UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO
PRO-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL
DOUTORADO ACADÊMICO EM CIÊNCIA ANIMAL

MARIA VALÉRIA DE OLIVEIRA SANTOS

ESTRATÉGIAS DE SELEÇÃO E CAPACITAÇÃO ESPERMÁTICA PARA A PRODUÇÃO *IN VITRO* DE EMBRIÕES EM CATETOS (*Pecari tajacu* LINNAEUS, 1758)

MOSSORÓ
2023

MARIA VALÉRIA DE OLIVEIRA SANTOS

ESTRATÉGIAS DE SELEÇÃO E CAPACITAÇÃO ESPERMÁTICA PARA A PRODUÇÃO *IN VITRO* DE EMBRIÕES EM CATETOS (*Pecari tajacu* LINNAEUS, 1758)

Tese apresentada ao Doutorado em Ciência Animal do Programa de Pós-Graduação em Ciência Animal da Universidade Federal Rural do Semi-Árido como requisito para obtenção do título de Doutora em Ciência Animal.

Linha de Pesquisa: Morfofisiologia e Biotecnologia Animal

Orientadora: Profa. Dra. Alexsandra Fernandes Pereira.

Co-orientador: Prof. Dr. Alexandre Rodrigues Silva.

MOSSORÓ

2023

MARIA VALÉRIA DE OLIVEIRA SANTOS

ESTRATÉGIAS DE SELEÇÃO E CAPACITAÇÃO ESPERMÁTICA PARA A PRODUÇÃO *IN VITRO* DE EMBRIÕES EM CATETOS (*Pecari tajacu* LINNAEUS, 1758)

Tese apresentada ao Doutorado em Ciência Animal do Programa de Pós-Graduação em Ciência Animal da Universidade Federal Rural do Semi-Árido como requisito para obtenção do título de Doutora em Ciência Animal.

Linha de Pesquisa: Morfofisiologia e Biotecnologia Animal

Defendida em: 24/02/2023

BANCA EXAMINADORA

_____

Profa. Dra. Alexsandra Fernandes Pereira (UFERSA)
Presidente e Orientadora

_____

Prof. Dr. André Furugen Cesar de Andrade (USP)
Membro Examinador

_____

Prof. Dr. Rodrigo Vasconcelos de Oliveira (UFRRJ)
Membro Examinador

_____

Prof. Dr. Ribrio Ivan Tavares Pereira Batista (UFF)
Membro Examinador

_____

Prof. Dr. Alexandre Rodrigues Silva (UFERSA)
Membro Examinado

O237e OLIVEIRA SANTOS, MARIA VALÉRIA DE.
Estratégias de seleção e capacitação espermática para a produção in vitro de embriões em catetos (Pecari tajacu Linnaeus, 1758) / MARIA VALÉRIA DE OLIVEIRA SANTOS. - 2023.
143 f. : il.

Orientadora: Alexsandra Fernandes Pereira.
Coorientador: Alexandre Rodrigues Silva.
Tese (Doutorado) - Universidade Federal Rural do Semi-árido, Programa de Pós-graduação em Ciência Animal, 2023.

1. Mamíferos silvestres. 2. Fertilização in vitro . 3. Processamento espermático . 4. Espermatozoides . 5. Agentes capacitantes. I. Fernandes Pereira, Alexsandra , orient. II. Rodrigues Silva, Alexandre , co-orient. III. Título.

Ficha catalográfica elaborada por sistema gerador automáto em conformidade com AACR2 e os dados fornecidos pelo) autor(a).
Biblioteca Campus Mossoró / Setor de Informação e Referência
Bibliotecária: Keina Cristina Santos Sousa e Silva
CRB: 15/120

O serviço de Geração Automática de Ficha Catalográfica para Trabalhos de Conclusão de Curso (TCC´s) foi desenvolvido pelo Instituto de Ciências Matemáticas e de Computação da Universidade de São Paulo (USP) e gentilmente cedido para o Sistema de Bibliotecas da Universidade Federal Rural do Semi-Árido (SISBI-UFERSA), sendo customizado pela Superintendência de Tecnologia da Informação e Comunicação (SUTIC) sob orientação dos bibliotecários da instituição para ser adaptado às necessidades dos alunos dos Cursos de Graduação e Programas de Pós-Graduação da Universidade.

A minha família, que é meu porto seguro, meu maior incentivo e minha força.

A Alexsandra Fernandes Pereira, pelos ensinamentos.

Dedico.

AGRADECIMENTOS

A Universidade Federal Rural do Semi-Árido (UFERSA) e ao Programa de Pós-Graduação em Ciência Animal (PPGCA) pela oportunidade e formação.

A Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pelo apoio financeiro.

Ao Laboratório de Biotecnologia Animal (LBA/UFERSA, responsável: Profa. Dra. Alexsandra Fernandes Pereira) e ao Laboratório de Conservação de Germoplasma Animal (LCGA/UFERSA, responsável: Prof. Dr. Alexandre Rodrigues Silva) por permitirem a utilização do espaço, equipamentos e reagentes para realização dos experimentos. Ao Centro de Multiplicação de Animais Silvestres (CEMAS/UFERSA) e a todos os funcionários que manejam os catetos e ajudaram nas coletas. Ao Abatedouro e Frigorífico Industrial de Mossoró S/A (AFIM) por disponibilizar os ovários suínos para realização deste trabalho.

A minha orientadora, Profa. Dra. Alexsandra Fernandes Pereira, que foi a minha melhor escolha profissional desde 2013 e que me transformou ano após ano na pessoa que sou hoje. Não tenho palavras para agradecer todo o tempo dedicado a mim e a esse projeto, a sua doação à ciência é admirável e inspiradora. Você consegue ser fortaleza e segurança ao mesmo tempo que é doce e amável com todos a sua volta, mesmo em situações desafiadoras. Obrigada pela confiança em todas as missões no decorrer dos anos, algumas que pareciam impossíveis, mas que juntas fomos capazes de cumprir. Muito obrigada também pela amizade e carinho que deixam as coisas mais fáceis e trazem leveza nessa correria da vida acadêmica.

Ao meu co-orientador, Prof. Dr. Alexandre Rodrigues Silva, pela contribuição em minha formação desde a graduação e por se disponibilizar para melhorar o desenvolvimento dos meus trabalhos. Muito obrigada pelos ensinamentos e lições que mudaram minha visão sobre a pesquisa e me trouxeram um grande amadurecimento.

Ao Prof. Dr. Moacir Franco de Oliveira, a quem sou extremamente grata por sempre estar presente nas coletas, sendo sempre amigável e fazendo as coisas darem certo. Muito obrigada pela confiança e colaboração.

Ao Prof. Dr. Vilceu Bordignon, que me recebeu da melhor forma possível na equipe do Large

Animal Research Unit (LARU), McGill. Muito obrigada pela oportunidade, pelos ensinamentos e parceria. Obrigada também a toda a equipe do LARU, especialmente Karina Gutierrez, Werner Giehl Glanzner, Mariana Priotto de Macedo e Zigomar da Silva.

A banca examinadora pela disponibilidade para avaliação do presente trabalho e por todas as contribuições.

A toda a equipe do LBA/UFERSA, obrigada por estarem sempre ajudando na obtenção dos resultados. A equipe do LCGA/UFERSA, pela disponibilidade e auxílio nas etapas de manipulação espermática, especialmente Andréa Maria da Silva que foi essencial para a execução deste trabalho.

Aos amigos que o LBA me apresentou: Érika Almeida Praxedes, Leonardo Vitorino Costa de Aquino e Lhara Ricarliany Medeiros de Oliveira. Vocês foram meu apoio em diversos momentos de pressão e nas loucuras da pós-graduação. Obrigada pela ajuda com esse trabalho, mas principalmente, pela amizade, pela escuta e companheirismo que irei guardar sempre no meu coração.

A minha família que é meu maior tesouro e tudo de mais importante que tenho nessa vida. A minha mãe, Maria de Fatima de Oliveira Santos, por seu amor incondicional e apoio constante, por ser meu maior exemplo de fé e honestidade. Ao meu pai, Francisco Valdenir dos Santos, por sempre confiar em mim e apoiar minhas decisões com muito amor e carinho. As minhas irmãs, Maria Vanessa de Oliveira Santos e Ana Paula de Oliveira Santos, pela amizade mais sincera e amor infinito, vocês são meu porto seguro. As minhas sobrinhas, Valeska Cristina de Oliveira e Anna Alessa de Oliveira, e meu sobrinho Gustavo de Oliveira, por me mostrarem a pureza do amor e a importância do tempo. Obrigada por suportarem minhas ausências, por serem sempre tão amáveis e confiarem em todas as minhas decisões, mesmo muitas vezes sem entender o que elas representam.

A Deus, por me abençoar todos os dias e ser meu guia.

