÃO DA ZONA PELUCIDA E DESENVOLVIMENTO DE EMBRIÕES BOVINOS PRODUZIDOS *IN VITRO*

Dissertação apresentada a Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciência Animal.

Presidente Prudente, 23 de março de 2022.

BANCA EXAMINADORA

_____

Prof. Dr. Anthony César de Souza Castilho
Universidade do Oeste Paulista – Unoeste
Presidente Prudente - SP

_____

Profª. Dra. Caliê Castilho Silvestre
Universidade do Oeste Paulista – Unoeste
Presidente Prudente - SP

_____

Profª. Dra. Raquel Zaneti Puelker
Progest Biotecnologia em Reprodução Animal
Botucatu - SP

AGRADECIMENTOS

A Deus, por ser essencial em minha vida e ter me dado saúde e força para superar os momentos de dificuldade, e esperança para acreditar que somos o percursor dos nossos próprios sonhos.

Aos meus pais, aos meus irmãos, aos meus sobrinhos e a toda minha família pelo amor, incentivo e apoio incondicional.

A minha equipe de projeto, Patrícia Kubo, Thainá Bacco, Priscila Santos, Fernanda Franchi e Sarah Nunes que foram fundamentais para a realização do mesmo, pela dedicação e engajamento durante a realização de todas as etapas do projeto.

Ao meu orientador, Prof. Dr. Anthony César de Souza Castilho pela oportunidade, confiança e suporte concedido.

Aos meus amigos, por todos os momentos que compartilhamos e pelo companheirismo ao longo desses anos.

À Universidade, ao corpo docente da pós-graduação do Ciência Animal, aos funcionários e todas as pessoas que contribuíram direta ou indiretamente para a conclusão da minha pós-graduação.

“O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – (Brasil) CAPES – Código de Financiamento 001”.

A todos o meu muito obrigada!

“Sem sonhos, a vida não tem brilho. Sem metas, os sonhos não têm alicerces. Sem prioridades, os sonhos não se tornam reais. Sonhe, trace metas, estabeleça prioridades e corra riscos para executar seus sonhos. Melhor é errar por tentar do que errar por omitir”

Augusto Cury

RESUMO

Efeito Da α-L-Fucosidase sobre a modulação da zona pelucida e desenvolvimento de embriões bovinos produzidos *in vitro*

A polispermia durante a fertilização *in vitro* ainda é uma importante limitação nos avanços da produção de embriões bovinos *in vitro*, resultando na redução do desenvolvimento embrionário, morte embrionária precoce ou aborto espontâneo. O oviduto é um orgão reprodutor com diversas funções, como transporte de gametas, maturação oocitária, fertilização, desenvolvimento inicial embrionário, além de ser responsável pela produção do fluido oviductal. A glicoproteína α-L-fucosidase (FUCA), uma glicosidase encontrada no fluído ovidutal de mamíferos, possui envolvimento na degradação hidrolítica da fucose, seu substrato presente na zona pelúcida (ZP), participando da ligação espermatozoide-oócito através das interações com glicanos complementares na superfície da ZP, atuando no controle da polispermia. Nosso objetivo foi investigar o efeito da adição da FUCA durante a pré-fertilização *in vitro* sobre a modulação da zona pelúcida, controle da polispermia, desenvolvimento embrionário e qualidade de blastocistos bovinos produzidos *in vitro*. No primeiro experimento, o efeito da FUCA (0,125 U/mL) foi avaliado durante toda a fertilização *in vitro* (FIV). No entanto, foi demonstrado que é embriotóxico por inibir completamente a formação de blastocisto. No segundo experimento, a FUCA (0,125 U/mL) foi testada como incubação de curto prazo antes da fertilização *in vitro* (etapa de pré-fertilização) por 30 min ou 2 h, o que demonstrou que o tratamento com a FUCA por 30 min resultou em endurecimento da ZP. No terceiro experimento, um tratamento pré-fertilização com FUCA (1 h) em diferentes concentrações (0, 0,0625 e 0,125 U/mL) mostrou que a FUCA (0,0625 U/mL) melhorou o endurecimento da ZP e tendeu a aumentar as taxas de fertilização monospérmica, mas não melhorou o rendimento e a qualidade do embrião. Em conjunto, foi demonstrado que a FUCA pode induzir o endurecimento da ZP pré-fertilização do oócito e pode melhorar o desempenho da fertilização monospérmica e esse efeito é dependente de ambas as variáveis (concentração de proteína e tempo de incubação).

Palavras-chave: Produção *in vitro* de embriões, polispermia, FUCA, expressão gênica, blastocisto.

ABSTRACT

Effect of α-L-Fucosidase on zona pellucida modulation and development of *in vitro* produced bovine embryos

Polyspermy during *in vitro* fertilization is still an important limitation in advances in in the production of bovine embryos *in vitro*, resulting in reduced embryonic development, early embryonic death or miscarriage. The oviduct is a reproductive organ with several functions, such as gamete transport, oocyte maturation, fertilization, early embryonic development, in addition to being responsible for the production of oviductal fluid. The glycoprotein α-L-fucosidase (FUCA), a glucosidase found in the oviductial fluid of mammals, is involved in the hydrolytic degradation of fucose, its substrate present in the zona pellucida (ZP), participating in the spermatozoa-oocyte connection through interactions with complementary glycans on the surface of the ZP, acting in the control of polyspermy. Our objective was to investigate the effect of the addition of FUCA during pre-IVF on the modulation of the zona pellucida, polyspermy control, embryonic development and quality of bovine blastocysts produced *in vitro*. In the frst experiment, the efect of FUCA (0.125 U/mL) was evaluated during the entire *in vitro* fertilization (IVF). However, it was demonstrated to be embryotoxic by completely inhibiting the blastocyst formation. In the second experiment, the FUCA (0.125 U/mL) was tested as short-term incubation before IVF (pre-fertilization step) for 30 min or 2 h, which demonstrated that FUCA treatment for 30 min resulted in ZP hardening. In the third experiment, a pre-fertilization FUCA treatment (1 h) at diferent concentrations (0, 0.0625, and 0.125 U/mL) showed that FUCA (0.0625 U/mL) improved pre-fertilization ZP hardening and tended to increase monospermic fertilization rates but did not improve embryo yield and quality. Together, it has been demonstrated that FUCA can induce oocyte pre-fertilization ZP hardening and might improve monospermic fertilization performance, and this efect is dependent on both variables (protein concentration and incubation time).

Keywords: *In vitro* embryo production, polyspermy, FUCA, gene expression, blastocyst.

LISTA DE SIGLAS

COC – Cumulus-oocyte complex

FUCA – a-L-fucosidase

IVC – *In vitro* cultive

IVEP – *In vitro* embryo production

IVF – *In vitro* fertilization

IVM – *In vitro* oocyte maturation

OF – Oviductal fluid

OVGP1 – Specific glycoprotein of oviduct

ZP – Zona pelúcida

SUMÁRIO

O presente artigo foi redigido e submetido segundos as normas da Revista Veterinary Research Communications e mantido na formatação original da revista.

Veterinary Research Communications
https://doi.org/10.1007/s11259-023-10291-y

RESEARCH

Pre-fertilization approach using α-l-fucosidase modulates zona pellucida hardening during bovine *in vitro* embryo production

Thainá Sallum Bacco Manssur[1] · Talita Raquel Cavichioli Sebastião[1] · Fernanda Fagali Franchi[2] · Priscila Helena dos Santos[2] · Eduardo Montanari Razza[2] · Sarah Gomes Nunes[2] · Anthony César de Souza Castilho[1] · Patricia Kubo Fontes[2]

Received: 19 October 2023 / Accepted: 28 December 2023

Abstract

The polyspermy occurrence is considerably lower under *in vivo* compared to *in vitro* embryo culture conditions, suggesting that the presence of some factors in the maternal environment is responsible for this. The α-L-fucosidase (FUCA) is a natural glycosidase present in the oviductal fluid, therefore, this study aimed at investigating the effect of adding FUCA to the hardening of the zona pellucida (ZP), polyspermy control, and embryonic yield and quality of bovine blastocysts produced *in vitro.* In the first experiment, the effect of FUCA (0.125 U/mL) was evaluated during the entire *in vitro* fertilization (IVF). However, it was demonstrated to be embryotoxic by completely inhibiting the blastocyst formation. In the second experiment, the FUCA (0.125 U/mL) was tested as short-term incubation before IVF (pre-fertilization step) for 30 min or 2 h, which demonstrated that FUCA treatment for 30 min resulted in ZP hardening. In the third experiment, a pre-fertilization FUCA treatment (1 h) at different concentrations (0, 0.0625, and 0.125 U/mL) showed that FUCA (0.0625 U/mL) improved pre-fertilization ZP hardening and tended to increase monospermic fertilization rates but did not improve embryo yield and quality. Together, it has been demonstrated that FUCA can induce oocyte pre-fertilization ZP hardening and might improve monospermic fertilization performance, and this effect is dependent on both variables (protein concentration and incubation time).

Keywords Polyspermy · FUCA · Blastocyst · Gene Expression · Cattle

Introduction

The efficiency of *in vitro* embryo production (IVEP) has not overcome the 40% blastocyst production success even after many years of progressive development (Lonergan et al. 2016). Among the challenges to improving this technology, polyspermic fertilization is the focus of this present study. Polyspermy is a pathological event that allows the penetration of more than one sperm within the oocyte during fertilization, resulting in low yield production and/or impaired embryo quality (Xia et al. 2001; Wang et al. 2003; Bijttebier et al. 2008). In cattle, the polyspermy rate during *in vitro* fertilization (IVF) ranges from 5 to 45% (Coy and Avilés 2010). Although the scenario for bovine IVF condition is better when compared to the pig IVF conditions (40–60% polyspermy incidence) (Coy and Romar 2002), yet, it is worse when compared to sheep, goats, hamsters, rabbits, and rats, of which the percentage of polyspermy is almost 20% (Coy and Avilés 2010). In natural conditions in mammals, the oviductal secretion (known as oviductal fluid – OF) has been described to be responsible for modifications in the zona pellucida (ZP) once the oocyte reaches the oviduct. These modifications have been demonstrated to result in the ZP-hardening even before sperm-oocyte interaction, therefore, described as "pre-fertilization ZP hardening" (Coy et al. 2008; Coy and Avilés 2010). From the observations in the *in vivo* conditions, some strategies were proposed to control the *in vitro* polyspermic fertilization. For instance,

✉ Patricia Kubo Fontes
pkfontes@gmail.com

[1] University of Western São Paulo (UNOESTE), Presidente Prudente, São Paulo State, Brazil

[2] Laboratory of Phytomedicines, Pharmacology and Biotechnology, Department of Pharmacology, Institute of Biosciences, University of São Paulo State (UNESP), Rua Prof. Antonio Celso Wagner Zanin, S/N, Zip Code: 18618689, Botucatu, São Paulo State, Brazil

Published online: 08 January 2024

Mondéjar et al. (Mondéjar et al. 2013) demonstrated that bovine oocytes incubated *in vitro* with OF presented higher monospermy levels, which was positively associated with the ZP hardening. Interestingly, improvement in the ZP hardening was also obtained by *in vitro* co-culture of oocytes with oviductal epithelial cells during the *in vitro* oocyte maturation, which also improved the monospermic fertilization rates (Davachi et al. 2016). Beyond, Ferraz et al. (Ferraz et al. 2017) described a completely abolished polyspermic fertilization in bovine oocytes when performing the *in vitro* fertilization process with oviductal epithelial cells co-culture microfluidic system. Given the above, it is certain that some OF component(s) are responsible for pre-fertilization ZP hardening, which is associated with control of polyspermic fertilization. However, no consensus in the identification of this oviductal factor(s) has been demonstrated yet.

Among the OF components responsible for the pre-fertilization ZP hardening, the oviductal-specific glycoprotein (OVGP1) was suggested as a possible candidate in cattle (Coy et al. 2008). Additionally, Mondéjar et al. (Mondéjar et al. 2013) also pointed out that the 78-kDa glucose-regulated protein (GRP78) regulates or participates in such mechanism in cattle and pigs. Later, Romero-Aguirregomezcorta et al. (Romero-Aguirregomezcorta et al. 2015) also evaluated the α-L-fucosidase (FUCA) as a candidate in the porcine model, however, they did not observe any improvement in the fertilization outcomes. In opposite to (Romero-Aguirregomezcorta et al. 2015), our research group has demonstrated that the α-L-fucosidase might develop some important role in the fertilization process in the bovine species. First, we demonstrated that the transcript levels of α-L-fucosidase (encoded by the *FUCA1* gene) were higher in the ipsilateral ampulla, the site where the fertilization process takes place (Fontes et al. 2018). Later, we also demonstrated that its transcript level is related to estradiol concentration, which was increased in the oviduct during the peri-ovulatory period (Fontes et al. 2019). Corroborating with our data, Carrasco et al. demonstrated that bovine oviductal fluid has glycosidase activity, including the α-L-fucosidase (Carrasco et al. 2008). Moreover, they suggested that α-L-fucosidase might develop a role in reproductive events, especially considering that among the glycosidases analyzed, the total α-L-fucosidase activity was the only one that increased significantly after ovulation (Carrasco et al. 2008).

Therefore, we hypothesize that FUCA is an OF component responsible for the pre-fertilization ZP hardening in bovine species, which, to the best of our knowledge, has not been evaluated yet. To test our hypothesis, our goal was to evaluate if FUCA added during the bovine *in vitro* fertilization could improve the ZP hardening and, later, evaluate if this modification contributes to increase in the monospermic fertilization, blastocyst formation yield, and *in vitro* embryo quality.

Material and Methods

Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) at the highest available purity.

Cumulus-oocyte complex (COC) recovery and *in vitro* embryo production (IVEP)

Ovaries from cows were collected from a local slaughterhouse. Immediately after the animals were slaughtered, the ovaries were placed in a thermal container containing sterile saline (NaCl 0.9% w/v) at 30° C and transported to the laboratory (one hour). Follicles of 3–8 mm diameter were aspirated using a needle and syringe. The recovered follicular fluid was deposited in a conical tube and kept at 35° C for cellular pelleting (10 min). COCs grades 1 and 2 (Stojkovic et al. 2001) were selected for all the experiments, essentially the oocytes with homogeneous cytoplasm or only small irregular pigmentation areas and multiple compact layers of cumulus cells.