Acima de tudo ame
como se fosse a única coisa que você sabe fazer
no fim do dia isso tudo
não significa nada
esta página
onde você está
seu diploma
seu emprego
o dinheiro
nada importa
exceto o amor e a conexão entre as pessoas
quem você amou
e com que profundidade você amou
como você tocou as pessoas à sua volta
e quanto você se doou a elas

Rupi Kaur

RESUMO

A produção de embriões por fertilização *in vitro* (FIV) possibilita uma abordagem científica, conservacionista e comercial dos catetos, mamíferos silvestres de importância biológica e com potencial de mercado. Contudo, informações sobre as variáveis envolvidas na seleção e capacitação espermática que antecede à FIV ainda são escassas em catetos. Assim, o objetivo foi aperfeiçoar o processamento espermático para a FIV em catetos. Para tanto, a presente tese foi dividida em duas etapas. A primeira etapa organizada em três experimentos teve como objetivo avaliar diferentes métodos de seleção espermática. No primeiro experimento, o efeito da centrifugação para remoção do plasma seminal antes do swim-up foi avaliado sobre a qualidade espermática. Neste experimento, o swim-up reduziu a motilidade (51,9 vs. 90,4%) e aumentou os níveis de espécies reativas de oxigênio em comparação com o controle (não selecionado), tendo a centrifugação prejudicado ainda mais a qualidade espermática. Este método não foi recomendado para catetos. No segundo experimento, as densidades do gradiente Percoll® (PG 45–90% e PG 35–70%) foram comparadas sobre os parâmetros de qualidade espermática. PG 45–90% melhoraram a velocidade média do trajeto (VAP; 96,8 vs. 69,7 µm/sec), velocidade linear (VSL; 88,4 vs. 56,0 µm/sec) e linearidade (LIN; 66,0 vs. 46,0%) em comparação ao grupo controle, enquanto PG 35–70% afetaram negativamente a motilidade e qualidade espermática. No terceiro experimento, o efeito do PG 45–90% e da lavagem por centrifugação (LC) foi avaliado sobre a cinética de motilidade espermática e fertilização heteróloga usando oócitos suínos. PG 45–90% melhoraram alguns parâmetros cinéticos de motilidade em comparação aos grupos controle e LC. Contudo, as taxas de clivagem (26,3 e 25,2%) e mórulas (10,5 e 8,1%) não diferiram entre os grupos. Assim, PG 45–90% e LC foram eficientes no isolamento de espermatozoides móveis de catetos, os quais foram capazes de fertilizar oócitos suínos e promover o desenvolvimento de embriões híbridos. Já a segunda etapa organizada em dois experimentos teve como objetivo determinar as condições de capacitação espermática, por meio da comparação do meio Tyrode albumina lactato piruvato (TALP) na ausência e na presença de heparina, cafeína e sua combinação por diferentes tempos de incubação (1, 3 e 6 h). No primeiro experimento, a influência das condições de capacitação foi avaliada sobre a qualidade espermática. Os parâmetros de motilidade progressiva (48,6 vs. 32,3%), retilinearidade (71,1 vs. 64,6%), LIN (40,7 vs. 35,2%) e espermatozoides rápidos (51,7 vs. 35,6%) foram superiores usando TALP com heparina em comparação ao TALP com cafeína. O meio TALP com heparina ou cafeína aumentaram a taxa de capacitação espermática em comparação com o TALP (75,5 vs. 75,9 vs. 55,0%, respectivamente), sendo a porcentagem máxima de capacitação alcançada após 3 h de incubação (77,5%). Adicionalmente, os espermatozoides submetidos à capacitação com heparina apresentaram menor estresse oxidativo após 1 h de incubação. Portanto, espermatozoides de catetos podem ser capacitados com cafeína ou heparina; mas a heparina melhorou a motilidade espermática, sendo recomendada para a capacitação espermática. No segundo experimento, a funcionalidade de espermatozoides capacitados em TALP com heparina foi avaliada comparando os tempos de incubação de 3 e 6 h. Não houve diferença entre os tempos de incubação na média de espermatozoides ligados à zona pelúcida de oócitos suínos (3 h: 47,0 vs. 6 h: 71,1 espermatozoides/oócito). Portanto, a incubação de gametas em TALP com heparina por 3 h pode ser eficiente para FIV na espécie. Em síntese, as condições de seleção e capacitação espermática foram estabelecidas e representam um avanço significativo para o desenvolvimento da FIV em catetos, permitindo sua utilização para a conservação e emprego comercial da espécie.

Palavras-chave: mamíferos silvestres, fertilização *in vitro*, processamento espermático, espermatozoides, agentes capacitantes.

ABSTRACT

The embryo production by *in vitro* fertilization (IVF) enables a scientific, conservationist and commercial approach to collared peccaries, wild mammals of biological importance and with market potential. However, the efficiency of IVF is directly influenced by variables that until now had not been established in collared peccaries. Thus, the aim was to improve sperm processing for IVF in peccaries. Therefore, this thesis was divided into two stages. The first stage, organized into three experiments, aimed to evaluate different sperm selection methods. In the first experiment, the effect of centrifugation to remove seminal plasma before the swim-up on sperm quality was evaluated. In this experiment, swim-up decreased motility (51.9 vs. 90.4%) and increased levels of reactive oxygen species compared to control (unselected), with centrifugation further impairing sperm quality. This method is not recommended for collared peccaries. In the second experiment, Percoll® gradient densities (PG 45–90% and PG 35–70%) were compared on sperm quality parameters. PG 45–90% improved velocity average pathway (VAP; 96.8 vs. 69.7 µm/sec), velocity straight line (VSL; 88.4 vs. 56.0 µm/sec) and linearity (LIN; 66.0 vs. 46.0%) compared to the control group, while PG 35–70% negatively affected sperm motility and quality. In the third experiment, the effect of PG 45–90% and washing by centrifugation (WC) was evaluated on sperm motility kinetics and heterologous fertilization using porcine oocytes. PG 45–90% improved some kinetic parameters of motility compared to control and WC groups. However, cleavage (26.3% and 25.2%) and morulae (10.5% and 8.1%) rates did not differ between groups. Thus, PG 45–90% and WC were efficient in isolating motile collared peccary sperm, which were able to fertilize porcine oocytes and promote the development of hybrid embryos. The second stage, organized into two experiments, aimed to determine the ideal conditions for sperm capacitation, by comparing Tyrode's albumin lactate pyruvate (TALP) medium in the absence and presence of heparin, caffeine and their combination for different incubation times (1, 3 and 6 h). In the first experiment, the influence of capacitating conditions was evaluated on sperm quality. Progressive motility parameters (48.6 vs. 32.3%), straightness (71.1 vs. 64.6%), LIN (40.7 vs. 35.2%) and rapid sperm (51.7 vs. 35.6%) were superior using TALP with heparin compared to TALP with caffeine. The TALP medium with heparin or caffeine increased the sperm capacitation rate compared to TALP (75.5 vs. 75.9 vs. 55.0%, respectively), with the maximum percentage of capacitation achieved after 3 h of incubation (77.5%). Additionally, sperm subjected to capacitation with heparin showed lower oxidative stress after 1 h of incubation. Therefore, collared peccary spermatozoa can be capacitated with caffeine or heparin; but heparin improved sperm motility, being recommended for sperm capacitation. In the second experiment, the functionality of spermatozoa capacitated in TALP with heparin was evaluated by comparing the incubation times of 3 and 6 h. There was no difference between incubation times in the average of spermatozoa bound to the zona pellucida of porcine oocytes (3 h: 47.0 vs. 6 h: 71.1 spermatozoa/oocyte). Therefore, incubation of gametes in TALP with heparin for 3 h may be efficient for IVF in this species. In summary, the conditions for sperm selection and capacitation were established and represent a significant advance for the development of IVF in peccaries, allowing its use for the conservation and commercial use of the species.

Keywords: wild mammals, *in vitro* fertilization, sperm processing, spermatozoa, capacitating agents.

LISTA DE FIGURAS

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

CAPÍTULO 2 – EMBRYO PRODUCTION BY *IN VITRO* FERTILIZATION IN WILD UNGULATES – PROGRESS AND PERSPECTIVES

CAPÍTULO 3 – EFFECTS OF DIFFERENT SELECTION TECHNIQUES ON PHYSIOLOGICAL AND FUNCTIONAL QUALITY OF SPERM COLLECTED FROM COLLARED PECCARIES

CAPÍTULO 4 – EFFECTS OF CAPACITATING MEDIA AND INCUBATION TIME ON COLLARED PECCARY SPERM QUALITY, ACROSOME REACTION, AND FERTILIZING ABILITY

LISTA DE QUADRO

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

LISTA DE TABELAS

CAPÍTULO 2 – EMBRYO PRODUCTION BY *IN VITRO* FERTILIZATION IN WILD UNGULATES – PROGRESS AND PERSPECTIVES

CAPÍTULO 3 – EFFECTS OF DIFFERENT SELECTION TECHNIQUES ON PHYSIOLOGICAL AND FUNCTIONAL QUALITY OF SPERM COLLECTED FROM COLLARED PECCARIES

CAPÍTULO 4 – EFFECTS OF CAPACITATING MEDIA AND INCUBATION TIME ON COLLARED PECCARY SPERM QUALITY, ACROSOME REACTION, AND FERTILIZING ABILITY

LISTA DE ABREVIATURAS E SIGLAS

° C	Grau Celsius
ALH	Amplitude of the lateral head
ANOVA	Variance analysis
ARTs	Assisted Reproductive Techniques
Av.	Avenida
BCF	Beat cross frequency
BSA	Bovine Serum Albumin
Ca^{2+}	Íon cálcio
cAMP	3’,5’ cyclic adenosine monophosphate
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CASA	Computer-assisted sperm assessment system
CEUA	Committee for Ethics in Animal Use
cm	Centímetro
CMXRos	MitoTracker Red®
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO_2	Dióxido de carbono
COC	*Cumulus*-oocyte complex
CTC	Chlortetracycline
D0	Day 0
D3	Day 3
D7	Day 7
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
et al.	E outros
FBS	Fetal bovine serum
Fig.	Figure
FSH	Follicle-stimulating hormone
g	G force
G	Gauge
g	Grama
GSH	Glutathione
h	Hour
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate

H_2O_2	Hydrogen peroxide
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis
ICMBio	Instituto Chico Mendes de Conservação da Biodiversidade
ICSI	Intracytoplasmic sperm injection
IU	International units
IUCN	International Union for Conservation of Nature
IVC	*In vitro* embryo culture
IVEP	*In vitro* embryo production
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
L	Litro
LC	Lavagem por centrifugação
LH	Luteinizing hormone
LIN	Linearity
M	Mol
mg	Miligrama
MII	Metaphase II
min	Minute
MIV	Maturação *in vitro*
mL	Mililitro
mm	Milímetro
mM	Milimolar
n	Número
P	Probabilidade de significância
PBS	Phosphate buffered saline
PG	Percoll gradient
pH	Hydrogen potential
PI	Propidium iodide
RN	Rio Grande do Norte
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SE	Standard error
SOF	Synthetic Oviductal Fluid
STR	Straightness
TALP	Tyrode's albumin lactate pyruvate

TALP-Caf	Tyrode's albumin lactate pyruvate and caffeine
TALP-CH	Tyrode's albumin lactate pyruvate, caffeine and heparin
TALP-Hep	Tyrode's albumin lactate pyruvate and heparin
TCM	199 Medium 199
UFERSA	Universidade Federal Rural do Semi-Árido
VAP	Velocity average pathway
VCL	Curvilinear
vs.	Versus
VSL	Velocity straight line
WC	Washing by centrifugation
µg	Micrograma
µL	Microlitro
µm	Micrometro
µM	Micromolar

LISTA DE SÍMBOLOS

@	Arroba
®	Marca registrada
%	Porcentagem
+	Soma
<	Menor que
=	Igual
>	Maior que
±	Mais ou menos
×	Multiplicação

SUMÁRIO

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1 INTRODUÇÃO

A produção de embriões por fertilização *in vitro* (FIV) tem sido útil não somente para o aumento da produtividade dos rebanhos (CHEN et al., 2022), mas também para a conservação da biodiversidade (HILDEBRANDT et al., 2018) e para a investigação fisiológica do desenvolvimento embrionário (WU et al., 2022). Devido a sua ampla aplicabilidade, essa estratégia vem sendo desenvolvida em mamíferos silvestres, sendo uma ferramenta para conservação e reprodução de importantes espécies, tais como os catetos. Esses animais possuem um significativo papel biológico, bem como possuem um potencial comercial interessante, tornando essencial o desenvolvimento adequado da FIV na espécie (MORAIS et al., 2022a).

No intuito de fomentar a reprodução de catetos, fornecendo estratégias para sua multiplicação em cativeiros, dois grupos de pesquisas da Universidade Federal Rural do Semi-Árido têm desenvolvido estratégias reprodutivas na espécie. Especificamente para a FIV, nossa equipe tem contribuído com pesquisas relativas à recuperação de oócitos imaturos, maturação *in vitro* de oócitos (BORGES et al., 2018, BORGES et al., 2020) e cultivo *in vitro* de embriões (BORGES et al., 2020). Além disso, a equipe do Laboratório de Conservação de Germoplasma Animal tem estabelecido protocolos de coleta (SOUZA et al., 2009), refrigeração (SOUZA et al., 2015), e criopreservação de sêmen (CASTELO et al., 2010a, b). Agora, diante desses passos já realizados, nosso objetivo foi desenvolver as condições de seleção e capacitação espermática, visando a produção de embriões nesta espécie.

Em geral, estudos sobre a fisiologia e as técnicas de reprodução assistida em catetos têm indicado que não é apropriado o emprego de protocolos usados em outras espécies sem estabelecimento prévio (SILVA et al., 2017). Assim, para a seleção espermática, a comparação de diferentes protocolos (swim-up, gradiente de Percoll e lavagem por centrifugação) permitiria a obtenção de um sistema de isolamento de espermatozoides móveis adequado para a FIV. Associado a isso, outros fatores podem influenciar o sucesso da FIV, como a escolha do agente capacitante e o tempo de incubação, especialmente quando em suínos, espécie doméstica filogeneticamente próxima aos catetos, há a influência destes fatores sobre a polispermia (OBERLENDER et al., 2016).

Portanto, o objetivo foi desenvolver estratégias de seleção e capacitação espermática para a produção de embriões por FIV em catetos.

2 REVISÃO DE LITERATURA

2.1 Aspectos biológicos e perspectivas comerciais dos catetos

Os catetos (**Figura 1**), também conhecidos como caititus ou porcos-do-mato, são artiodátilos de médio porte, com peso em torno de 15–28 kg, comprimento de 84–106 cm e altura de 30–50 cm (TABER et al., 2011). Esse mamífero pertence a subordem Suina e a família Taiaçuídea (DESBIEZ et al., 2012). Os catetos são amplamente distribuídos no continente americano, onde podem ser encontrados em diversos habitats, abrangendo múltiplas condições climáticas e intensidades de presença humana, em virtude de adaptações fisiológicas, comportamentais e dieta variada (MARTÍNEZ-GUTIÉRREZ et al., 2017; SILVEIRA; PACHECO, 2018). Nesses habitats, os catetos vivem em grupos familiares mistos e estáveis, com adultos e juvenis de ambos os sexos (ROMERO et al., 2013). Eles apresentam importante papel biológico na manutenção dos ecossistemas como dispersores de sementes (PÉREZ-IRINEO; SANTOS-MORENO, 2016) e presas de grandes carnívoros (SCOGNAMILLO et al., 2003), podendo ser considerados indicadores de qualidade ambiental (DESBIEZ et al., 2012).

Figura 1. Catetos pertencentes ao Centro de Multiplicação de Animais Silvestres, da Universidade Federal Rural do Semi-Árido.

De acordo com a União Internacional para a Conservação da Natureza (IUCN), os

catetos são classificados internacionalmente como uma espécie menos preocupante (GONGORA et al., 2011). Contudo, ao contrário de outros biomas brasileiros, na Mata Atlântica, o cateto é classificado como quase ameaçado. Já em nosso bioma Caatinga, onde seu quantitativo populacional requer atenção, as principais ameaças incluem construção de parques eólicos, alteração de habitat, caça, retaliações, fogo e mineração (DESBIEZ et al., 2011).

Neste cenário, estratégias de conservação *in situ* (SILVEIRA; PACHECO, 2018) e *ex situ* (MOREIRA et al., 2022) têm sido desenvolvidas visando reduzir maiores perdas populacionais. Além dos catetos, outras duas espécies pertencem a família Tayassuidae: queixada (*Tayassu pecari* Link, 1795), o qual também ocorre no Brasil e é classificado como vulnerável; e taguá (*Catagonus wagneri* Rusconi, 1930) que está em risco de extinção e é encontrado no Paraguai, Bolívia e Argentina. Tal informação pode inferir que o cateto poderia também ser empregado como modelo experimental destas espécies da mesma família, os quais possuem um quantitativo populacional bem mais reduzido.

No que diz respeito aos aspectos comerciais, o consumo de carne silvestre é uma prática comum em algumas comunidades do Brasil, onde esse produto pode ser encontrado em mercados locais ou obtido por meio da caça (EL BIZRI et al., 2020). Nesse sentido, os catetos representam uma importante fonte de proteína para algumas comunidades e são visados para a caça em virtude do interesse em sua carne e couro, que apresentam ótima qualidade e potencial de mercado (BODMER, 1990; NUNES et al., 2019). Na região Norte do Brasil, os catetos são frequentemente caçados para consumo de subsistência, mas em outras regiões do Brasil, como no Sudeste, esta carne é comercializada como exótica para preparações gastronômicas (MORAIS et al., 2022).

Contudo, o consumo de produtos provenientes de caça pode incentivar o comércio ilegal, que associado ao desmatamento e a fragmentação de habitats, causam a diminuição populacional das espécies (SANTOS et al., 2009). Por essa razão, a criação de mamíferos silvestres em escala comercial pode ser considerada uma estratégia de conservação, pois aumenta o estoque populacional e diminui a pressão da caça e do tráfico, bem como representa uma alternativa para geração de renda para produtores rurais (ALBUQUERQUE et al., 2016).

Os catetos apresentam todas as características necessárias para que sejam criados em cativeiro, ou seja, não estão na lista de animais ameaçados ou em risco de extinção e são capazes de se adaptar, crescer e se reproduzir nesse sistema de criação (ALBUQUERQUE et al., 2016). Esses animais têm boa produtividade em cativeiro, com uma média de 1,03 nascimentos/fêmea/ano e 1,85 crias/fêmea/ano (MAYOR et al., 2007). Essa espécie é onívora na natureza, mas em cativeiro se adapta bem a ração comercial para suínos, que pode ser complementada com grãos, frutas, vegetais, raízes e forragens (ALBUQUERQUE et al., 2002;

NOGUEIRA FILHO, 1999). Além disso, o manejo dos catetos é previsto pelo Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) pelo Decreto no. 6.099, de 26 de abril de 2007 (no. 02001.005418/2007-11).

Portanto, diante do interesse comercial de produtos derivados do cateto no mercado nacional e internacional, a criação em cativeiro nos locais onde esses animais são encontrados em maior quantidade pode ser usada de forma econômica e sustentável (MAYOR et al., 2007; SANTOS et al., 2009). Existem poucos registros de criação comercial de catetos, especialmente em virtude do baixo retorno econômico ocasionado por desconhecimento do mercado e das práticas de manejo mais adequadas para o desenvolvimento desses animais (NOGUEIRA FILHO et al., 2014).

No Brasil, mais especificamente no Mercado Municipal de São Paulo, a carne crua de cateto é comercializada por aproximadamente R$ 110,00/kg (US$ 20,68/kg), o que significa um custo três vezes maior em comparação a carne suína (MORAIS et al., 2022a). No mercado internacional, o couro apresenta valor elevado na Europa, onde um par de luvas tem sido vendido entre €150 e €500 (SANTOS et al., 2009; ALBUQUERQUE et al., 2016). Além disso, estima-se que os produtos oriundos do cateto podem ser mais valorizados com o aumento do seu desempenho zootécnico (NOGUEIRA FILHO et al., 2014). Adicionalmente, a carne de cateto possui excelente qualidade de sabor e textura, alto teor de proteínas (20,96%) e baixo teor de lipídios totais (6,7–7,0%), que gera interesse para o desenvolvimento de novos alimentos (MORAIS et al., 2022a). Morais et al. (2022b) desenvolveram salsichas frescas usando carne de cateto com adição de fibras dietéticas que tiveram uma ótima aceitação dos consumidores (87%) e alta intenção de compra (4 de uma escala de 1–5).

Nesse sentido, torna-se interessante o desenvolvimento de estudos para conhecer esta espécie, visando aumentar sua produtividade em cativeiro e incrementar seu interesse comercial, permitindo uma abordagem científica e conservacionista. Assim, pesquisas voltadas para conhecer e aprimorar a reprodução de catetos são de grande relevância, especialmente para animais nascidos e criados em cativeiros (SILVA et al., 2017).

2.2 Aspectos reprodutivos dos catetos

Diferentes estudos têm sido desenvolvidos em catetos visando conhecer a fisiologia reprodutiva de machos (PEIXOTO et al., 2016) e fêmeas (SILVA et al., 2016). Esse conhecimento é o primeiro passo para o desenvolvimento das Técnicas de Reprodução Assistida (TRAs), uma vez que permite buscar similaridades com espécies domésticas que apresentam protocolos estabelecidos para TRAs. Em catetos, a espécie doméstica

filogeneticamente mais próxima é a suína, que embora seja de uma família distinta pode ser usada para fins de comparação como modelo inicial (**Figura 2**). Por outro lado, similaridades reprodutivas entre catetos e ruminantes têm sido observadas e podem ser úteis no desenvolvimento das TRAs (MOREIRA et al., 2019).

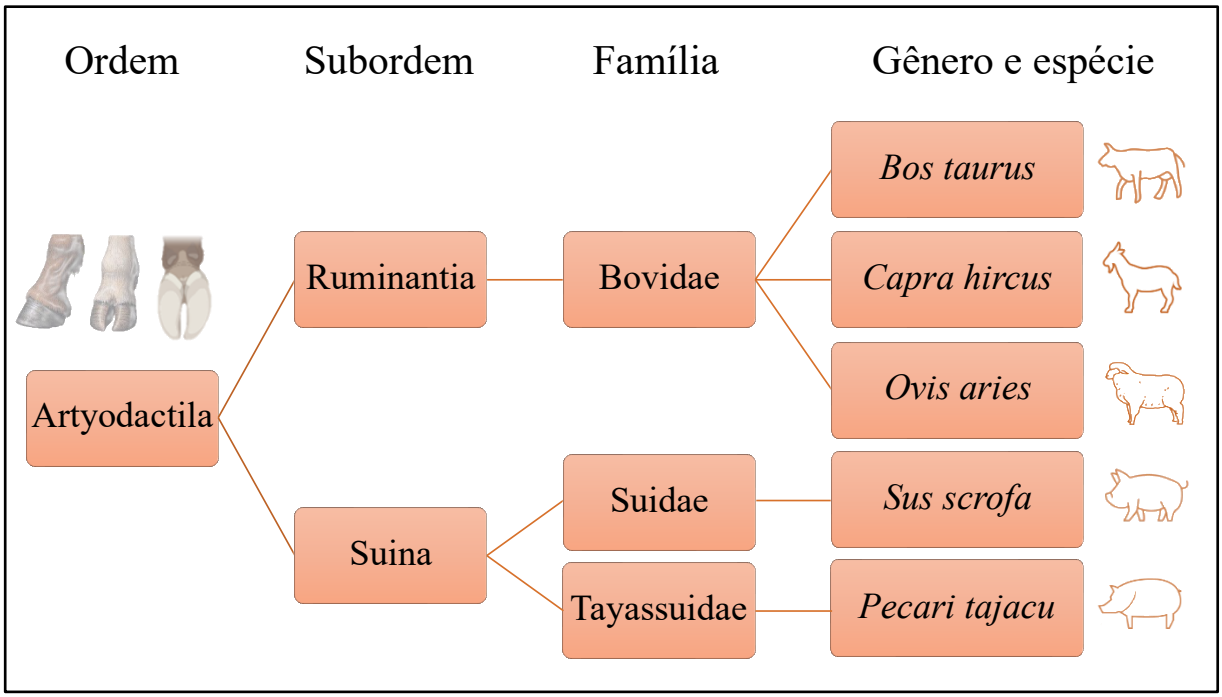

Figura 2. Taxonomia de catetos e espécies domésticas filogeneticamente próximas, tais como suínos, bovinos, caprinos e ovinos.

Inicialmente, a vida reprodutiva de catetos machos e fêmeas começa entre 8 e 16 meses de idade sem época preferencial para a reprodução (GARCIA et al., 2009). Nas fêmeas, o período de gestação tem duração média de 145 dias, sendo observados um ou duas crias por parto, sendo a taxa de ovulação de aproximadamente dois oócitos por ciclo (MAYOR et al., 2004). A duração do ciclo estral é em média 21,0 dias, sendo em média 6 dias para a fase estrogênica e 15 dias para a fase progesterônica (MAIA et al., 2014a).

Já a população estimada de folículos pré-antrais em cada ovário é de 33273,45, sendo a maioria dos folículos classificados como primordiais (91,56%), seguido dos primários (6,29%) e dos secundários (2,15%) (LIMA et al., 2013). Em relação aos folículos antrais, o número médio de folículos totais por fêmea na fase folicular do ciclo estral é de 30,4, dos quais 10,8 são pequenos (0,5 mm), 14,4 médios (< 2,5 mm) e 5,2 grandes (>2,5 mm) (MAYOR et al., 2006). Quando às fêmeas foram estimuladas hormonalmente com PG600®, o número médio de folículos antrais totais variou de 25,0 a 35,3 folículos/fêmea, evidenciando que o protocolo de estimulação ainda deve ser otimizado (VIANA et al., 2020).

Além destas pesquisas voltadas para a caracterização reprodutiva das fêmeas (LIMA et al., 2013), estudos foram desenvolvidos quanto às biotécnicas de monitoramento do estro (MAIA et al., 2014a), sincronização do estro (MAIA et al., 2014b), inseminação artificial (PEIXOTO et al., 2019), criopreservação de tecido ovariano (LIMA et al., 2019), estimulação ovariana (VIANA et al., 2020) e cultivo de folículos pré-antrais (CAMPOS et al., 2021). Todos estes estudos têm enfatizado a necessidade de mais investigações quanto à reprodução de catetos. Adicionalmente, uma vez que estes trabalhos foram realizados utilizando a espécie suína como modelo para os protocolos, foi possível concluir que apesar de algumas similaridades, não se deve extrapolar o conhecimento acerca dos suínos para os catetos (CAMPOS et al., 2019).

No que se refere ao macho, o tamanho dos testículos é de aproximadamente 5,4 cm de comprimento, 2,8 cm de largura e 2,6 cm de espessura (SONNER et al., 2004), bem como o peso testicular médio é de 23,7 g, onde 12,8% do parênquima testicular é ocupado por células de Leyding (COSTA et al., 2010). A espermatogênese dos catetos é considerada de alta eficiência na produção espermática, apresentando um ciclo espermatogênico com duração aproximada de 12,3 dias e a produção diária é em torno de $23,4 \times 10^6$ espermatozoides/g de testículo (COSTA et al., 2010). O sêmen de catetos possui três frações, sendo uma pobre em células, constituída de secreção das glândulas acessórias, uma rica em células espermáticas, e uma fração gel (GARCIA et al., 2009). Os espermatozoides de cateto (**Figura 3**) apresentam cabeça com forma discretamente alongada, com a base mais fina do que o ápice, sendo a cauda inserida na região posterior. A cabeça tem comprimento e largura média de $6,34 \times 4,20$ µm, enquanto o comprimento da peça intermediaria é de 13,29 µm e da cauda é de 32,25 µm, totalizando em média 50,69 µm de comprimento (SOUSA et al., 2013).

Diversos estudos foram realizados visando a caracterização reprodutiva dos machos e o desenvolvimento de TRAs, tais como a colheita de sêmen (CASTELO et al., 2010a), métodos de análise da qualidade do sêmen (SOUSA et al., 2013), criopreservação de espermatozoides (SOUZA et al., 2016), resfriamento (SANTOS et al., 2021) e criopreservação de tecido testicular (SILVA et al., 2021). Tal cenário tem reforçado que a cada passo e novo conhecimento adquirido sobre a fisiologia reprodutiva dos catetos, mais pesquisas podem progredir para técnicas mais avançadas, tais como a manipulação de gametas para produção de embriões por FIV.