After selection, the COCs were submitted to *in vitro maturation* (IVM). COCs were deposited in four-well plates (45–50 COCs/well) containing 10 µL/COC of maturation medium and kept in an incubator (highly humid atmosphere, 5.5% CO_2, 38.5°C) for 24 h. The maturation medium used was bicarbonate-based TCM199 supplemented with Bovine Serum Albumin (BSA, 4 mg/mL), pyruvate (0.22 mg/mL), amikacin (75 µg/mL), and recombinant human FSH (0.1 UI/ml).

For *in vitro fertilization* (IVF), groups of 20–25 matured COCs were transferred to 90 µL of fertilization medium composed of Tyrode's Lactate Stock (TL-Stock) added BSA (6 mg/mL), pyruvate (0.2 mM), amikacin (75 µg/mL), heparin (30 µg/mL), and PHE solution (20 µM Penicillamine, 10 µM Hypotaurine, and 1 µM Epinephrine). For fertilization, bull semen straws kept in liquid nitrogen were used. The semen was thawed in water at 36°C for 30 s. Then, the live sperm were selected by centrifugation in percoll gradient concentration (45 and 90%) and subjected to motility and concentration assessment. The sperm were diluted to a final concentration of 1×10^6 sperm/mL with IVF media and 6µL of this solution was added in each 90 µL IVF drop previously with the matured COCs. IVF medium was covered with silicone oil and COCs and sperm were kept in the IVF medium for 18 h in the incubator (highly humid atmosphere, 5.5% CO_2, 38.5°C).

Subsequently, the presumable zygotes (PZ) were subjected to cumulus cell removal by vortex and transferred to *in vitro* culture (IVC) medium (10 µL/PZ) in four-well plates (25 PZ/well). The IVC medium used was SOFaa (Synthetic Oviduct Fluid) supplemented by Bovine Fetal

Serum (FBS, 2.5%), BSA (5 mg/mL), pyruvate (0.2 mM), and amikacin (75 µg/mL). The PZ was cultured for 8 days in a high-humidity atmosphere, with 5% CO_2, 5% O_2, and 90% N_2, at 38.5°C.

Treatment with α-L-Fucosidase

The α-L-fucosidase protein (FUCA, F-5884; Sigma-Aldrich) was used in all the experiments. The enzyme was obtained as a suspension in 3.2 M ammonium sulfate containing 10 mM sodium phosphate monobasic, and 10 mM citrate, with a specific activity of ≥ 2 U per mg protein (Biuret). First, the enzyme was diluted in TL-Stock, aliquoted, and kept at -80°C in a stock concentration of 0.25 U/µL. The FUCA treatments were always performed by adding the FUCA to the IVF medium. Three experiments were designed, as described below (Fig. 1):

Experiment 1: The effect of FUCA during the entire IVF

After IVM, the COCs were divided into two groups for IVF: the control group (no FUCA added) and FUCA (0.125 U/ml), the treatment was kept during the entire IVF period (18 h). Subsequently, the PZ followed to IVC. The blastocyst rate was assessed on the last culture day (D8). This experiment was performed in two replicates, 160 COCs in total (n = 40 COCs/group/replicate). The FUCA concentration (0.125 U/mL) was selected based on the OF glycosidase activity described in bovine oviductal fluid (Carrasco et al. 2008) (Fig. 1).

Experiment 2: Short-time effect of FUCA during a pre-fertilization period

Considering the clear toxic effect of the FUCA incubation during the entire IVF observed in experiment 1, we decided to apply another strategy by reducing the COC's time exposure to FUCA treatment. Since our hypothesis is that the FUCA might be responsible for the pre-fertilization ZP hardening, we reduced the incubation time simulating the period that the COC is in the oviduct before the sperm reaches the fertilization site, which has also been the strategy of other studies that evaluated the pre-fertilization ZP hardening (Romero-Aguirregomezcorta et al. 2015; Coy et al. 2008; Coy and Avilés 2010).

Therefore, after IVM, the COCs were submitted to pre-fertilization treatment for 30 min or 2 h with 0 (control group) or FUCA (0.125 U/mL), resulting in four groups (control-30 min, control-2 h, FUCA-30 min, and FUCA-2 h). After treatment, the COCs were submitted to ZP digestion time (experiment 2.1), performed in four independent replicates, 160 COCs in total (n = 10 COCs/group/replicate), or submitted to *in vitro* embryo production (experiment 2.2) for blastocyst rate assessment on the last culture day (D8), performed in six independent replicates, 240 COCs in total (n = 10 COCs/group/replicate) (Fig. 1).

Experiment 3: The effect of FUCA in different concentrations during pre-fertilization

Since the pre-fertilization treatment (30 min incubation) presented a positive ZP hardening effect (experiment 2), we hypothesized that decreasing the FUCA concentration and increasing the incubation time could improve embryo development.

Therefore, after IVM, COCs were submitted to pre-fertilization treatment for 1 h, and different FUCA concentrations were tested: 0 (control group), 0.0625 U/mL of FUCA, and 0.125 U/mL of FUCA. After treatment, the COCs were washed and divided into three experiments: experiment 3.1, the COCs were submitted to ZP digestion time (n = 10 COCs/group/replicate) performed in three independent replicates, 30 COCs in total per replicate, experiment 3.2, the COCs were submitted to IVF and the monospermic fertilization rate was evaluated (n = 10 COCs/group/replicate) in three independent replicates, 30 COCs in total per replicate, last, in experiment 3.3, the COCs were submitted to *in vitro* embryo production (n = 25–30 COCs/group/replicate) performed in three independent replicates, 90 COCs in total per replicate. On the last culture day (D8), the blastocyst rate was analyzed and embryos at the blastocyst stage were stored at -80°C for gene expression analysis (Fig. 1).

ZP digestion time

After treatments, the COCs were submitted to cumulus cell removal by vortex, washed in PBS Ca^{+2}/Mg^{+2} free, and incubated in 50 µl of 0.5% (w/v) pronase (P6911, Sigma-Aldrich) solution in PBS according to the protocol established by Coy et al. (2008). The ZP digestion process was monitored continuously and individually for each oocyte after pronase treatment using a stereomicroscope (20 × and 40x). The dissolution time of the zona pellucida of each oocyte was recorded individually, measuring the interval between the time the oocyte was placed in the pronase solution until the ZP was no longer visible. This allowed accurate measurement of the time required for each oocyte to digest its ZP.

Evaluation of monospermic fertilization rate

Frozen sperm were thawed at 37 °C for 30 s and live sperm were selected by centrifugation in a percoll gradient concentration (45 and 90%). After, semen was stained with MitoTracker green FM® (250 nM solution) by incubation in the fertilization medium for 30 min (37°C). The stained

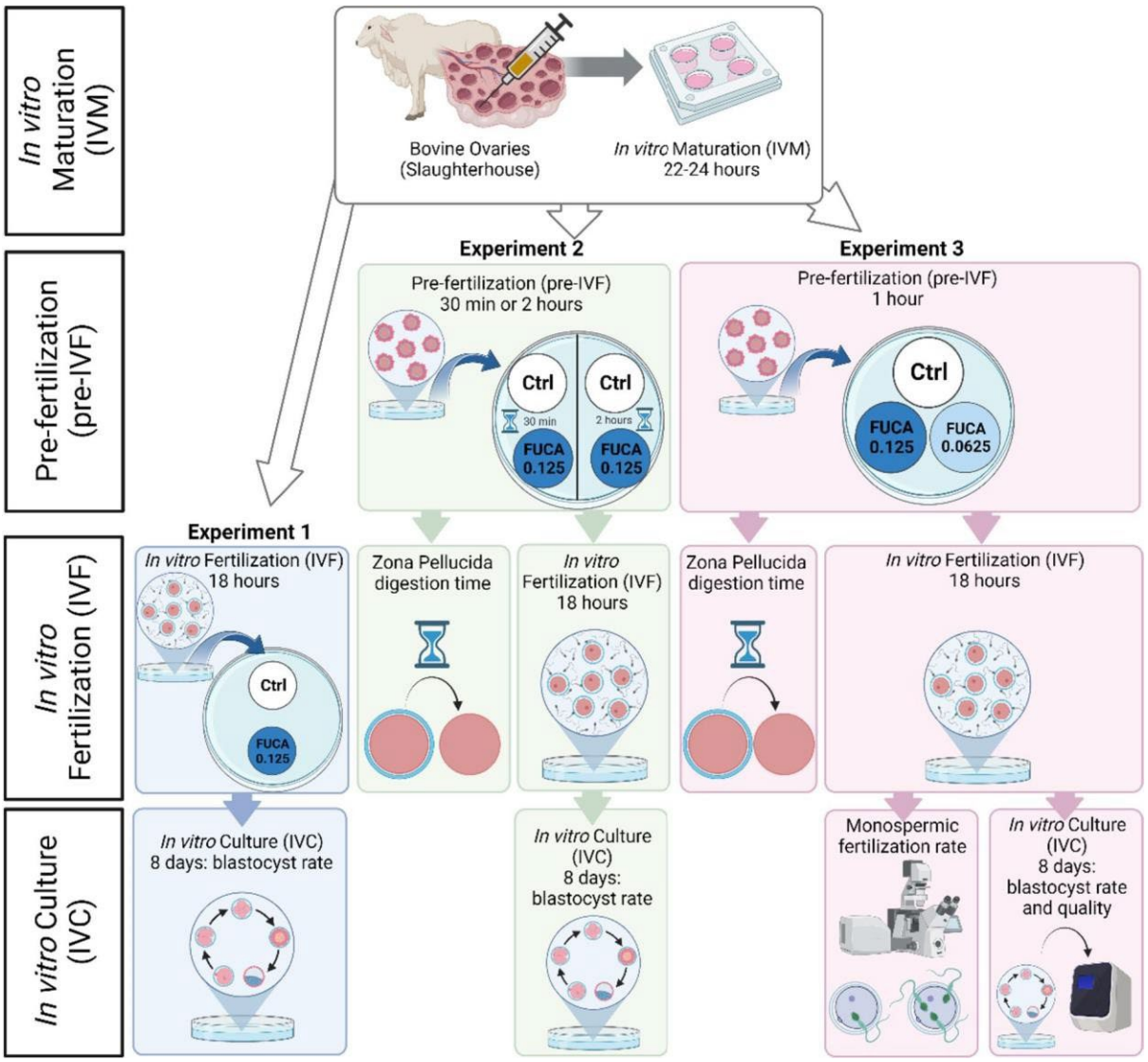


Fig. 1 Experimental design. This study was divided into three experi- ments, for that, all the bovine cumulus-oocyte complexes (COCs) were obtained from ovaries from a slaughterhouse and submitted to *in vitro* maturation (IVM) for 22 to 24 h. Experiment 1 (blue): evalu- ation of FUCA effect during the entire *in vitro* fertilization (IVF) period, the COCs and sperm were incubated in IVF media with 0 (control group) or FUCA (0.125 U/mL) for 18 h. After, the presum- able zygotes (PZ) were *in vitro* cultured (IVC) for 8 days for blas- tocyst rates assessment. Experiment 2 (green): evaluation of FUCA effect for short-time during a pre-fertilization period. The matured COCs were submitted to pre-fertilization treatment for 30 min or 2 h with 0 (control group) or FUCA (0.125 U/mL), resulting in four groups (control-30 min, control-2 h, FUCA-30 min, and FUCA-2 h). After treatment, COCs were submitted to *zona pellucida* (ZP) digestion time evaluation or COCs followed with IVF (18 h) and IVC for 8 days for blastocyst rate assessment. Experiment 3 (pink): evaluation of FUCA effect in different concentrations during pre-fertilization. The matured COCs were submitted to pre-fertilization treatment for 1 h with 0 (control group) and two concentrations of FUCA (0.0625 and 0.125 U/mL). After treatment, COCs were submitted to ZP digestion time evaluation, COCs followed with IVF (18 h) for the evaluation of monospermic fertilization, or COCs followed with IVF (18 h) and IVC for 8 days for blastocyst rate assessment and embryo quality evaluation by mRNA abundance analysis. Figure created with BioRender (BioRender.com)

sperm were washed three times and evaluated for motility and concentration. The sperm were diluted to a final concentration of 1×10^6 sperm/mL with IVF media. After pre-fertilization treatments, COCs were transferred to a 90 µL IVF drop covered by silicone oil, 6µL of stained sperm solution (1×10^6 sperm/mL) was added in each 90 µL drop IVF, and gametes were incubated for 18 h (highly humid atmosphere, 5.5% CO_2, 38.5°C).

After IVF, the PZs were submitted to cumulus cell removal and fixed in 4% paraformaldehyde for 20 min at room temperature. Next, the PZ were washed, stained with Hoechst (for 30 min at room temperature, 1% w/v

Hoechst 33,342; Sigma-Aldrich in PBS), and mounted on glass slides, according to Ferraz et al. (Ferraz et al. 2017). The oocytes were evaluated by Laser Scanning confocal fluorescence microscopy by an SPE-II TCS system (Leica Microsystems GmbH, Wetzlar, Germany) that is connected to a semi-automatic inverted DMI4000 microscope (Leica) with a magnification lens objective of 20 × and 40 × NA 1.25. For Hoescht 33,342 the parameters were settled as 350/461 nm (Excitation/Emission) and for Mitotracker Green as 490/516 nm (Excitation/Emission). Images of each PZ were obtained in z-stack (1 μm/stack) of the entire PZ and exported by using LAS X software. Images were analyzed using ImageJ 1.53t software (U. S. National Institutes of Health, Bethesda, MD, USA).

Penetration was classified by the number of pronuclei and sperm midpieces within the ooplasm. Monospermic penetration was determined as two pronuclei and one sperm midpiece. Polyspermic penetration was characterized when two or more sperm midpieces were identified in the ooplasm and more than two pronuclei in an oocyte. Andunfertilized oocyte was represented by the presence of one pronucleus without the presence of a sperm midpiece.

Gene expression in bovine blastocyst

For the evaluation of gene expression, five pools of three expanded blastocysts were analyzed per treatment. The total RNA of each sample was extracted using PicoPure® RNA Isolation Kit (ThermoFisher Scientific) according to the manufacturer's instructions with the addition of the DNAse treatment step. The total RNA concentrations (Nanodrop, ND-2000®) and the integrity of the RNA (Agilent 2100 Bioanalyzer) were analyzed, only samples with RIN (RNA Integrity Number) above seven (scale from 0 to 10) were used. The reverse transcription was done using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Quantification of mRNA was performed by RT-qPCR using the Power Sybr® Green Master Mix detection system (ThermoFisher Scientific), in a final volume reaction of 20μL (1μL of cDNA, 10μL Power Sybr Green Master Mix, 1.2μL of each primer at 300 mM and 6.6μL of water). The cycling conditions were 95°C for 10 min for initial denaturation, followed by 40 cycles of 95°C for 10 s and 1 min at 60°C for priming and amplicon extension.

To choose the most stable reference gene for detailed analyses of blastocyst, peptidylprolyl isomerase A (*PPIA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Ribosomal Protein L30 (*RPL30*), and Actin Beta (*ACTB*) amplification profiles were compared using the geNorm applet Microsoft Excel. The most stable reference gene was *RPL30*. The ΔΔCt method with efficiency correction was used to calculate relative expression values genes/*RPL30*) for each target gene, using one control sample as a calibrator.

The relative abundance of genes linked to embryonic competence described in the literature was evaluated: plur ipotency (Oct amer-binding transcr iption factor 4—*OCT4*), differentiation and implantation (Placenta Associated 8—*PLAC8*—and Caudal Type Homeobox 2— *CDX2*), embryonic development (Superoxide dismutase 2—*SOD2* —and Vascular endothelial growth factor A—*VEGF*), and apoptosis regulator (BCL2 Associated X—*BAX*) (Heid et al. 1994; Paula-Lopes et al. 1998; Luo et al. 2002; Rizos et al. 2003; Clempson et al. 2011; Rickelt et al. 2011; Hirayama et al. 2012; Machado et al. 2012; Kim et al. 2012; Sakurai et al. 2012; Ozawa et al. 2012; Cebrian-Serrano et al. 2013). All the primer sequences, primer concentration per reaction, and annealing primer temperature are presented in Table 1.

Table 1 RT-qPCR primer sequences of the genes

Gene	Forward sequence (5' - 3')	Reverse sequence (5' - 3')	Concentration (mM)	Temperature (°C)
PPIA	5'GCCATGGAGCGCTTTGG	CCACAGTCAGCAATGGTGATCT	300	60
GAPDH	GGCGTGAACCACGAGAAGTATAA	CCCTCCACGATGCCAAAG	200	60
RPL30	TGGTGTCCATCACTACAGTGGCAA	ACCAGTCTGTTCTGGCATGCTTCT	300	60
ATCB	GCGTGGCTACAGCTTCACC	TTGATGTCACGGACGATTTC	300	60
OCT4	GTTTTGAGGCTTTGCAGCTC	TCTCCAGGTTGCCTCTCACT	300	60
PLAC8	GACTGGCAGACTGGCATCTT	CTCATGGCGACACTTGATCC	300	60
CDX2	TGGAGCTGGAGAAGGAGTTTCACT	TCCTTCGCTCTGCGGTTCTGAAAT	300	56
SOD2	GATGCCTTTCTAGTCCTATTC	TCAGTTCACCTGCTACATT	300	60
VEGF	CCCAGATGAGATTGAGTTCATTTT	ACCGCCTCGGCTTGTCAC	300	60
BAX	TGTTTTCTGACGGCAACTTCA	CGAAGGAAGTCCAATGTCCAG	300	60

Statistical analysis

First, data was tested for normality distribution and homogeneity of variances with transformation to logarithm, if necessary, to perform parametric analysis. The blastocyst rates were transformed into arcsine. In experiment 2, the effect of FUCA treatment on the ZP digestion time and embryo production rate was tested by Student t-test. In experiment 3, the effect of FUCA in the ZP digestion time, penetration rate, embryo production rate, and mRNA abundance were tested by ANOVA, followed by comparison by Tukey test, when applied. Data are presented as mean ± standard error mean (SEM). Differences were considered significant when $P < 0.05$. All analyses were performed using the JMP statistical program, version 7.0 (SAS).

Results

Experiment 1: The effect of FUCA during the entire IVF process

In the first experiment, FUCA (0.125 U/mL) was added to the *in vitro* fertilization (IVF) media, and COCs and spermatozoa were incubated with it during the entire IVF process. After 18 h post-fertilization (hpf), the presumptive zygotes (PZ) from the control group (no FUCA added to IVF) presented the expected morphology (compact cumulus cells around the PZ (Fig. 2A/2a), while the PZ from the treated group (FUCA) presented a pattern of disperse cumulus cells, as represented in Fig. 2B/2b. The PZ of both groups followed the *in vitro* culture (IVC) for blastocyst development evaluation. As a result, after 192 hpf, an expected blastocyst yield was recorded in the control group (38% blastocyst/COC, Fig. 2C), while no blastocyst formation in the FUCA group (Fig. 2D). Statistics analysis was not performed (only two replicates were performed), however, due to the clear lethal toxicity, we decided to stop experiment 1 and apply another strategy to evaluate the effect of FUCA. For that, we decided to reduce the incubation time of FUCA, as presented in experiment 2.

Experiment 2: The effect of FUCA during a pre-fertilization period

Due to the possible embryotoxic effect of long-period FUCA treatment (experiment 1), a shorter incubation period was included between the *in vitro* maturation and *in vitro* fertilization, named as the pre-fertilization step. For that, matured COCs were incubated with FUCA (0.125 U/mL) for either 30 min or 2 h before following the fertilization process. After, the ZP hardening was estimated by the duration of the pronase digestion assay. A longer time was necessary to digest the ZP of treated oocytes (30 min, 0.125 U/mL FUCA) when compared to the control group (30 min incubation in control media), suggesting that FUCA might contribute to the ZP hardening ($P < 0.0001$, Fig. 3A and Supplementary Table 1). No difference in the ZP hardening was observed in COCs treated for 2 h ($P > 0.05$, Fig. 3A, Supplementary Table 1). When evaluating the embryo production, no difference was observed neither in the cleavage (evaluated at 96 hpf, 30 min: $P = 0.21$, 2 h: $P = 0.31$, Fig. 3B, Supplementary Table 1) nor in the blastocyst rates (evaluated at 192 hpf, 30 min: $P = 0.34$, 2 h: $P = 0.1$, Fig. 3C, Supplementary Table 1).

Experiment 3: The effect of FUCA in different concentrations during pre-fertilization

Since the pre-fertilization treatment (30 min incubation) presented a positive ZP hardening effect (experiment 2), we hypothesized that decreasing the FUCA concentration and increasing the incubation time could improve embryo development. Therefore, in experiment 3, matured COCs were incubated with 0 (control group) and two concentrations of FUCA: 0.0625 U/mL of FUCA and 0.125 U/mL of FUCA for one hour during the pre-fertilization step. As a result, indeed the time for pronase ZP digestion was longer in the 0.0625 U/mL group compared to the 0 (control) and 0.125 U/mL FUCA ($P = 0.0007$, Fig. 4A and Supplementary Table 2). Interestingly, when performing the incubation for 1 h, no difference was observed when comparing the groups 0 (control) and 0.125 U/mL FUCA ($P > 0.05$, Fig. 4A and Supplementary Table 2), contrasting the result observed in experiment 2 when incubations were performed for 30 min (Fig. 3A), suggesting that both variables (concentration and incubation time) are relevant for the FUCA effect. Next, the relation between the ZP hardening and oocyte fertilization was evaluated by the sperm penetration assay. There was no difference in the monospermic, polyspermic, and non-fertilized oocyte rates when comparing the groups ($P > 0.05$; Fig. 4B, C and Supplementary Table 2). However, a tendency ($P = 0.067$) of higher monospermic penetration was observed when comparing the control and 0.0625 U/mL FUCA groups (Fig. 4B, C), suggesting that the ZP hardening could increase the monospermic fertilization. This tendency, however, did not impact positively the blastocyst yield after *in vitro* embryo production ($P > 0.05$, Fig. 3D and Supplementary Table 2), nor improve the embryo quality evaluated by the relative mRNA abundance of genes known as embryo quality markers (*VEGF, SOD2, OCT4, PLAC8, CDX2,* and *BAX*, Fig. 4E, $P > 0.05$).

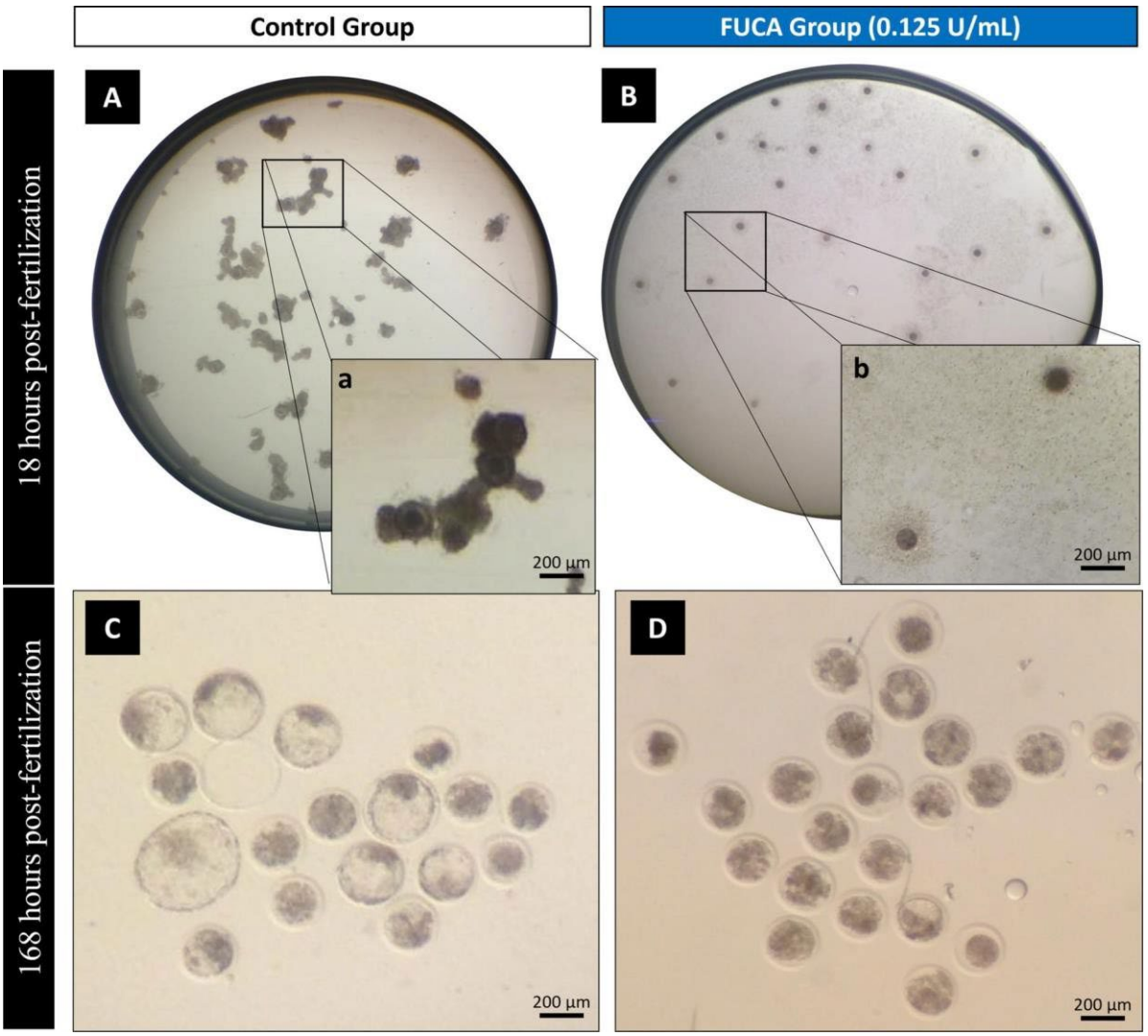


Fig. 2 Experiment 1: COCs and sperm incubation with FUCA (0.125 U/mL) for 18 h during *in vitro* fertilization (n = 2 replicates, 40 COCs/group/replicate). Top panel: presumptive zygotes at 18 h post- fertilization (hpf) at (**A**) control group (no FUCA) and (**B**) FUCA group (0.125 U/mL), the inlets represent a magnification of the structures of (**a**) control and (**b**) FUCA groups. Bottom panel: blastocyst rate at 192 hpf at (**C**) control group (38% blastocyst/COC) and (**D**) FUCA group (no blastocyst developed). COCs: cumulus oocyte complexes, FUCA: α-L-Fucosidase

Discussion

In the present study, an experimental design composed of three experiments was arranged to evaluate the effect of α-L-fucosidase in the bovine *in vitro* fertilization process. The effect of FUCA was tested during different incubation times and at different concentrations to understand its effect on the female gamete's final maturation and during the *in vitro* fertilization. Our main finding was that FUCA works in the pre-fertilization ZP hardening, which effect is dependent on the incubation time and the protein concentration. Moreover, this ZP hardening tended to increase monospermic fertilization but did not improve the yield and quality of *in vitro* embryo production.