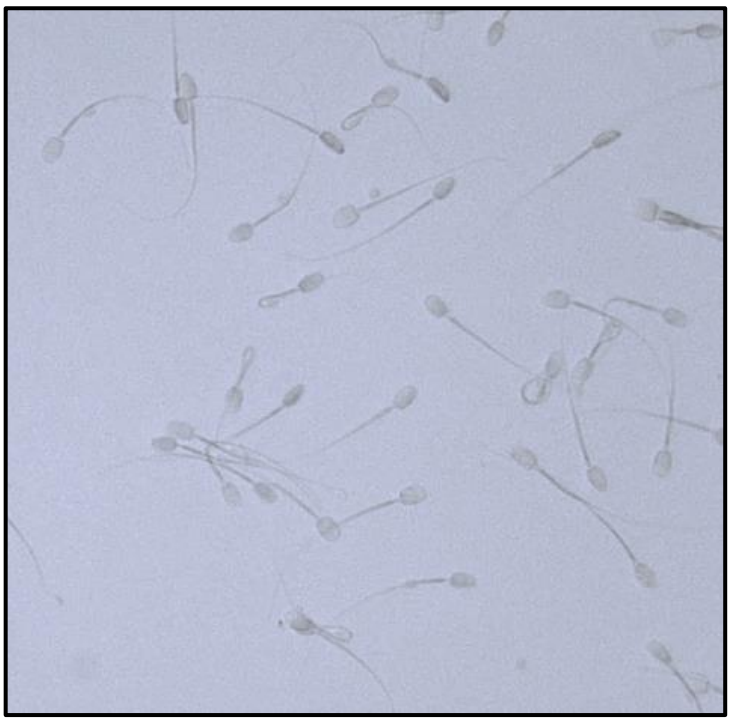

Figura 3. Espermatozoides de cateto visualizados sob microscopia de campo claro (40×).

2.2.1 Estado da arte da manipulação *in vitro* de oócitos derivados de folículos antrais de catetos

Em catetos, os primeiros ensaios de colheita de oócitos imaturos foram realizados com ovários derivados de fêmeas *post-mortem* não estimuladas, usando um sistema de aspiração de seringa (5 mL) e agulha 21G (BORGES et al., 2018). Neste estudo, uma média de 6,6 oócitos foram recuperados por fêmea, dos quais 3,6 foram considerados viáveis. Posteriormente, Borges et al. (2020) demonstraram que a estimulação hormonal ovariana com PG600® (400,00 UI de gonadotrofina sérica da égua prenhe e 200,00 UI de gonadotrofina coriônica humana) possibilitou uma maior taxa de recuperação de oócitos viáveis (**Figura 4**), ou seja, 16,2 oócitos por fêmea e 12,1 oócitos viáveis por fêmea.

Além disso, estudos foram realizados visando o estabelecimento das condições de manipulação de oócitos para produção *in vitro* de embriões. Uma vez que o ponto de partida para a maioria das biotécnicas reprodutivas *in vitro* é a maturação oocitária, inicialmente foi avaliado o tempo de maturação (24 vs. 48 h) desses oócitos (BORGES et al., 2018). No estudo, oócitos apresentaram melhores taxas de expansão das células do *cumulus* (100% vs. 46,2%) e maturação nuclear (presença de placa metafásica: 76,9% vs. 19,0%) após 48 h de maturação (BORGES et al., 2018).

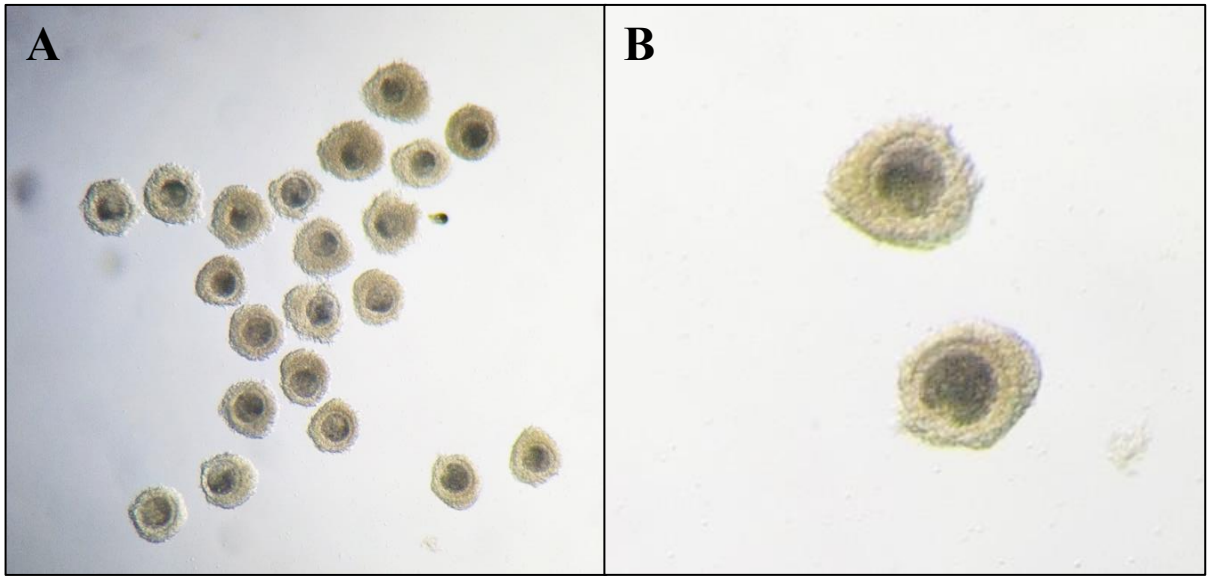

Figura 4. Ovários imaturos derivados de catetos adultos. **(A)** Oócitos visualizados em 10×. **(B)** Oócitos visualizados em 20×.

Posteriormente, pesquisas quanto ao meio de maturação mostraram que a utilização do fator de crescimento epidermal (EGF) não influenciou o desenvolvimento meiótico em oócitos de catetos, mas teve efeito positivo sobre os parâmetros morfométricos oocitários (BORGES et al., 2020). Além disso, oócitos são capazes de se desenvolver como embriões de qualidade em meio fluido sintético do oviduto (SOF) após ativação artificial, alcançando 24% de blastocistos (BORGES et al., 2020). Apesar desses resultados ainda poderem ser otimizados, com essas informações já seria possível a realização da produção *in vitro* de embriões por FIV. Contudo, o maior desafio em relação a isso ainda é a disponibilidade dos gametas, uma vez que a colheita de oócitos de catetos *in vivo* ainda não foi descrita.

2.2.2 Estado da arte da manipulação de espermatozoides de catetos *in vitro*

Os primeiros passos de manipulação de espermatozoides *in vitro* de catetos foram realizados a partir do estabelecimento da eletroejaculação como metodologia para obtenção de sêmen (SOUZA et al., 2009; CASTELO et al., a,b). A partir destes estudos, aspectos seminais puderam ser descritos, sendo observado em dez animais os valores médios: 3,5 mL de volume, $0,8 \times 10^9$ espermatozoide/mL, 4,2 de vigor (escala de 1–5), 85,1% de motilidade, 79,9% de morfologia normal, e 86,3% de viabilidade (PEIXOTO et al., 2012). Outras técnicas foram ainda desenvolvidas para obtenção de espermatozoides, tais como a coleta a partir da cauda do epidídimo, a qual permitiu a obtenção de 57,8% de espermatozoides móveis, com vigor de 2,9 e viabilidade de 68,2% (BEZERRA et al., 2014).

Paralelamente, ao longo dos anos, técnicas para análise da qualidade espermática, permitindo uma maior acurácia na comparação de técnicas de manipulação do sêmen e desenvolvimento de TRAs foram desenvolvidas. Assim, métodos de avaliação da integridade funcional da membrana por meio do teste hiposmótico (SANTOS et al., 2013), análise morfológica e morfométrica (SOUSA et al., 2013), concentração espermática (SILVA et al., 2014), integridade da membrana usando fluoróforos (SOUZA et al., 2016), e teste de ligação dos espermatozoides de catetos a substratos heterólogos, tais como oócitos suínos domésticos e membrana perivitelina da gema de ovo de galinha (CAMPOS et al., 2017) foram aperfeiçoados e validados para catetos.

A criopreservação de espermatozoides foi realizada para a espécie inicialmente usando protocolos desenvolvidos em suínos, os quais posteriormente foram aperfeiçoados para atender as especificidades da espécie (CASTELO et al. 2010a,b). Em geral, as condições de criopreservação estabelecidas foram: diluente à base de Tris acrescido de frutose ou glicose (CASTELO et al., 2010b), podendo ser suplementado com 20% de gema de ovo (ALVES et al., 2013), lipoproteínas de baixa densidade (SOUZA et al., 2015) ou extrato de *Aloe vera* (SOUZA et al., 2016) como crioprotetores associados ao glicerol. Os trabalhos mais recentes utilizaram Tris-frutose, 20% de gema de ovo e 3% de glicerol, e estabeleceram o uso de 70 µg/mL do antibiótico gentamicina (MOREIRA et al., 2022) e 0,1% do detergente dodecilsulfato de sódio (MOREIRA et al., 2023).