The pre-fertilization ZP hardening has been proposed by many studies to be one of the mechanisms related to the polyspermic fertilization control in bovine (Mondéjar et al. 2013; Coy et al. 2008; Coy and Avilés 2010), porcine (Mondéjar et al. 2013; Coy et al. 2008; Coy and Avilés 2010), ovine (Davachi et al. 2016), and murine (Körschgen et al. 2017). Besides the pre-fertilization ZP hardening, polyspermic fertilization is also controlled by the maternal reproductive tract, which regulates the amount of sperm that arrives at the fertilization site, and the oocyte's cortical granules, which increases ZP resistance by the proteolytic digestion that occurs shortly after fertilization (Coy et al. 2008; Coy and Avilés 2010). Among these three mechanisms related to polyspermic fertilization, the pre-fertilization ZP hardening

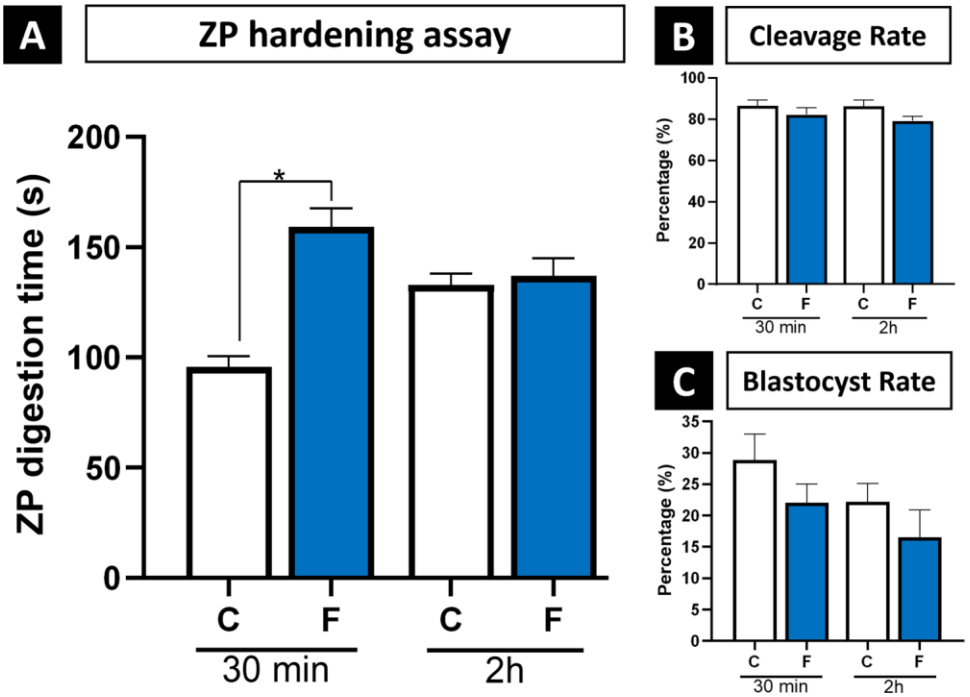


Fig. 3 Experiment 2: FUCA treatment (0.125 U/mL) for a shorter period (30 min or 2 h). **A** Zona pellucida (ZP) hardening assay: matured COCs treated with 0 (control group—C) or 0.125 U/mL of FUCA (F) were denuded and submitted to pronase digestion assay, the time for total ZP digestion of each oocyte was recorded and data were compared by Student's t-test (data showed as mean ± standard error deviation, in seconds, n = 4 replicates, 10 COCs/group/replicate, 160 COCs in total). After FUCA treatment the COCs were washed, fertilized, and followed to IVC, and the **B** cleavage and **C** blastocyst rates were compared between groups (Student's t-test, and data showed as mean ± standard error deviation of %, n = 6 replicates, 10 COCs/group/replicate, 240 COCs in total). * indicates statistical significance ($P < 0.05$). C: control group, F: FUCA group, min: minutes, h: hour

holds more attention due to the possibility of applying this approach for the success of *in vitro* fertilization conditions. Therefore, the identification of the molecules that participate in the ZP modification is very relevant.

Brown and Jones (1987) stated that oviductal glycoproteins induced by the estrus interact with ZP in swine oocytes. A few years later, it was described that an oviductal glycoprotein was able to alter the biochemical properties of the ZP in hamster oocytes (Oikawa et al. 1988). Later studies demonstrated that some glycoproteins, such as oviduct-specific glycoprotein (OVGP1) and heparin-like glycosaminoglycans (GAGs), present in the bovine OF, also bind to ZP and increase its resistance to enzymatic digestion and sperm penetration, which reduces the polyspermic incidence and improves the IVF efficiency (Buhi 2002; Coy et al. 2008; Coy and Avilés 2010; Algarra et al. 2016; González-Brusi et al. 2020). In recent studies, the metalloproteinase ovastacin was elected to cause a pre-fertilization ZP hardening in mice (Körschgen et al. 2017). Moreover, when oocytes were incubated with OF (Mondéjar et al. 2013; Bragança et al. 2021) or co-cultured with oviductal epithelial cells (Davachi et al. 2016; Ferraz et al. 2017) an increase in the monospermic fertilization was also observed. This component(s) might even be located in extracellular vesicles (EVs), which are small extracellular compartments responsible for carrying proteins, bioactive lipids, RNAs, and DNAs contents that cover intercellular communication events (Zaborowski et al. 2015), as demonstrated by Alcântara-Neto et al. (2020), which observed decrease of polyspermy incidence in pigs when EVs from OF were added in the IVF medium.

Still, there is no consensus on the identification of the molecule related to the pre-fertilization ZP hardening. Which could be species-specific and might also be controlled by multiple factors, instead of a single-molecule based. Yet, the most expected factor to be related to this role would be a component of the oviductal fluid highly abundant in the ampulla (the fertilization site) during the peri-ovulatory period. Corroborating with this hypothesis, a study of our research group identified higher *FUCA1* transcript levels in the ipsilateral ampulla compared to the contralateral one day after ovulation (the location and period when the fertilization occurs), suggesting that *FUCA1* might be important for the fertilization process (Fontes et al. 2018). Moreover, another study also demonstrated the presence and activity of FUCA in the bovine oviductal fluid (Carrasco et al. 2008). Among all the glycosidases identified in the bovine OF, the α-L-fucosidase was the only glycosidase whose total activity increased significantly after ovulation (Carrasco et al. 2008).

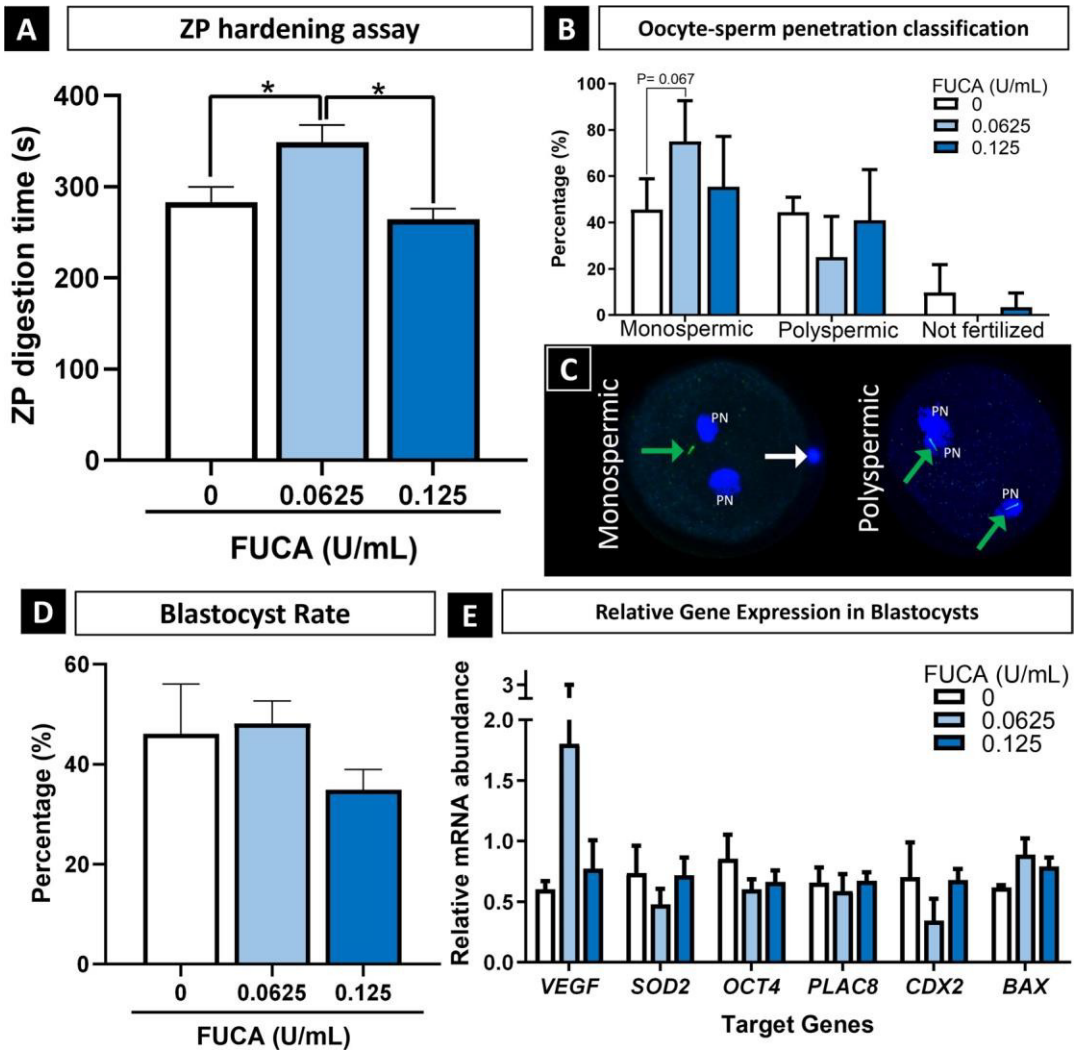


Fig. 4 Experiment 3: pre-fertilization treatment with FUCA for 1 h with different FUCA concentrations (0, 0.0625, and 0.125 U/mL). **A** Zona pellucida (ZP) hardening assay: matured COCs treated with FUCA (0. 0.0625, or 0.125 U/mL FUCA) were denuded and sub- mitted to pronase treatment, the time for total ZP digestion of each oocyte was recorded (n = 3 replicates, 10 COCs/group/replicate, 90 COCs in total). **B** Sperm penetration assay: after pre-fertilization treatment with FUCA, the COCs were submitted to IVF with sperm pre-stained with Mitrotacker® Green, 18 h after IVF, the presump- tive zygotes were denuded and stained with Hoechst to DNA staining. All presumptive zygotes were classified as monospermic (one sperm tail – green – and two pronuclear material – PN, blue), polyspermic (two or more sperm penetration), or non-fertilized (when only one or no PN was identified) (n = 3 replicates, 10 COCs/group/replicate, 90 COCs in total). **C** Representative picture of a monospermic and polyspermic penetration, PN: pronucleus, green arrow: sperm tail, white arrow: polar corpuscle. **D** Blastocyst rate at 192 h post-fertilization (n = 3 replicates, 30 COCs/group/replicate, 270 COCs in total). **E** Relative mRNA abundance of the genes (*VEGF, SOD2, OCT4, PLAC8, CDX2*, and *BAX*) analyzed by RT-qPCR in the blastocyst's samples (n = 5 samples/group, each sample is a pool of 3 blastocysts). All the analyses were analyzed by ANOVA followed by Tukey's test and data are presented as mean ± standard error deviation. * indicates statistical significance ($P < 0.05$)

Therefore, at first, we tested the effect of FUCA during the entire IVF process, which showed a clear embryotoxic effect by completely preventing embryo development. For this experiment, the FUCA concentration was similar to that reported in bovine OF (Carrasco et al. 2008). One possible reason for the toxic effect might be related to the static system used in our study, which might have overexposed the oocytes to the enzymatic FUCA activity. The impairment effect of high exposure levels when culturing in a static system has been reported before for amino acid concentrations in IVC media for bovine embryo production, in which the same components turned advantageous when they were 1:2 diluted (Santos et al. 2021). Therefore, our next approach was to reduce the incubation time to decrease the over-exposure FUCA effect. Indeed, after incubating oocytes with FUCA for no longer than 2 h, we observed an expected *in vitro* embryo development rate (experiment 2). However, the incubation with FUCA for 30 min increased

the pre-fertilization ZP hardening, while incubation for 2 h had no impact on this feature. Moreover, when submitting these oocytes to embryo production, FUCA treatment had no effect on the cleavage and blastocyst rates, suggesting the pre-fertilization ZP hardening does not play a role in improving embryo development. Regardless of the ZP hardening, the presence of the FUCA effect for 30 min of incubation, but an absence of the FUCA effect for the two hours of incubation might be related to the reversibility of the ZP hardening. One theory that sustains the reversibility of ZP hardening is due to the specificity of enzymes that modulate different glycoproteins present in the ZP composition (Mondéjar et al. 2013). Therefore, proteolytic enzymes can present a cyclic action, building and undoing the chemical modifications that result in the ZP hardening (Kolbe and Holtz 2005; Mondéjar et al. 2013). Corroborating with the dynamism of the OF composition, a study of our group demonstrated that the *FUCA1* and *FUCA2* transcript levels might be estradiol-dependent. When analyzing the ampulla segments of cows submitted to ovarian superstimulation protocol, higher *FUCA1* and *FUCA2* levels were detected when compared to synchronized cows (single ovulation), which was also correlated to the higher estradiol level in the oviductal segments, demonstrating that the FUCA presence is dynamic and modulated by the hormonal milieu (Fontes et al. 2019). To confirm the hypothesis of reversibility of ZP hardening, Coy et al. (2008) demonstrated that bovine and porcine oocytes incubated for 30 min with oviductal fluid presented high ZP digestion time. However, the ZP digestion time sharply decreased 15 min after the oocytes were removed from the oviductal fluid and transferred to an IVF media, and the ZP digestion time continued decreasing steadily when longer incubation times on IVF media were tested (Coy et al. 2008). Another hypothesis for the absence of effect for the two hours of incubation observed in our study is a possible suitable toxic effect of 2 h of incubation, which was not enough to completely block embryo development (as in experiment 1) but resulted in no FUCA effect in the pre-fertilization ZP hardening. Still, the hypothesis that the ZP resistance to proteinases is reversible is the most likely, since the ZP is required to lysis for the hatching process of the blastocyst during its development in the uterus. Last, a third strategy to evaluate the effect of FUCA in the bovine *in vitro* embryo production was to test different concentrations of the protein. For this third experiment, the incubation time was settled to one hour, based on other studies that also evaluated 1-h pre-fertilization incubation time (Romero-Aguirregomezcorta et al. 2015; Phopin et al. 2012). In our study, we demonstrated that slightly increasing the incubation time (from 30 min to 1 h) and decreasing the concentration (from 0.125 U/mL to 0.0625 U/mL) improved the pre-fertilization ZP hardening time when compared to the control group and tended to present a greater monospermic fertilization rate (75% in FUCA 0.0625 U/mL *versus* 45.7% in Control). Yet, the blastocyst rate was not different between treatments (experiment 3). Even though the blastocyst yield was not improved by the treatments, we hypothesized that the embryo quality could have been better when compared to the control. For that, we submitted the embryos for gene abundance evaluation of transcripts known as embryo quality markers. Nonetheless, the FUCA treatments had no effect on blastocyst production quality, including the marker for pluripotency (OCT4), differentiation and implantation (PLAC8, CDX2), embryonic development (SOD2, VEGF) and the expression of BAX (Heid et al. 1994; Paula-Lopes et al. 1998; Luo et al. 2002; Rizos et al. 2003; Clempson et al. 2011; Rickelt et al. 2011; Hirayama et al. 2012; Machado et al. 2012; Kim et al. 2012; Sakurai et al. 2012; Ozawa et al. 2012; Cebrian-Serrano et al. 2013). Blocking the polyspermy is necessary for fertilization and embryo survival, as it prevents the penetration of two or more sperm into an oocyte, which would result in abnormal embryonic development, leading to death or physiological conditions of early spontaneous abortion (Ducibella 1996; Coy and Avilés 2010). Taking it all together, in our study we observed that FUCA might have the function of controlling pre-fertilization ZP hardening in bovine species, however, it did not translate into a significant positive effect on polyspermy control and embryo development. Our study corroborates with other studies that evaluated the FUCA effect on oocyte penetration in porcine (Romero-Aguirregomezcorta et al. 2015) and mice (Phopin et al. 2012). Even though the study on porcine did not evaluate embryo production (Romero-Aguirregomezcorta et al. 2015), our results showed the same pattern observed in mice, in which the embryo production presented no improvement by the FUCA effect (Phopin et al. 2012). Still, we also have to highlight some limitations of our study. First, the limited number of COCs analyzed in here could influence the generalization of the results, as a larger sample size could offer a more comprehensive and robust view of the effects of FUCA on the pre-fertilization ZP hardening and embryonic development. Furthermore, we only performed one analytical test to verify the ZP hardening (pronase digestion time assay), while other tests could provide more detailed insights into the biochemical and structural changes induced in the ZP. Last, it is important to be aware that the oviductal fluid has a complex composition of factors, that might be relevant to performing all biological modulation in the gametes. In this study, we apply the strategy of evaluating one single protein, isolating the possible synergism and interrelations of the multiple factors from the oviductal fluid.

In conclusion, FUCA can induce oocyte pre-fertilization ZP hardening and might improve monospermic fertilization performance for the bovine *in vitro* embryo production system. From a future perspective, with the current information

on the FUCA effect and being aware that the OF composition is complex and dynamic, we might have to associate more OF factors in different moments of IVEP to be able to improve the embryo produced *in vitro*.

Supplementary Information The online version contains supplemen- tary material available at https://doi.org/10.1007/s11259-023-10291-y.

Acknowledgements This study was supported by the São Paulo Research Foundation (FAPESP – grant numbers: # 2019/12223-0, # 2016/25685-4, and #2018/06674-7). It was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior e Brasil (CAPES) e Finance Code 001.

Author contributions PKF and ACSC designed the research. TSBM, TRCS, FFF, PHS, EMR, SGN, and PKF collected the samples and per- formed the investigation and formal analysis. TSBM, TRCS, and PKF wrote the original draft. ACSC acquired and administrated the funding. ACSC and PKF supervised. PKF manuscript review and edition. All authors reviewed, read, and approved the manuscript.

Data Availability The datasets generated during and/or analyzed dur- ing the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Conflicts of Interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References

Abascal I, Skalaban SR, Grimm KM, Avile M, Angel J, Martianez-Menarguez JA, Castells MT, Ballesta J, Alhadeff JA (1998) Alteration of the isoform composition of plasma-membrane-associated rat sperm α-L-fucosidase during late epididymal maturation : comparative characterization of the acidic and neutral isoforms. Biochem J. https://doi.org/10.1042/bj3330201

Alcântara-Neto AS, Fernandez-Rufete M, Corbin E, Tsikis G, Uzbekov R, Garanina AS, Coy P, Almiñana C, Mermillod P (2020) Oviduct fluid extracellular vesicles regulate polyspermy during porcine in vitro fertilisation. Reprod Fertil Dev. https://doi.org/10.1071/RD19058

Algarra B, Han L, Soriano-Úbeda C, Avilés M, Coy P, Jovine L, Jiménez-Movilla M (2016) The C-terminal region of OVGP1 remodels the zona pellucida and modifies fertility parameters. Sci Rep. https://doi.org/10.1038/SREP32556

Algarra B, Maillo V, Avilés M, Gutiérrez-Adán A, Rizos D, Jiménez-Movilla M (2018) Effects of recombinant OVGP1 protein on in vitro bovine embryo development. J Reprod Dev. https://doi.org/10.1262/JRD.2018-058

Alhadeff JA, Khunsook S, Choowongkomon K, Baney T, Heredia V, Tweedie A, Bean B (1999) Characterization of human semen α-L-fucosidases. Mol Hum Reprod. https://doi.org/10.1093/molehr/5.9.809

Avile M, Abascal I, Martínez-Menárguez A, Castells T, Skalaban SR, Ballesta J, Alhadeff JA (1996) Immunocytochemical localization and biochemical characterization of a novel plasma membrane-associated, neutral pH optimum α-L-fucosidase from rat testis and epididymal spermatozoa. Biochem J. https://doi.org/10.1042/bj3180821

Bijttebier J, Van Soom A, Meyer E, Mateusen B, Maes D (2008) Preovulatory follicular fluid during in vitro maturation decreases polyspermic fertilization of cumulus-intact porcine oocytes. In vitro maturation of porcine oocytes. Theriogenology. https://doi.org/10.1016/j.theriogenology.2008.04.046

Bragança GM, Alcântara-Neto AS, Batista RITP, Brandão FZ, Freitas VJF, Mermillod P, Souza-Fabjan JMG (2021) Oviduct fluid during IVF moderately modulates polyspermy in in vitro-produced goat embryos during the non-breeding season. Theriogenology. https://doi.org/10.1016/j.theriogenology.2021.03.022

Brown CR, Jones R (1987) Binding of zona pellucida proteins to a boar sperm polypeptide of M r 53000 and identification of zona moieties involved. Development. https://doi.org/10.1242/dev.99.3.333

Buhi WC (2002) Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. Reproduction. https://doi.org/10.1530/REP.0.1230355

Carrasco LC, Coy P, Avilés M, Gadea J, Romar R (2008) Glycosidase determination in bovine oviducal fluid at the follicular and luteal phases of the oestrous cycle. Reprod Fertil Dev. https://doi.org/10.1071/RD08113

Cebrian-Serrano A, Salvador I, García-Roselló E, Pericuesta E, Pérez-Cerezales S, Gutierrez-Adán A, Coy P, Silvestre MA (2013) Effect of the bovine oviductal fluid on in vitro fertilization, development and gene expression of in vitro-produced bovine blastocysts. Reprod Domest Anim. https://doi.org/10.1111/J.1439-0531.2012.02157.X

Clempson AM, Pollott GE, Brickell JS, Bourne NE, Munce N, Wathes DC (2011) Evidence that leptin genotype is associated with fertility, growth, and milk production in Holstein cows. J Dairy Sci. https://doi.org/10.3168/JDS.2010-3626

Cordova A, Perreau C, Schmaltz-Panneau B, Locatelli Y, Ponsart C, Mermillod P (2013) Use of an in vitro model in bovine to evidence a functional and molecular dialogue between preimplantation embryo and oviduct epithelial cells. Gynecol Obstet Fertil. https://doi.org/10.1016/J.GYOBFE.2013.07.015

Coy P, Avilés M (2010) What controls polyspermy in mammals, the oviduct or the oocyte? Biol Rev. https://doi.org/10.1111/j.1469-185X.2009.00117.x

Coy P, Romar R (2002) In vitro production of pig embryos: a point of view. Reprod Fertil Dev. https://doi.org/10.1071/RD01102

Coy P, Cánovas S, Mondéjar I, Saavedra MD, Romar R, Grullón L, Matás C, Avilés M (2008) Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. Proc Natl Acad Sci U S A. https://doi.org/10.1073/PNAS.0804422105

Davachi ND, Zare Shahneh A, Kohram H, Zhandi M, Shamsi H, Hajiyavand AM, Saadat M (2016) Differential influence of ampullary and isthmic derived epithelial cells on zona pellucida hardening and in vitro fertilization in ovine. Reprod Biol. https://doi.org/10.1016/J.REPBIO.2015.11.002

Ducibella T (1996) The cortical reaction and development of activation competence in mammalian oocytes. Hum Reprod Update. https://doi.org/10.1093/HUMUPD/2.1.29

Ferraz MAMM, Henning HHW, Costa PF, Malda J, Melchels FP, Wubbolts R, Stout TAE, Vos PLAM, Gadella BM (2017) Improved bovine embryo production in an oviduct-on-a-chip system: prevention of poly-spermic fertilization and parthenogenic activation. Lab Chip. https://doi.org/10.1039/C6LC01566B

Fontes PK, Ereno RL, Peixoto AR, Carvalho RF, Scarano WR, Trinca LA, Barros CM, Castilho ACS (2018) Can the antral follicular count modulate the gene expression of bovine oviducts in Aberdeen angus and nelore heifers? PLoS ONE. https://doi.org/10.1371/journal.pone.0202017

Fontes PK, Razza EM, Pupulim AGR, Barros CM, de Souza Castilho AC (2019) Equine chorionic gonadotropin increases estradiol levels in the bovine oviduct and drives the transcription of genes related to fertilization in superstimulated cows. Mol Reprod Dev. https://doi.org/10.1002/mrd.23243

González-Brusi L, Algarra B, Moros-Nicolás C, Izquierdo-Rico MJ, Avilés M, Jiménez-Movilla M (2020) A Comparative View on the Oviductal Environment during the Periconception Period. Biomolecules. https://doi.org/10.3390/BIOM10121690

Hancock LW, Raab LS, Aronson NN (1993) Synthesis and Processing of Rat Sperm-Associated α-L-Fucosidase. Biol Reprod. https://doi.org/10.1095/BIOLREPROD48.6.1228

Heid HW, Schmidt A, Zimbelmann R, Schäfer S, Winter-Simanowski S, Stumpp S, Keith M, Figge U, Schnölzer M, Franke WW (1994) Cell type-specific desmosomal plaque proteins of the plakoglobin family: plakophilin 1 (band 6 protein). Differentiation. https://doi.org/10.1046/J.1432-0436.1995.5820113.X

Hirayama H, Ushizawa K, Takahashi T, Sawai K, Moriyasu S, Kageyama S, Miura R, Matsui M, Fukuda S, Naito A, Fujii T, Minamihashi A (2012) Differences in apoptotic status in the bovine placentome between spontaneous and induced parturition. J Reprod Dev. https://doi.org/10.1262/JRD.2012-043

Jauhiainen A, Vanha-Perttula T (1986) alpha-L-Fucosidase in the reproductive organs and seminal plasma of the bull. Biochim Biophys Acta. https://doi.org/10.1016/0304-4165(86)90123-6

Khunsook S, Alhadeff JA, Bean BS (2002) Purification and characterization of human seminal plasma alpha-L-fucosidase. Mol Hum Reprod. https://doi.org/10.1093/MOLEHR/8.3.221

Kim EY, Park MJ, Park HY, Noh EJ, Noh EH, Park KS, Lee JB, Jeong CJ, Riu KZ, Park SP (2012) Improved Cloning Efficiency and Developmental Potential in Bovine Somatic Cell Nuclear Transfer with the Oosight Imaging System. Cell Reprogram. https://doi.org/10.1089/CELL.2011.0103

Kolbe T, Holtz W (2005) Differences in proteinase digestibility of the zona pellucida of in vivo and in vitro derived porcine oocytes and embryos. Theriogenology. https://doi.org/10.1016/J.THERIOGENOLOGY.2004.07.023

Körschgen H, Kuske M, Karmilin K, Yiallouros I, Balbach M, Floehr J, Wachten D, Jahnen-Dechent W, Stöcker W (2017) Intracellular activation of ovastacin mediates pre-fertilization hardening of the zona pellucida. Mol Hum Reprod. https://doi.org/10.1093/MOLEHR/GAX040

Lonergan P, Fair T, Forde N, Rizos D (2016) Embryo development in dairy cattle. Theriogenology. https://doi.org/10.1016/J.THERIOGENOLOGY.2016.04.040

Luo H, Kimura K, Aoki M, Hirako M (2002) Vascular Endothelial Growth Factor (VEGF) Promotes the Early Development of Bovine Embryo in the Presence of Cumulus Cells. J Vet Med Sci. https://doi.org/10.1292/JVMS.64.967

Machado GM, Caixeta ES, Lucci CM, Rumpf R, Franco MM, Dode MAN (2012) Post-hatching development of bovine embryos in vitro: the effects of tunnel preparation and gender. Zygote. https://doi.org/10.1017/S0967199411000086

Mondéjar I, Martínez-Martínez I, Avilés M, Coy P (2013) Identification of potential oviductal factors responsible for zona pellucida hardening and monospermy during fertilization in mammals. Biol Reprod. https://doi.org/10.1095/BIOLREPROD.113.111385

Oikawa T, Sendai Y, Kurata S-I, Yanagimachi R (1988) A glycoprotein of oviductal origin alters biochemical properties of the zona pellucida of hamster egg. Gamete Res. https://doi.org/10.1002/MRD.1120190202

Ozawa M, Sakatani M, Hankowski KE, Terada N, Dobbs KB, Hansen PJ (2012) Importance of culture conditions during the morula-to-blastocyst period on capacity of inner cell-mass cells of bovine blastocysts for establishment of self-renewing pluripotent cells. Theriogenology. https://doi.org/10.1016/J.THERIOGENOLOGY.2012.05.020

Papi M, Sylla L, Parasassi T, Brunelli R, Monaci M, Maulucci G, Missori M, Arcovito G, Ursini F, De Spirito M (2009) Evidence of elastic to plastic transition in the zona pellucida of oocytes using atomic force spectroscopy. Appl Phys Lett. https://doi.org/10.1063/1.3107265

Paula-Lopes FF, De Moraes AAS, Edwards JL, Justice JE, Hansen PJ (1998) Regulation of preimplantation development of bovine embryos by interleukin-1beta. Biol Reprod. https://doi.org/10.1095/BIOLREPROD59.6.1406

Phopin K, Nimlamool W, Bartlett MJ, Bean BS (2012) Distribution, crypticity, stability, and localization of α-L-fucosidase of mouse cauda epididymal sperm. Mol Reprod Dev. https://doi.org/10.1002/MRD.22016

Rickelt S, Moll I, Franke WW (2011) Intercellular adhering junctions with an asymmetric molecular composition: desmosomes connecting Merkel cells and keratinocytes. Cell Tissue Res. https://doi.org/10.1007/S00441-011-1260-0

Rizos D, Gutiérrez-Adán A, Pérez-Garnelo S, De la Fuente J, Boland MP, Lonergan P (2003) Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. Biol Reprod. https://doi.org/10.1095/BIOLREPROD.102.007799

Romar R, Cánovas S, Matás C, Gadea J, Coy P (2019) Pig in vitro fertilization: Where are we and where do we go? Theriogenology. https://doi.org/10.1016/J.THERIOGENOLOGY.2019.05.045