Contudo, a criopreservação foi associada a um aumento na quantidade de vesículas acrossomais que podem estar relacionadas à criocapacitação, a qual consiste em uma aceleração no processo de capacitação provocada pelo procedimento de criopreservação (BEZERRA et al., 2018). Ainda, por meio de microscopia de transmissão, o mesmo estudo observou distensões na membrana e perda de mitocôndrias que são características que podem afetar negativamente a eficiência da FIV. Adicionalmente, Maia et al. (2018) observaram que as condições ambientais não afetaram a qualidade do sêmen fresco; contudo, a congelabilidade espermática foi afetada durante o período seco no clima semiárido, ocasionando diminuição na qualidade espermática após descongelação.

Além disso, espermatozoides de catetos demonstraram sensibilidade à centrifugação e baixa longevidade após criopreservação (CAMPOS et al., 2014). Castelo et al. (2010a) observaram que a centrifugação (3000 rpm/3 min em solução de descongelação de Beltsville) antes ou após a congelação/descongelação de sêmen reduziu a motilidade espermática e aumentou os defeitos morfológicos. Bezerra et al. (2014) observaram uma diminuição na viabilidade de espermatozoides frescos obtidos do epidídimo após centrifugação a 3×g por 10 min.

Portanto, considerando esses aspectos inerentes da espécie, é clara a necessidade de estabelecer cuidadosamente as condições de manipulação e preparação dos espermatozoides para a FIV. Campos et al. (2017) demonstraram que espermatozoides frescos de catetos foram capazes de se ligar adequadamente a zona pelúcida de oócitos imaturos suínos, bem como penetrá-los. Esse resultado tem sido importante, pois mostrou que oócitos suínos podem ser usados para avaliação da fertilidade espermática a partir de FIV heteróloga.

2.3 Catetos e espécies filogeneticamente próximas

Uma vez que os catetos são mamíferos silvestres onde a disponibilidade de amostras é limitada, ter modelos experimentais para guiar o desenvolvimento de protocolos auxilia no uso mais assertivo das amostras disponíveis. Neste cenário, considerando a filogenia dos catetos em comparação à espécies domésticas, é possível afirmar que os catetos são mais próximos aos suínos, seguidos dos ruminantes (caprinos, ovinos e bovinos). Por essa razão, alguns estudos consideram o modelo suíno para catetos. Contudo, os resultados têm demonstrado que em alguns aspectos esses animais têm mais similaridades com ruminantes, bem como apresentam características exclusivas (**Quadro 1**).

Quadro 1. Características reprodutivas gerais de catetos, suínos, caprinos, ovinos e bovinos.

Característica	**Cateto[a]**	**Suíno**	**Caprino**	**Ovino**	**Bovino**
Número de cromossomos	30	38	60	54	60
		Fêmea[b]			
Maturidade sexual (meses)	8–14	5–7	6–8	6–8	12–15
Duração do ciclo estral (dias)	21–28	19–21	21	16–17	21–22
Taxa de ovulação (folículos)	1–2	11–24	1–2	1–2	1
Período de gestação (dias)	145	114	150	148	278
		Machos[c]			
Maturidade sexual (meses)	11	5–7	6–8	6–8	9–12
Ciclo espermatogênico (dias)	12,3	8,6	10,6	10,6	13,5
Espermatogênese completa (dias)	55,1	38,7	47,7	47,7	60,8
Produção diária espermática (espermatozoide/g de testículo) × 10^6	23,4	24–27	30	22,8[d]	11–13

[a]Revisado por Garcia et al. (2009). [b]Hafez e Hafez (2000). [c]Revisado por França et al. (2005).

[d]Cardoso; Queiroz (1988).

A população de folículos primordiais de catetos é muito baixa (33 273,45 por ovário) quando comparada aos valores relatados para suínos (420 000 por par de ovários), provavelmente em virtude da diferente taxa de ovulação e recrutamento folicular entre essas espécies (GOSDEN; TELFER, 1987; MAYOR et al., 2006). Por outro lado, em caprinos, um número semelhante foi relatado, os quais apresentam 32 204 folículos primordiais por ovário (LUCCI et al., 1999). Nos oócitos, assim como em suínos, foi observada a presença de gotas lipídicas citoplasmáticas (LIMA et al., 2013), bem como o tempo de 48 h de maturação *in vitro* foi mais eficiente, semelhante ao tempo de suínos (BORGES et al., 2018).

Em geral, a espermatogênese dos catetos é considerada muito semelhante à dos suínos, os quais apresentam similaridades histológicas em várias regiões do epidídimo, mas em catetos são encontrados muitos espermatozoides imaturos na cabeça do epidídimo, diferentemente dos suínos (OLIVEIRA et al., 2016). Já a composição do plasma seminal do cateto é diferente dos suínos e tem semelhanças com bovinos e ovinos em termos de concentração de cálcio e magnésio (MOREIRA et al., 2019).

A sensibilidade dos espermatozoides de catetos à centrifugação e criopreservação é semelhante a espécie suína (CASTELO et al., 2010a; DEORI et al., 2022). Por outro lado, protocolos de armazenamento de sêmen específicos para ruminantes são mais eficazes em catetos do que os protocolos adaptados de suínos (CASTELO et al., 2010b; SOUZA et al., 2015). Além disso, os catetos apresentam uma expressão distintamente alta de isoformas de clusterina e uma quantidade menor de espermadesinas no plasma seminal em comparação a suínos (SANTOS et al., 2014). Portanto, embora não seja possível extrapolar os protocolos de espécies domésticas para catetos, ainda assim a experiência com tais espécies pode contribuir para o desenvolvimento dos protocolos para esse mamífero silvestre.

2.4 Estratégias para o desenvolvimento da FIV em catetos

Diante de características dos espermatozoides de catetos, bem como conhecendo as similaridades desse mamífero com suínos e ruminantes, foram traçadas algumas estratégias para o estabelecimento da FIV. Em geral, a eficiência da FIV depende das etapas de seleção e capacitação espermática, bem como da co-incubação com oócitos (OHLWEILER et al., 2020). Nesse sentido, a sensibilidade à centrifugação e baixa longevidade após condições de estresse observada em espermatozoides de catetos são características que merecem atenção (CASTELO et al., 2010a; CAMPOS et al., 2014). Isso porque estes são aspectos importantes na definição

dos protocolos de seleção espermática para a FIV. Essa etapa é essencial na separação de gametas mais competentes e de melhor qualidade (MATÁS et al., 2011) e, para tanto, o swim-up, gradiente de Percoll e a lavagem por centrifugação são técnicas frequentemente empregadas em suínos e ruminantes (CRUZ et al., 2016; OHLWEILER et al., 2020), sendo observados resultados distintos com a utilização dos métodos nessas espécies (ARIAS et al., 2017). Portanto, tais técnicas apresentam vantagens e desvantagens, bem como diferentes protocolos de execução, sendo necessário estudos que identifiquem a melhor eficiência para espermatozoides de catetos.

Após a seleção, para garantir a adequada interação oócito-espermatozoide é imprescindível que ocorra a capacitação espermática que resulta na hiperativação da motilidade e aquisição da capacidade para sofrer a reação acrossômica (HARRISON; GADELLA, 2005; SALICIONI et al., 2007). Assim, para estimular a capacitação, alguns suplementos são adicionados aos meios de FIV, tais como a cafeína que é muito empregada em suínos (ROMAR et al., 2019), e a heparina amplamente usada em ruminantes (CUNHA et al., 2019). Uma vez que espermatozoides de catetos podem se comportar de forma similar a estas diferentes espécies, é importante comparar os agentes capacitantes mais usados para definir as melhores condições de incubação. Adicionalmente, é necessário verificar o tempo de co-incubação dos gametas *in vitro*, uma vez que em suínos a alta taxa de polispermia é um problema recorrente que prejudica o desenvolvimento embrionário (OBERLENDER et al., 2016).

3 JUSTIFICATIVA

A fertilização *in vitro* (FIV) tem sido empregada na reprodução de mamíferos em virtude de suas aplicações e por favorecer um amplo conhecimento acerca da fisiologia reprodutiva. Para mamíferos silvestres, a FIV pode ser determinante não somente para a conservação e para auxiliar no conhecimento da espécie, mas também para o aumento da produtividade no sentido zootécnico, especialmente em animais, como os catetos, que já possuem uma boa adaptação a cativeiro e potencial de mercado. Nesse sentido, a FIV pode acelerar a geração de crias e ampliar a capacidade produtiva de animais com melhores características zootécnicas, além de permitir o controle de todo o procedimento para a obtenção de embriões, gerando informações que podem subsidiar outras técnicas *in vitro* e *in vivo*.

Contudo, para utilização com sucesso de uma biotécnica reprodutiva, como a FIV, que apresenta diferentes etapas e requerimentos, é necessário estabelecer os parâmetros que influenciam no seu resultado. Nesse contexto, algumas questões referentes aos espermatozoides podem influenciar diretamente o resultado da FIV e ainda não foram estabelecidas para catetos. Dentre essas questões, tem-se a etapa de seleção espermática, bem como a capacitação induzida por agentes capacitantes adicionados ao meio de cultivo que são fundamentais para a interação entre os gametas. Além disso, existem variações nos tempos para a co-incubação de gametas e que, portanto, influenciam diretamente na eficiência da fertilização.