Romero-Aguirregomezcorta J, Matás C, Coy P (2015) α-L-fucosidase enhances capacitation-associated events in porcine spermatozoa. Vet J. https://doi.org/10.1016/J.TVJL.2014.11.006

Sakurai T, Bai H, Bai R, Arai M, Iwazawa M, Zhang J, Konno T, Godkin JD, Okuda K, Imakawa K (2012) Coculture system that mimics in vivo attachment processes in bovine trophoblast cells. Biol Reprod. https://doi.org/10.1095/BIOLREPROD.112.100180/2513806

Santos ÉC, Fonseca Junior AM, Lima CB, Ispada J, Silva JVA, Milazzotto MP (2021) Less is more: Reduced nutrient concentration during in vitro culture improves embryo production rates and morphophysiology of bovine embryos. Theriogenology. https://doi.org/10.1016/J.THERIOGENOLOGY.2021.07.010

Song XX, Yang XZ, Iga K, Niwa K (1999) Binding of Lectins to the Zona Pellucida of In Vitro Matured Pig Oocytes and Sperm-Oocyte Interaction In Vitro. J Mamm Ova Res. https://doi.org/10.1274/JMOR.16.23

Srivastava PN, Farooqui AA, Gould KG (1981) Studies on hydrolytic enzymes of chimpanzee semen. Biol Reprod. https://doi.org/10.1095/BIOLREPROD25.2.363

Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Gonçalves PB, Wolf E (2001) Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. Biol Reprod. https://doi.org/10.1095/BIOLREPROD64.3.904

Tulsiani DRP (2006) Glycan-modifying enzymes in luminal fluid of the mammalian epididymis: an overview of their potential role in sperm maturation. Mol Cell Endocrinol. https://doi.org/10.1016/J.MCE.2005.12.025

Venditti JJ, Donigan KA, Bean BS (2007) Crypticity and functional distribution of the membrane associated alpha-L-fucosidase of human sperm. Mol Reprod Dev. https://doi.org/10.1002/MRD.20666

Venditti JJ, Swann JM, Bean BS (2010) Hamster sperm-associated alpha-L-fucosidase functions during fertilization. Biol Reprod. https://doi.org/10.1095/BIOLREPROD.109.076695

Wang WH, Day BN, Wu GM (2003) How does polyspermy happen in mammalian oocytes? Microsc Res Tech. https://doi.org/10.1002/JEMT.10346

Xia P, Wang Z, Yang Z, Tan J, Qin P (2001) Ultrastructural study of polyspermy during early embryo development in pigs, observed by scanning electron microscope and transmission electron microscope. Cell Tissue Res. https://doi.org/10.1007/S004410000315

Zaborowski MP, Balaj L, Breakefield XO, Lai CP (2015) Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. Bioscience. https://doi.org/10.1093/BIOSCI/BIV084

ANEXO A – NORMAS DA REVISTA VETERINARY RESEARCH COMMUNICATIONS

Instructions for Authors

Authorship Policy

Authorship should incorporate and should be restricted to those who have contributed substantially to the work in one or more of the following categories:

• Conceived of or designed study

• Performed research

• Analyzed data

• Contributed new methods or models

• Wrote the paper

Types of Articles

All manuscripts should be presented preferably in Times New Roman font, double-spaced, using A4 paper size. Please use the automatic page and line numbering function to number the pages and lines in your document and number the lines in a single continuous sequence.

The journal accepts the following types of articles:

1. Research
2. Brief Report
3. Review
4. Correspondence
5. Case Reports

1. Research

Research, also known as Regular Articles should be as concise as possible and structured into the following sections;

a. Abstract of 150-250 words giving a synopsis of the findings presented and the conclusions reached. The Abstract should be submitted as a single continuous paragraph without subdivisions.
b. Introduction stating the purpose of the work
c. Materials and Methods
d. Results
e. Discussion (including also a short paragraph as conclusions)

f. Acknowledgements
g. Statement of Animal Ethics, including the number of the relevant Ethical Committee's protocol, where appropriate.
h. Conflict of Interest Statement
i. References

2. Brief Report

Also known as Short Communications, reports original scientific data. It should not typically exceed approximately 2000 words in the main text and no more than 3 figures/Tables and 20 references. As for regular articles, the abstract should contain between 150-250 words. All sections in a short communication should follow the regular article style (sections b-i). If necessary, a minimum number of sub-headings may be included if it adds clarity to the article.

3. Review

Review articles will be welcomed. However, authors considering the submission of review articles are advised to consult the Editor-in-Chief in advance.

4. Correspondence

Denotes arresting and timely comments on material published in the journal as well as anything of likely interest to the readers, such as policy debates and community announcements. Opinions are welcome as long as they are factually based. The expected length is about 1500-2000 words, a maximum of ten references, and no more than two tables or figures.

5. Case Reports

Emphasize distinctive instances involving animal patients that exhibit unforeseen diagnoses, treatment outcomes, or clinical courses. Additionally, study reports concerning significant programs or policy interventions pertinent to veterinary sciences are welcome. The report should be carefully structured under three distinct headings: Background, Case Presentation, and the Discussion and Conclusions section, which are presented together. Manuscripts incorporating a thorough evaluation of the study's processes and impact, along with recommendations for the future, will generally receive favorable consideration.

It is the author's responsibility to ensure that submitted manuscripts comply with journal format as indicated in the current instructions to authors and free sample articles on the springer.com journal homepage.

Manuscript Submission

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its

publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Online Submission

Please follow the hyperlink “Submit manuscript” and upload all of your manuscript files following the instructions given on the screen.

Source Files

Please ensure you provide all relevant editable source files at every submission and revision. Failing to submit a complete set of editable source files will result in your article not being considered for review. For your manuscript text please always submit in common word processing formats such as .docx or LaTeX.

Submitting Declarations

Please note that Author Contribution information and Competing Interest information must be provided at submission via the submission interface. Only the information submitted via the interface will be used in the final published version. Please make sure that if you are an editorial board member and also a listed author that you also declare this information in the Competing Interest section of the interface.

Please see the relevant sections in the submission guidelines for further information on these statements as well as possible other mandatory statements.

Title Page

Please make sure your title page contains the following information.

Title

The title should be concise and informative.

Author information

- The name(s) of the author(s)
- The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country
- A clear indication and an active e-mail address of the corresponding author
- If available, the 16-digit ORCID of the author(s)

If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

Large Language Models (LLMs), such as ChatGPT, do not currently satisfy our authorship criteria. Notably an attribution of authorship carries with it accountability for the work, which cannot be effectively applied to LLMs. Use of an LLM should be properly documented in the Methods section (and if a Methods section is not available, in a suitable alternative part) of the manuscript. The use of an LLM (or other AI-tool) for "AI assisted copy editing" purposes does not need to be declared. In this context, we define the term "AI assisted copy editing" as AI-assisted improvements to human-generated texts for readability and style, and to ensure that the texts are free of errors in grammar, spelling, punctuation and tone. These AI-assisted improvements may include wording and formatting changes to the texts, but do not include generative editorial work and autonomous content creation. In all cases, there must be human accountability for the final version of the text and agreement from the authors that the edits reflect their original work.

Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

For life science journals only (when applicable)

- Trial registration number and date of registration for prospectively registered trials
- Trial registration number and date of registration, followed by “retrospectively registered”, for retrospectively registered trials

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Text

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX. We recommend using Springer Nature's LaTeX template.

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

Additional remark:

Please use the automatic page and line numbering function to number the pages and lines in your document and number the lines in a single continuous sequence.

For revised manuscripts, please include a ‘clean’ version of your manuscript as well as a ‘tracked changes or highlighted’ version of your manuscript. This allows Editors and potential reviewers to quickly assess where the changes have been made within the revised file. Depending upon the extent of the revisions it may be easier for the ‘tracked changes’ version of your manuscript to be highlighted with changes rather than using the tracked changes feature in Word Documents. In your point by point response, please specify the page and / or line where each change has been made.

References

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

Reference list entries should be alphabetized by the last names of the first author of each work. Please alphabetize according to the following rules: 1) For one author, by name of author, then chronologically; 2) For two authors, by name of author, then name of coauthor, then chronologically; 3) For more than two authors, by name of first author, then chronologically.

If available, please always include DOIs as full DOI links in your reference list (e.g. “https://doi.org/abc”).

- Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal’s name according to the ISSN List of Title Word Abbreviations, see

ISSN LTWA

If you are unsure, please use the full journal title.

Statements & Declarations

The following statements must be included in your submitted manuscript under the heading 'Statements and Declarations'. This should be placed after the References section. Please note that submissions that do not include required statements will be returned as incomplete.

Funding

Please describe any sources of funding that have supported the work. The statement should include details of any grants received (please give the name of the funding agency and grant number).

Example statements:

“This work was supported by […] (Grant numbers […] and […]). Author A.B. has received research support from Company A.”

“The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.”

Competing Interests

Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Interests within the last 3 years of beginning the work (conducting the research and preparing the work for submission) should be reported. Interests outside the 3-year time frame must be disclosed if they could reasonably be perceived as influencing the submitted work.

Example statements:

“Financial interests: Author A and B declare they have no financial interests. Author C has received speaker and consultant honoraria from Company M. Dr. C has received speaker honorarium and research funding from Company M and Company N. Author D has received travel support from Company O. Non-financial interests: Author D has served on advisory boards for Company M and Company N.”

“The authors have no relevant financial or non-financial interests to disclose.”

Please refer to the “Competing Interests” section below for more information on how to complete these sections.

Author Contributions

Authors are encouraged to include a statement that specifies the contribution of every author to the research and preparation of the manuscript.

Example statement:

"All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [full name], [full name] and [full name]. The first draft of the manuscript was written by [full name] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript."

Please refer to the "Authorship Principles " section below for more information on how to complete this section.

Data Availability

This journal encourages authors to provide an optional statement of data availability in their article. Data Availability Statements should include information on where data supporting the results reported in the article can be found, including, where applicable, hyperlinks to publicly archived datasets analysed or generated during the study. Data availability statements can also indicate whether data are available on request from the authors and where no data are available, if appropriate.

Example statements:

"The datasets generated during and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT LINK TO DATASETS]"

"The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request."

Please refer to the "Research Data Policy and Data Availability" section below for more information on how to complete this section.

In addition to the above, manuscripts that report the results of studies involving humans and/or animals should include the following declarations:

Ethics approval

Authors of research involving human or animal subjects should include a statement that confirms that the study was approved (or granted exemption) by the appropriate institutional and/or national research ethics committee (including the name of the ethics committee and reference number, if available). For research involving animals, their data or biological material, authors should supply detailed information on the ethical treatment of their animals in their submission. If a study was granted exemption or did not require ethics approval, this should also be detailed in the manuscript.

"This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No).”

"This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required."

For detailed information on relevant ethical standards and criteria, please refer to the sections on "Research involving human participants, their data or biological material", "Research involving animals, their data or biological material".

Consent to participate

For all research involving human subjects, freely-given, informed consent to participate in the study must be obtained from participants (or their parent or legal guardian in the case of children under 16) and a statement to this effect should appear in the manuscript.

Example statement:

"Informed consent was obtained from all individual participants included in the study."

"Written informed consent was obtained from the parents."

Please refer to the section on "Informed Consent" for additional help with completing this information.

Consent to publish

Individuals may consent to participate in a study, but object to having their data published in a journal article. If your manuscript contains any individual person's data in any form (including any individual details, images or videos), consent for publication must be obtained from that person, or in the case of children, their parent or legal guardian. This is in particular applicable to case studies. A statement confirming that consent to publish has been received from all participants should appear in the manuscript.

Example statement:

"The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 1b and 1c."

Please refer to the section on "Informed Consent" for additional help with completing this information.

Tables

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

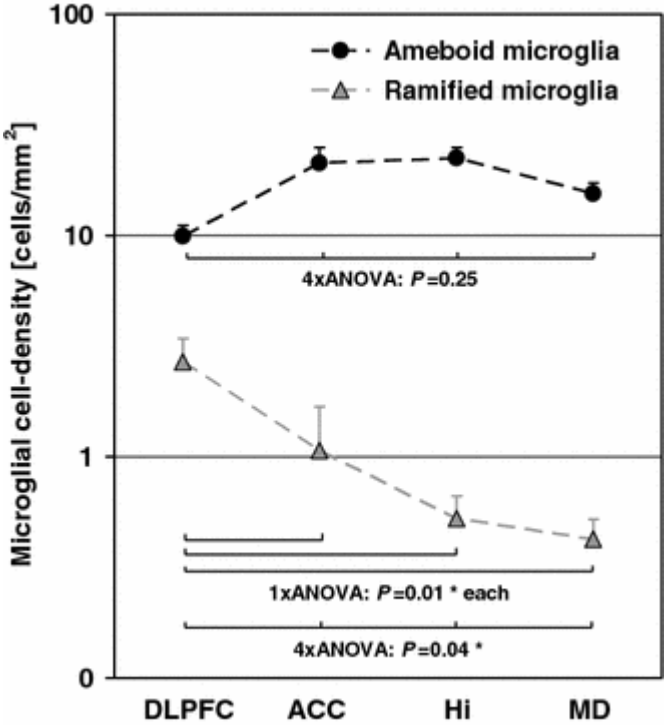
Artwork and Illustrations Guidelines

Electronic Figure Submission

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.