Portanto, antes da aplicação da FIV em catetos, a seleção espermática, a capacitação e o tempo de co-incubação devem ser otimizados. Espera-se a partir disso ser possível utilizar a FIV em catetos de forma eficiente e contribuir com sua produtividade e conservação a partir da obtenção de embriões em boa quantidade e qualidade.

4 HIPÓTESES CIENTÍFICAS

4.1 A remoção do plasma seminal por centrifugação antes do swim-up melhora a eficiência da seleção de espermatozoides de catetos.

4.2 Os espermatozoides de catetos podem ser selecionados mais eficientemente usando a técnica de gradiente de Percoll 45–90% em comparação aos protocolos de swim-up e lavagem por centrifugação;

4.3 A capacitação de espermatozoides de catetos ocorre com maior eficiência em meio suplementado com cafeína, bem como o tempo de co-incubação de gametas por 3 h é suficiente para ligação dos espermatozoides na zona pelúcida;

4.4 Oócitos suínos podem ser usados para avaliação da fertilidade de espermatozoides de catetos, suportando o desenvolvimento embrionário inicial.

5 OBJETIVOS

5.1 Objetivo Geral

Aperfeiçoar as etapas de seleção, capacitação e co-incubação de espermatozoides de catetos para a fertilização *in vitro*.

5.2 Objetivos específicos

5.2.1 Estudar o efeito da remoção do plasma seminal sobre parâmetros espermáticos após seleção de espermatozoides de catetos por swim-up.

5.2.2 Avaliar diferentes gradientes de Percoll (35–70% *vs*. 45–90%) sobre parâmetros espermáticos de catetos.

5.2.3 Comparar os métodos de swim-up, gradiente de Percoll e lavagem por centrifugação para seleção de espermatozoides de catetos sobre os parâmetros espermáticos e capacidade de fertilização heteróloga;

5.2.4 Analisar a influência de agentes capacitantes (cafeína vs. heparina vs. cafeína associado a heparina) sobre os espermatozoides de catetos quanto aos parâmetros espermáticos

5.2.5 Verificar capacidade de ligação de espermatozoides de catetos à oócitos heterólogos em diferentes tempos de incubação em meio capacitante.

REFERÊNCIAS

ALBUQUERQUE, N.I.; DIAS, H.; GUIMARÃES, D.D.A.; PENDU, Y.L.; GARCIA, A.; KAHWAGE, P.; SELIGMANN, I. **Criação de caititus em cativeiro:** sistema intensivo de produção na Amazônia Oriental. Embrapa Amazônia Oriental-Livro técnico (INFOTECA-E). Brasília, DF: Embrapa, 2016, 108 p.

ALBUQUERQUE, N.I.D.; OHASHI, O.M.; GUIMARÃES, D.A.; LE PENDU, Y.; DIAS, H.T. Alternativas de sistema de produção de caititu (*Tayassu tajacu*) para a pequena agricultura na Amazonia. In: **Livro de resultados dos projetos de pesquisa dirigida (PPDs)**. Brasília-DF: Subprograma de Ciência e Tecnologia, 2002, p. 107–112.

ALVES, H.M.; OLIVEIRA, I.R.S.; CASTELO, T.S.; LIMA, G.L.; SOUZA, A.L.P.; MOREIRA, M.A.P.; PAULA, V.V.; SILVA, A.R. Comparison of different glycerol and egg yolk concentrations added to tris-based extender for the collared peccaries (*Tayassu tajacu*) semen freezing. **Reproduction in Domestic Animals**, v. 48, p. 506–511. 2013

ARIAS, M.E.; ANDARA, K.; BRIONES, E.; FELMER, R. Bovine sperm separation by Swim-up and density gradients (Percoll and BoviPure): Effect on sperm quality, function and gene expression. **Reproductive Biology**, v. 17, p. 126–132, 2017.

BEZERRA, J.A.B.; SILVA, A.M.; PEIXOTO, G.C.X.; SILVA, M.A.; OLIVEIRA, M.F.; SILVA, A.R. Influence of recovery method and centrifugation on epididymal sperm from collared peccaries (*Pecari tajacu* Linnaeus, 1758). **Zoological Science**, v. 31, p. 338–342, 2014.

BEZERRA, L.G.P.; SOUZA, A.L.P.; SILVA, H.V.R.; VASCONCELOS, F.R.; MOURA, A.D.A.A.; PEREIRA, A.F.; OLIVEIRA, M.F.; SILVA, A.R. Ultrastructural description of fresh and frozen/thawed sperm derived from collared peccaries (*Pecari tajacu* Linnaeus, 1,758). **Microscopy Research and Technique**, v. 81, p. 1301–1309, 2018.

BODMER, R.E. Responses of ungulates to seasonal inundations in the Amazon floodplain. **Journal of Tropical Ecology**, v. 6, p. 191–201, 1990.

BORGES, A.A.; SANTOS, M.V.O.; NASCIMENTO, L. E.; LIRA, G.P.O.; PRAXEDES, É.A.; OLIVEIRA, M. F.; SILVA, A.R.; PEREIRA, A.F. Production of collared peccary (*Pecari tajacu* Linnaeus, 1758) parthenogenic embryos following different oocyte chemical activation and *in vitro* maturation conditions. **Theriogenology**, v. 142, p. 320–327, 2020.

BORGES, A.A.; SANTOS, M.V.O.; QUEIROZ NETA, L.B.; OLIVEIRA, M.F.; SILVA, A.R.; Pereira, A.F. *In vitro* maturation of collared peccary (*Pecari tajacu* Linnaeus, 1758) oocytes after different incubation times. **Pesquisa Veterinária Brasileira**, v. 38, p. 1863–1868, 2018.

CAMPOS, L.B; SILVA, M.A.; BEZERRA, J. A.B.; CASTELO, T.S.; PEIXOTO, G. C.X.; SILVA, A.R. Survival of collared peccaries (*Pecari tajacu*) sperm after freezing-thawing using different extenders. **Acta Scientiae Veterinariae,** v. 42, p. 1–8, 2014.

CAMPOS, L.B.; SILVA, A.M.; PRAXEDES, E.C.G.; BEZERRA, L.G.P.; LINS, T.L.B.G.; MENEZES, V.G.; MATOS, M.H.T.; LIMA, G.L.; RODRIGUES, A.P.R.; SILVA, A.R. Vitrification of collared peccary ovarian tissue using open or closed systems and different intracellular cryoprotectants. **Cryobiology**, v. 91, p. 77–83, 2019.

CAMPOS, L.B.; SILVA, A.M.; PEIXOTO, G.C.X.; SOUZA, A.L.P.; CASTELO, T.S.; MAIA, K.M.; PEREIRA, A.F.; SILVA, A.R. Estimating the binding ability of collared peccary (*Pecari tajacu* Linnaeus, 1758) sperm using heterologous substrates. **Theriogenology**, v. 92, p. 57–62, 2017b.

CAMPOS, L.B.; SILVA, A.M.; PRAXEDES, É.C.; BEZERRA, L.G.; FREITAS, J.L.; MELO, L.M.; PEREIRA, A.F.; FIGUEIREDO, J.R.; SILVA, A.R. Effect of growth differentiation factor 9 (GDF-9) on *in vitro* development of collared peccary preantral follicles in ovarian tissues. **Animal Reproduction Science**, v. 226, p. 106717, 2021.

CARDOSO, F.M.; QUEIROZ, G.F. Duration of the cycle of the seminiferous epithelium and daily sperm production of Brazilian hairy rams. **Animal Reproduction Science**, v. 17, p.77–84, 1988.

CASTELO, T.S.; BEZERRA, F.S.B.; LIMA, G.L.; ALVES, H.M.; OLIVEIRA, I.R.S.; SANTOS, E.A.A.; PEIXOTO, G.C.X.; SILVA, A.R. Effect of centrifugation and sugar supplementation on the semen cryopreservation of captive collared peccaries (*Tayassu tajacu*). **Cryobiology**, v. 61, p. 275–279, 2010a.

CASTELO, T.S.; BEZERRA, F.S.B.; SOUZA, A.L.P.; MOREIRA, M.A.P.; PAULA, V.V.; OLIVEIRA, M.F.; SILVA, A.R. Influence of the thawing rate on the cryopreservation of semen from collared peccaries using Tris-based extenders. **Theriogenology**, v.74, p.1060–1065, 2010b.

CHEN, Y.; WANG, K.; ZHANG, S. Osteopontin enhances sperm capacitation and in vitro fertilization efficiency in boars. **Journal of Animal Science and Technology**, v. 64, p. 235–246, 2022.

COSTA, G.M.J.; LEAL, M.L.; SILVA, J.V.; FERREIRA, A.C.S.; GUIMARÃES, D.A.; FRANÇA, L.R. Spermatogenic cycle length and sperm production in a feral pig species (collared peccary, *Tayassu tajacu*). **Journal of Andrology**, v. 31, p.221–230, 2010.

CRUZ, R.S.; GIULIANO, S.M.; GAMBAROTTA, M.C.; MORRELL, J.M.; ABRAHAM, M.C.; MIRAGAYA, M.H.; CARRETERO, M.I. Comparison of different methods of sperm selection of llama raw semen. **Animal Reproduction Science**, v. 173, p. 8–12, 2016.

CUNHA, A.; CARVALHO, J.O.; GUIMARÃES, A.; LEME, L.O.; CAIXETA, F.M.; VIANA, J.; DODE, M. Bovine epididymal spermatozoa treatment for i*n vitro* fertilization: Heparin accelerates fertilization and enables a reduction in coincubation time. **Plos one**, v. 14, p. e0209692, 2019.