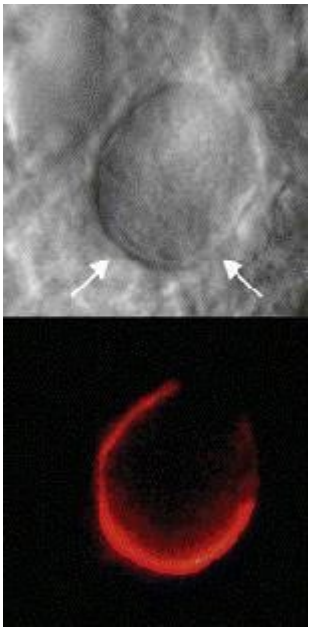
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Line Art



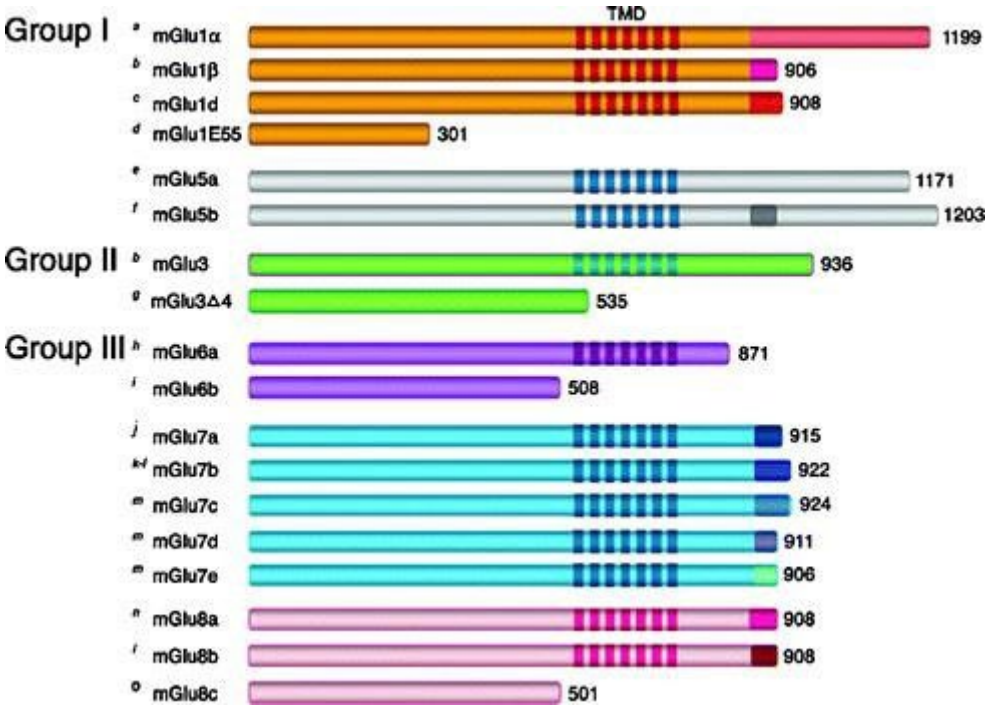
- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art



- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

Combination Art



- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

Color Art

- Color art is free of charge for online publication.
- If black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent.
- If the figures will be printed in black and white, do not refer to color in the captions.
- Color illustrations should be submitted as RGB (8 bits per channel).

Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).
- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.
- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

Figure Numbering

- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
- Figure parts should be denoted by lowercase letters (a, b, c, etc.).
- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures,"A1, A2, A3, etc." Figures in online appendices [Supplementary Information (SI)] should, however, be numbered separately.

Figure Captions

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.

- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

Figure Placement and Size

- Figures should be submitted within the body of the text. Only if the file size of the manuscript causes problems in uploading it, the large figures should be submitted separately from the text.
- When preparing your figures, size figures to fit in the column width.
- For large-sized journals the figures should be 84 mm (for double-column text areas), or 174 mm (for single-column text areas) wide and not higher than 234 mm.
- For small-sized journals, the figures should be 119 mm wide and not higher than 195 mm.

Permissions

If you include figures that have already been published elsewhere, you must obtain permission from the copyright owner(s) for both the print and online format. Please be aware that some publishers do not grant electronic rights for free and that Springer will not be able to refund any costs that may have occurred to receive these permissions. In such cases, material from other sources should be used.

Accessibility

In order to give people of all abilities and disabilities access to the content of your figures, please make sure that

- All figures have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware)
- Patterns are used instead of or in addition to colors for conveying information (colorblind users would then be able to distinguish the visual elements)
- Any figure lettering has a contrast ratio of at least 4.5:1

Generative AI Images

Please check Springer's policy on generative AI images and make sure your work adheres to the principles described therein.

Supplementary Information (SI)

Springer accepts electronic multimedia files (animations, movies, audio, etc.) and other supplementary files to be published online along with an article or a book

chapter. This feature can add dimension to the author's article, as certain information cannot be printed or is more convenient in electronic form.

Before submitting research datasets as Supplementary Information, authors should read the journal's Research data policy. We encourage research data to be archived in data repositories wherever possible.

Submission

- Supply all supplementary material in standard file formats.
- Please include in each file the following information: article title, journal name, author names; affiliation and e-mail address of the corresponding author.
- To accommodate user downloads, please keep in mind that larger-sized files may require very long download times and that some users may experience other problems during downloading.

Audio, Video, and Animations

- Aspect ratio: 16:9 or 4:3
- Maximum file size: 2 GB
- Minimum video duration: 1 sec
- Supported file formats: avi, wmv, mp4, mov, m2p, mp2, mpg, mpeg, flv, mxf, mts, m4v, 3gp

Text and Presentations

- Submit your material in PDF format; .doc or .ppt files are not suitable for long-term viability.
- A collection of figures may also be combined in a PDF file.

Spreadsheets

- Spreadsheets should be submitted as .csv or .xlsx files (MS Excel).

Specialized Formats

- Specialized format such as .pdb (chemical), .wrl (VRML), .nb (Mathematica notebook), and .tex can also be supplied.

Collecting Multiple Files

- It is possible to collect multiple files in a .zip or .gz file.

Numbering

- If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.
- Refer to the supplementary files as “Online Resource”, e.g., "... as shown in the animation (Online Resource 3)", “... additional data are given in Online Resource 4”.
- Name the files consecutively, e.g. “ESM_3.mpg”, “ESM_4.pdf”.

Captions

- For each supplementary material, please supply a concise caption describing the content of the file.

Processing of supplementary files

- Supplementary Information (SI) will be published as received from the author without any conversion, editing, or reformatting.

Accessibility

In order to give people of all abilities and disabilities access to the content of your supplementary files, please make sure that

- The manuscript contains a descriptive caption for each supplementary material
- Video files do not contain anything that flashes more than three times per second (so that users prone to seizures caused by such effects are not put at risk)

Generative AI Images

Please check Springer’s policy on generative AI images and make sure your work adheres to the principles described therein.

Ethical Responsibilities of Authors

This journal is committed to upholding the integrity of the scientific record. As a member of the Committee on Publication Ethics (COPE) the journal will follow the COPE guidelines on how to deal with potential acts of misconduct.

Authors should refrain from misrepresenting research results which could damage the trust in the journal, the professionalism of scientific authorship, and ultimately the entire scientific endeavour. Maintaining integrity of the research and its presentation is helped by following the rules of good scientific practice, which include*:

- The manuscript should not be submitted to more than one journal for simultaneous consideration.

- The submitted work should be original and should not have been published elsewhere in any form or language (partially or in full), unless the new work concerns an expansion of previous work. (Please provide transparency on the re-use of material to avoid the concerns about text-recycling ('self-plagiarism').
- A single study should not be split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time (i.e. 'salami-slicing/publishing').
- Concurrent or secondary publication is sometimes justifiable, provided certain conditions are met. Examples include: translations or a manuscript that is intended for a different group of readers.
- Results should be presented clearly, honestly, and without fabrication, falsification or inappropriate data manipulation (including image based manipulation). Authors should adhere to discipline-specific rules for acquiring, selecting and processing data.
- No data, text, or theories by others are presented as if they were the author's own ('plagiarism'). Proper acknowledgements to other works must be given (this includes material that is closely copied (near verbatim), summarized and/or paraphrased), quotation marks (to indicate words taken from another source) are used for verbatim copying of material, and permissions secured for material that is copyrighted.

Important note: the journal may use software to screen for plagiarism.

- Authors should make sure they have permissions for the use of software, questionnaires/(web) surveys and scales in their studies (if appropriate).
- Research articles and non-research articles (e.g. Opinion, Review, and Commentary articles) must cite appropriate and relevant literature in support of the claims made. Excessive and inappropriate self-citation or coordinated efforts among several authors to collectively self-cite is strongly discouraged.
- Authors should avoid untrue statements about an entity (who can be an individual person or a company) or descriptions of their behavior or actions that could potentially be seen as personal attacks or allegations about that person.
- Research that may be misapplied to pose a threat to public health or national security should be clearly identified in the manuscript (e.g. dual use of research). Examples include creation of harmful consequences of biological agents or toxins, disruption of immunity of vaccines, unusual hazards in the use of chemicals, weaponization of research/technology (amongst others).
- Authors are strongly advised to ensure the author group, the Corresponding Author, and the order of authors are all correct at submission. Adding and/or deleting authors during the revision stages is generally not permitted, but in some cases may be warranted. Reasons for changes in authorship should be

explained in detail. Please note that changes to authorship cannot be made after acceptance of a manuscript.

*All of the above are guidelines and authors need to make sure to respect third parties rights such as copyright and/or moral rights.

Upon request authors should be prepared to send relevant documentation or data in order to verify the validity of the results presented. This could be in the form of raw data, samples, records, etc. Sensitive information in the form of confidential or proprietary data is excluded.

If there is suspicion of misbehavior or alleged fraud the Journal and/or Publisher will carry out an investigation following COPE guidelines. If, after investigation, there are valid concerns, the author(s) concerned will be contacted under their given e-mail address and given an opportunity to address the issue. Depending on the situation, this may result in the Journal's and/or Publisher's implementation of the following measures, including, but not limited to:

- If the manuscript is still under consideration, it may be rejected and returned to the author.
- If the article has already been published online, depending on the nature and severity of the infraction:

  - an erratum/correction may be placed with the article

  - an expression of concern may be placed with the article

  - or in severe cases retraction of the article may occur.

The reason will be given in the published erratum/correction, expression of concern or retraction note. Please note that retraction means that the article is **maintained on the platform**, watermarked "retracted" and the explanation for the retraction is provided in a note linked to the watermarked article.

- The author's institution may be informed
- A notice of suspected transgression of ethical standards in the peer review system may be included as part of the author's and article's bibliographic record.

Fundamental errors

Authors have an obligation to correct mistakes once they discover a significant error or inaccuracy in their published article. The author(s) is/are requested to contact the journal and explain in what sense the error is impacting the article. A decision on how to correct the literature will depend on the nature of the error. This may be a

correction or retraction. The retraction note should provide transparency which parts of the article are impacted by the error.

Suggesting / excluding reviewers

Authors are welcome to suggest suitable reviewers and/or request the exclusion of certain individuals when they submit their manuscripts. When suggesting reviewers, authors should make sure they are totally independent and not connected to the work in any way. It is strongly recommended to suggest a mix of reviewers from different countries and different institutions. When suggesting reviewers, the Corresponding Author must provide an institutional email address for each suggested reviewer, or, if this is not possible to include other means of verifying the identity such as a link to a personal homepage, a link to the publication record or a researcher or author ID in the submission letter. Please note that the Journal may not use the suggestions, but suggestions are appreciated and may help facilitate the peer review process.

Authorship principles

These guidelines describe authorship principles and good authorship practices to which prospective authors should adhere to.

Authorship clarified

The Journal and Publisher assume all authors agreed with the content and that all gave explicit consent to submit and that they obtained consent from the responsible authorities at the institute/organization where the work has been carried out, **before** the work is submitted.

The Publisher does not prescribe the kinds of contributions that warrant authorship. It is recommended that authors adhere to the guidelines for authorship that are applicable in their specific research field. In absence of specific guidelines it is recommended to adhere to the following guidelines*:

All authors whose names appear on the submission

1) made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work;

2) drafted the work or revised it critically for important intellectual content;

3) approved the version to be published; and

4) agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

* Based on/adapted from:

ICMJE, Defining the Role of Authors and Contributors,

Transparency in authors' contributions and responsibilities to promote integrity in scientific publication, McNutt at all, PNAS February 27, 2018

Disclosures and declarations

All authors are requested to include information regarding sources of funding, financial or non-financial interests, study-specific approval by the appropriate ethics committee for research involving humans and/or animals, informed consent if the research involved human participants, and a statement on welfare of animals if the research involved animals (as appropriate).

The decision whether such information should be included is not only dependent on the scope of the journal, but also the scope of the article. Work submitted for publication may have implications for public health or general welfare and in those cases it is the responsibility of all authors to include the appropriate disclosures and declarations.

Data transparency

All authors are requested to make sure that all data and materials as well as software application or custom code support their published claims and comply with field standards. Please note that journals may have individual policies on (sharing) research data in concordance with disciplinary norms and expectations.

Role of the Corresponding Author

One author is assigned as Corresponding Author and acts on behalf of all co-authors and ensures that questions related to the accuracy or integrity of any part of the work are appropriately addressed.

The Corresponding Author is responsible for the following requirements:

- ensuring that all listed authors have approved the manuscript before submission, including the names and order of authors;

- managing all communication between the Journal and all co-authors, before and after publication;*
- providing transparency on re-use of material and mention any unpublished material (for example manuscripts in press) included in the manuscript in a cover letter to the Editor;
- making sure disclosures, declarations and transparency on data statements from all authors are included in the manuscript as appropriate (see above).

* The requirement of managing all communication between the journal and all co-authors during submission and proofing may be delegated to a Contact or Submitting Author. In this case please make sure the Corresponding Author is clearly indicated in the manuscript.

Author contributions

In absence of specific instructions and in research fields where it is possible to describe discrete efforts, the Publisher recommends authors to include contribution statements in the work that specifies the contribution of every author in order to promote transparency. These contributions should be listed at the separate title page.

Examples of such statement(s) are shown below:

• Free text:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [full name], [full name] and [full name]. The first draft of the manuscript was written by [full name] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Example: CRediT taxonomy:

• Conceptualization: [full name], …; Methodology: [full name], …; Formal analysis and investigation: [full name], …; Writing - original draft preparation: [full name, …]; Writing - review and editing: [full name], …; Funding acquisition: [full name], …; Resources: [full name], …; Supervision: [full name],….

For **review articles** where discrete statements are less applicable a statement should be included who had the idea for the article, who performed the literature search and data analysis, and who drafted and/or critically revised the work.

For articles that are based primarily on the **student's dissertation or thesis**, it is recommended that the student is usually listed as principal author:

A Graduate Student's Guide to Determining Authorship Credit and Authorship Order, APA Science Student Council 2006

Affiliation

The primary affiliation for each author should be the institution where the majority of their work was done. If an author has subsequently moved, the current address may additionally be stated. Addresses will not be updated or changed after publication of the article.

Changes to authorship

Authors are strongly advised to ensure the correct author group, the Corresponding Author, and the order of authors at submission. Changes of authorship by adding or deleting authors, and/or changes in Corresponding Author, and/or changes in the sequence of authors are **not** accepted **after acceptance** of a manuscript.

- **Please note that author names will be published exactly as they appear on the accepted submission!**

Please make sure that the names of all authors are present and correctly spelled, and that addresses and affiliations are current.

Adding and/or deleting authors at revision stage are generally not permitted, but in some cases it may be warranted. Reasons for these changes in authorship should be explained. Approval of the change during revision is at the discretion of the Editor-in-Chief. Please note that journals may have individual policies on adding and/or deleting authors during revision stage.

Author identification

Authors are recommended to use their ORCID ID when submitting an article for consideration or acquire an ORCID ID via the submission process.

Deceased or incapacitated authors

For cases in which a co-author dies or is incapacitated during the writing, submission, or peer-review process, and the co-authors feel it is appropriate to include the author, co-authors should obtain approval from a (legal) representative which could be a direct relative.

Authorship issues or disputes

In the case of an authorship dispute during peer review or after acceptance and publication, the Journal will not be in a position to investigate or adjudicate. Authors will be asked to resolve the dispute themselves. If they are unable the Journal reserves the right to withdraw a manuscript from the editorial process or in case of a published paper raise the issue with the authors' institution(s) and abide by its guidelines.

Confidentiality

Authors should treat all communication with the Journal as confidential which includes correspondence with direct representatives from the Journal such as Editors-in-Chief and/or Handling Editors and reviewers' reports unless explicit consent has been received to share information.

Compliance with Ethical Standards

To ensure objectivity and transparency in research and to ensure that accepted principles of ethical and professional conduct have been followed, authors should include information regarding sources of funding, potential conflicts of interest (financial or non-financial), informed consent if the research involved human participants, and a statement on welfare of animals if the research involved animals.

Authors should include the following statements (if applicable) in a separate section entitled "Compliance with Ethical Standards" when submitting a paper:

- Disclosure of potential conflicts of interest
- Research involving Human Participants and/or Animals
- Informed consent

Please note that standards could vary slightly per journal dependent on their peer review policies (i.e. single or double blind peer review) as well as per journal subject discipline. Before submitting your article check the instructions following this section carefully.

The corresponding author should be prepared to collect documentation of compliance with ethical standards and send if requested during peer review or after publication.

The Editors reserve the right to reject manuscripts that do not comply with the above-mentioned guidelines. The author will be held responsible for false statements or failure to fulfill the above-mentioned guidelines.

Competing Interests

Authors are requested to disclose interests that are directly or indirectly related to the work submitted for publication. Interests within the last 3 years of beginning the work (conducting the research and preparing the work for submission) should be reported. Interests outside the 3-year time frame must be disclosed if they could reasonably be perceived as influencing the submitted work. Disclosure of interests provides a complete and transparent process and helps readers form their own judgments of potential bias. This is not meant to imply that a financial relationship with an organization that sponsored the research or compensation received for consultancy work is inappropriate.

Editorial Board Members and Editors are required to declare any competing interests and may be excluded from the peer review process if a competing interest exists. In addition, they should exclude themselves from handling manuscripts in cases where there is a competing interest. This may include – but is not limited to – having previously published with one or more of the authors, and sharing the same institution as one or more of the authors. Where an Editor or Editorial Board Member is on the author list we recommend they declare this in the competing interests section on the submitted manuscript. If they are an author or have any other competing interest regarding a specific manuscript, another Editor or member of the Editorial Board will be assigned to assume responsibility for overseeing peer review. These submissions are subject to the exact same review process as any other manuscript. Editorial Board Members are welcome to submit papers to the journal. These submissions are not given any priority over other manuscripts, and Editorial Board Member status has no bearing on editorial consideration.

Interests that should be considered and disclosed but are not limited to the following:

Funding: Research grants from funding agencies (please give the research funder and the grant number) and/or research support (including salaries, equipment, supplies, reimbursement for attending symposia, and other expenses) by organizations that may gain or lose financially through publication of this manuscript.

Employment: Recent (while engaged in the research project), present or anticipated employment by any organization that may gain or lose financially through publication of this manuscript. This includes multiple affiliations (if applicable).

Financial interests: Stocks or shares in companies (including holdings of spouse and/or children) that may gain or lose financially through publication of this manuscript; consultation fees or other forms of remuneration from organizations that may gain or lose financially; patents or patent applications whose value may be affected by publication of this manuscript.

It is difficult to specify a threshold at which a financial interest becomes significant, any such figure is necessarily arbitrary, so one possible practical guideline is the following: "Any undeclared financial interest that could embarrass the author were it to become publicly known after the work was published."

Non-financial interests: In addition, authors are requested to disclose interests that go beyond financial interests that could impart bias on the work submitted for publication such as professional interests, personal relationships or personal beliefs (amongst others). Examples include, but are not limited to: position on editorial board, advisory board or board of directors or other type of management relationships; writing and/or consulting for educational purposes; expert witness; mentoring relations; and so forth.

Primary research articles require a disclosure statement. Review articles present an expert synthesis of evidence and may be treated as an authoritative work on a subject. Review articles therefore require a disclosure statement. Other article types such as editorials, book reviews, comments (amongst others) may, dependent on their content, require a disclosure statement. If you are unclear whether your article type requires a disclosure statement, please contact the Editor-in-Chief.

Please note that, in addition to the above requirements, funding information (given that funding is a potential competing interest (as mentioned above)) needs to be disclosed upon submission of the manuscript in the peer review system. This information will automatically be added to the Record of CrossMark, however it is **not added** to the manuscript itself. Under 'summary of requirements' (see below) funding information should be included in the '**Declarations**' section.

Summary of requirements

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Funding' and/or 'Competing interests'. Other declarations include Ethics approval, Consent, Data, Material and/or Code availability and Authors' contribution statements.

Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

When all authors have the same (or no) conflicts and/or funding it is sufficient to use one blanket statement.

Examples of statements to be used when funding has been received:

- Partial financial support was received from [...]
- The research leading to these results received funding from […] under Grant Agreement No[…].
- This study was funded by […]
- This work was supported by […] (Grant numbers […] and […]

Examples of statements to be used when there is no funding:

- The authors did not receive support from any organization for the submitted work.
- No funding was received to assist with the preparation of this manuscript.
- No funding was received for conducting this study.
- No funds, grants, or other support was received.

Examples of statements to be used when there are interests to declare:

- **Financial interests:** Author A has received research support from Company A. Author B has received a speaker honorarium from Company W and owns stock in Company X. Author C is consultant to company Y.

  Non-financial interests: Author C is an unpaid member of committee Z.

- **Financial interests:** The authors declare they have no financial interests.

  Non-financial interests: Author A is on the board of directors of Y and receives no compensation as member of the board of directors.

- **Financial interests:** Author A received a speaking fee from Y for Z. Author B receives a salary from association X. X where s/he is the Executive Director.

  Non-financial interests: none.

- **Financial interests:** Author A and B declare they have no financial interests. Author C has received speaker and consultant honoraria from Company M and Company N. Dr. C has received speaker honorarium and research funding from Company M and Company O. Author D has received travel support from Company O.

  Non-financial interests: Author D has served on advisory boards for Company M, Company N and Company O.

Examples of statements to be used when authors have nothing to declare:

- The authors have no relevant financial or non-financial interests to disclose.
- The authors have no competing interests to declare that are relevant to the content of this article.

- All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.
- The authors have no financial or proprietary interests in any material discussed in this article.

Authors are responsible for correctness of the statements provided in the manuscript. See also Authorship Principles. The Editor-in-Chief reserves the right to reject submissions that do not meet the guidelines described in this section.

Research involving animals, their data or biological material

The welfare of animals (vertebrate and higher invertebrate) used for research, education and testing must be respected. Authors should supply detailed information on the ethical treatment of their animals in their submission. For that purpose they may use the ARRIVE checklist which is designed to be used when submitting manuscripts describing animal research.

For studies involving client-owned animals, authors must also document informed consent from the client or owner and adherence to a high standard (best practice) of veterinary care.

Authors are recommended to comply with:

• The International Union for Conservation of Nature (IUCN) Policy Statement on Research Involving Species at Risk of Extinction and consult the IUCN red list index of threatened species.

• Convention on the Trade in Endangered Species of Wild Fauna and Flora

When reporting results authors should indicate:

• … that the studies have been approved by a research ethics committee at the institution or practice at which the studies were conducted. Please provide the name of ethics committee and relevant permit number;

• … whether the legal requirements or guidelines in the country and/or state or province for the care and use of animals have been followed.

Researchers from countries without any legal requirements or guidelines voluntarily should refer to the following sites for guidance:

– The Basel Declaration describes fundamental principles of using animals in biomedical research

– The International Council for Laboratory Animal Science (ICLAS) provides ethical guidelines for researchers as well as editors and reviewers

– The Association for the study of Animal Behaviour describes ethical guidelines for the treatment of animals in research and teaching

– The International Association of Veterinary Editors' Consensus Author Guidelines on Animal Ethics provide guidelines for authors on animal ethics and welfare

Researchers may wish to consult the most recent (ethical) guidelines available from relevant taxon-oriented professional societies.

If a study was granted exemption or did not require ethics approval, this should also be detailed in the manuscript.

Summary of requirements

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Ethics approval'.

Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

Examples of statements to be used when ethics approval has been obtained:

• All procedures involving animals were in compliance with the European Community Council Directive of 24 November 1986, and ethical approval was granted by the Kocaeli University Ethics Committee (No. 29 12 2014, Kocaeli, Turkey).

• All procedures performed in the study were in accordance with the ARVO Statement for Use of Animals in Ophthalmic Vision and Research. The ethical principles established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised 2011) were followed. The research protocol was approved by the Ethics Committee on Animal Use (Protocol No. 06174/14) of FCAV/Unesp, Jaboticabal.

• This study involved a questionnaire-based survey of farmers as well as blood sampling from their animals. The study protocol was assessed and approved by Haramaya University, research and extension office. Participants provided their verbal informed consent for animal blood sampling as well as for the related survey questions. Collection of blood samples was carried out by veterinarians adhering to the regulations and guidelines on animal husbandry and welfare.

• All brown bear captures and handling were approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (Application C18/15) and the Swedish Environmental Protection Agency in compliance with Swedish laws and regulations.

• The ethics governing the use and conduct of experiments on animals were strictly observed, and the experimental protocol was approved by the University of Maiduguri Senate committee on Medical Research ethics. Proper permit and consent were obtained from the Maiduguri abattoir management, before the faecal samples of the cattle and camels slaughtered in this abattoir were used for this experiment.

Examples of statements to be used when no ethical approval is required/exemption granted:

• No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

• As the trappings of small mammals were conducted as part of regular pest control measures in accordance with the NATO Standardized Agreement 2048 "Deployment Pest and Vector Surveillance and Control ", no approval by an ethics committee was required.

• All experiments have been conducted as per the guidelines of the Institutional Animal Ethics Committee, Department of Zoology, Utkal University, Bhubaneswar, Odisha, India. However, the insect species used in this study is reared for commercial production of raw silk materials, as a part of agro-based industry. Therefore, use of this animal in research does not require ethical clearance. We have obtained permission from the office of Research officer sericulture, Baripada, Orissa, India for the provision of infrastructure and support for rearing of silkworm both in indoor and outdoor conditions related to our study to promote sericulture practices.

Authors are responsible for correctness of the statements provided in the manuscript. See also Authorship Principles. The Editor-in-Chief reserves the right to reject submissions that do not meet the guidelines described in this section.

Research Data Policy and Data Availability Statements

This journal follows Springer Nature research data policy. Sharing of all relevant research data is strongly encouraged and authors must add a Data Availability Statement to original research articles.

Research data includes a wide range of types, including spreadsheets, images, textual extracts, archival documents, video or audio, interview notes or any specialist formats generated during research.

Data availability statements

All original research must include a data availability statement. This statement should explain how to access data supporting the results and analysis in the article, including links/citations to publicly archived datasets analysed or generated during the study. Please see our full policy here.

If it is not possible to share research data publicly, for instance when individual privacy could be compromised, this statement should describe how data can be accessed and any conditions for reuse. Participant consent should be obtained and documented prior to data collection. See our guidance on sensitive data for more information.

When creating a data availability statement, authors are encouraged to consider the minimal dataset that would be necessary to interpret, replicate and build upon the findings reported in the article.

Further guidance on writing a data availability statement, including examples, is available at:

Data availability statements

Data repositories

Authors are strongly encouraged to deposit their supporting data in a publicly available repository. Sharing your data in a repository promotes the integrity, discovery and reuse of your research, making it easier for the research community to build on and credit your work.

See our data repository guidance for information on finding a suitable repository.

We recommend the use of discipline-specific repositories where available. For a number of data types, submission to specific public repositories is mandatory.

The journal encourages making research data available under open licences that permit reuse. The journal does not enforce use of particular licences in third party repositories. You should ensure you have necessary rights to share any data that you deposit in a repository.

Data citation

The journal recommends that authors cite any publicly available data on which the conclusions of the paper rely. This includes data the authors are sharing alongside their publication and any secondary data the authors have reused. Data citations should include a persistent identifier (such as a DOI), should be included in the reference list using the minimum information recommended by DataCite (Dataset Creator, Dataset Title, Publisher [repository], Publication Year, Identifier [e.g. DOI, Handle, Accession or ARK]) and follow journal style.

Research data and peer review

If the journal that you are submitting to uses double-anonymous peer review and you are providing reviewers with access to your data (for example via a repository link, supplementary information or data on request), it is strongly suggested that the authorship in the data is also anonymised. There are data repositories that can assist with this and/or will create a link to mask the authorship of your data.

Support with research data policy

Authors who need help understanding our data sharing policy, finding a suitable data repository, or organising and sharing research data can consult our Research Data Helpdesk for guidance.

After acceptance

Upon acceptance, your article will be exported to Production to undergo typesetting. Shortly after this you will receive two e-mails. One contains a request to confirm your affiliation, choose the publishing model for your article, as well as to arrange rights and payment of any associated publication cost. A second e-mail containing a link to your article's proofs will be sent once typesetting is completed.

Article publishing agreement

Depending on the ownership of the journal and its policies, you will either grant the Publisher an exclusive licence to publish the article or will be asked to transfer copyright of the article to the Publisher.

Offprints

Offprints can be ordered by the corresponding author.

Color illustrations

Online publication of color illustrations is free of charge. For color in the print version, authors will be expected to make a contribution towards the extra costs.

Proof reading

The purpose of the proof is to check for typesetting or conversion errors and the completeness and accuracy of the text, tables and figures. Substantial changes in content, e.g., new results, corrected values, title and authorship, are not allowed without the approval of the Editor.

After online publication, further changes can only be made in the form of an Erratum, which will be hyperlinked to the article.

Online First

The article will be published online after receipt of the corrected proofs. This is the official first publication citable with the DOI. After release of the printed version, the paper can also be cited by issue and page numbers.