DEORI, S.; NTALLARIS, T.; WALLGREN, M.; MORRELL, J.M.; JOHANNISSON, A. Comparison of single layer centrifugation and magnetic activated cell sorting for selecting viable boar spermatozoa after thawing. **Livestock Science**, v. 257 p. 104853, 2022.

DESBIEZ, A.L.J.; KEUROGHLIAN, A.; MELLO BEISIEGEL, B.; MEDICI, E.P.; GATTI, A.; PONTES, A.R.M.; DE ALMEIDA, L.B. Avaliação do risco de extinção do cateto *Pecari tajacu* Linnaeus, 1758, no Brasil. **Biodiversidade Brasileira**, v. 1, p. 74–83, 2012.

EL BIZRI, H.R.; MORCATTY, T.Q.; VALSECCHI, J.; MAYOR, P.; RIBEIRO, J.E.S.; VASCONCELOS, C.F.A.; OLIVEIRA, J.S.; FURTADO, K.M.; FERREIRA, U.C.; MIRANDA, C.F.S.; SILVA, C.H.; LOPES, V.L.; LOPES, G.P.; FLORINDO, C.C.F.; CHAGAS, R.C.; NIJMAN, V.; FA, J.E. Urban wild meat consumption and trade in central **Amazonia. Conservation Biology**, v. 34, p. 438–448, 2020.

FRANÇA, L.R.; AVELAR, G.F.; ALMEIDA, F.F. Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. **Theriogenology**, v. 63, p. 300–318, 2005.

GARCIA, A.R.; KAHWAGE, P.R.; OHASHI, O.M. Aspectos reprodutivos de caititus (*Tayassu tajacu*). Embrapa Amazônia Oriental-Artigo em periódico indexado (ALICE), 2009. **Revista Brasileira de Reprodução Animal,** v.33, p.71–81, 2009.

GONGORA, J.; REYNA-HURTADO, R.; BECK, H.; TABER, A.; ALTRICHTER, M.; KEUROGHLIAN, A. 2011. *Pecari tajacu*. The IUCN Red List of Threatened Species 2011: e.T41777A10562361. Disponível em <http://dx.doi.org/10.2305/IUCN.UK.2011-2.RLTS.T41777A10562361.en.> Acesso em: 27 de julho de 2022.

GOSDEN, R.G.; TELFER, E. Numbers of follicles and oocytes in mammalian ovaries and their allometric relationships. Journal of Zoology, v. 211, p. 169–175, 1987.

HAFEZ, E.S. E.; HAFEZ, B. Reproduction in Farm Animals. 7th Edition. Wiley-Blackwell, 2000.

HARRISON, R.A.P.; GADELLA, B.M. Bicarbonate-induced membrane processing in sperm capacitation. **Theriogenology**, v. 63, p. 342–351, 2005.

HILDEBRANDT, T.B.; HERMES, R.; COLLEONI, S.; DIECKE, S.; HOLTZE, S.; RENFREE, M.B.; STEJSKAL, J.; HAYASHI, K.; DRUKKER, M.; LOI, P.; GÖRITZ, F.; LAZZARI, G.; GALLI, C. Embryos and embryonic stem cells from the white rhinoceros. **Nature Communications**, v. 9, p. 1–9, 2018.

INTERNATIONAL UNION FOR CONSERVATION OF NATURE AND NATURAL RESOURCES (IUCN) Red List of Threatened Species. Disponível em < http://www.iucnredlist.org/details/41777/0> Acesso em: 08 de junho de 2022.

LIMA, G.L.; SANTOS, E.A.A.; LUZ, V.B.; RODRIGUES, A.P.R.; SILVA, A.R. Morphological characterization of the ovarian preantral follicle population of collared peccaries (*Tayassu tajacu* Linnaeus, 1758). **Anatomia, Histologia, Embryologia**, v. 42, p. 304–311, 2013.

LIMA, G.L.; LUZ, V.B.; LUNARDI, F.O.; SOUZA, A.L.; PEIXOTO, G.C.; RODRIGUES, A.P.R.; OLIVEIRA, M.F.; SANTOS, R.R.; SILVA, A.R. Effect of cryoprotectant type and concentration on the vitrification of collared peccary (*Pecari tajacu*) ovarian tissue. **Animal Reproduction Science**, v. 205, p. 126–133, 2019.

MAIA, K.M.; PEIXOTO, G.C.; CAMPOS, L.B.; BEZERRA, J.A.B.; RICARTE, A.R.; MOREIRA, N.; OLIVEIRA, M.F.; SILVA, A.R. Estrus cycle monitoring of captive collared peccaries (*Pecari tajacu*) in semiarid conditions. **Pesquisa Veterinária Brasileira**, v. 34, p. 1115–1120, 2014a.

MAIA, K.M.; PEIXOTO, G.C.X.; CAMPOS, L.B.; SILVA, A.M.; CASTELO, T.S.; RICARTE, A.R.F.; SILVA, A.R. Estrous synchronization in captive collared peccaries (*Pecari tajacu*) using a prostaglandin F2α analog. **Zoological Science**, v. 31, p. 836–839, 2014b.

MAIA, K.M.; SOUZA, A.L.; PRAXEDES, E.C.; BEZERRA, L.G.; SILVA, A.M.; CAMPOS, L.B.; MOREIRA, S.S.J., APOLINÁRIO, C.A.C.; SOUZA, JR. J.B.F.; SILVA, A.R. Environmental factors related to a semiarid climate influence the freezability of sperm from collared peccaries (*Pecari tajacu* Linnaeus, 1758). **Biopreservation and Biobanking**, v. 16, p. 186–190, 2018.

MARTÍNEZ-GUTIÉRREZ, P.G.; MARTÍNEZ-MEYER, E.; PALOMARES, F.; FERNÁNDEZ, N. Niche centrality and human influence predict range wide variation in population abundance of a widespread mammal: The collared peccary (*Pecari tajacu*). **Diversity and Distributions**, v. 24, p. 103–115, 2017.

MATÁS, C.; VIEIRA, L.; GARCÍA-VÁZQUEZ, F.A.; AVILÉS-LÓPEZ, K.; LÓPEZ-ÚBEDA, R.; CARVAJAL, J.A.; GADEA, J. Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function. **Animal Reproduction Science**, v. 127, p. 62–72, 2011.

MAYOR, P.; FENECH, M.; BODMER, R.E.; LOPEZ-BEJAR, M. Ovarian features of the wild collared peccary (*Tayassu tajacu*) from the northeastern Peruvian Amazon. **General and Comparative Endocrinology,** v. 147, p. 268–275, 2006.

MAYOR, P.; GUIMARAES, D.A.; LE PENDU, Y.; SILVA, J.V.; JORI, F.; LÓPEZ-BÉJAR, M. Reproductive performance of captive collared peccaries (*Tayassu tajacu*) in the eastern Amazon. **Animal Reproduction Science**, v. 102, p. 88–97, 2007.

MAYOR, P.; JORI, F.; LOPEZ-BEJAR. M. Anatomicohistological characteristics of the tubular genital organs of the female collared peccary (*Tayassu tajacu*) from the northeastern Amazon. **Anatomia, Histologia, Embryologia**, v. 33, p. 65–74, 2004.

MORAIS, B.H.S.; LIMA, A.J.M.; COSTA, J.S.; MAYOR, P.; ALBUQUERQUE, N.I.; CHISTÉ, R.C.; GUIMARÃES, D.A.A. Use of wildlife as an alternative protein source: Collared peccary meat. **Meat Science**, v. 192, p. 108895, 2022a.

MORAIS, B.H.S.; LIMA, A.J.M.; ALBUQUERQUE, N.I.; CHISTÉ, R.C.; GUIMARÃES, D.A.A. Bacteriological, physicochemical, and sensory characteristics of collared peccary sausages (*Pecari tajacu*) with added dietary fibers. **Food Science and Technology**, v. 42, p. e04922, 2022b.

MOREIRA, S.S.; SILVA, A.M.; PRAXEDES, É.C.; CAMPOS, L.B.; SANTOS, C.S.; SOUZA, A.L.P.; PEREIRA, A.F.; SOUZA-JÚNIOR, J.B.F.; COSTA, L.M.C.; SILVA, A.R. Composition of collared peccary seminal plasma and sperm motility kinetics in semen obtained during dry and rainy periods in a semiarid biome. **Animal Reproduction Science**, v. 211, p. 91–101, 2019.

MOREIRA, S.S.J.; SANTOS, C.S.; CASTELO, T.S.; BEZERRA, L.G.P.; PRAXEDES, É.C.G.; MATOS, T.M.; SOUZA-JUNIOR, J.B.F.; FEIJÓ, F.M.C.; COMIZZOLI, P.; SILVA, A.R. Investigating the need for antibiotic supplementation to the extender used for semen cryopreservation in collared peccaries. **Frontiers in Veterinary Science**, v. 9, p. 954921, 2022.

MOREIRA, S.S.; SILVA, A.M.; PEREIRA, A.G.; SANTOS, R.P.; DANTAS, M.R.; SOUZA-JÚNIOR, J.B.; SNOECK, P.P.; SILVA, A.R. Effect of detergents based on sodium dodecyl sulfate on functional metrics of frozen–thawed collared peccary (*Pecari tajacu*) Semen. **Animals**, v. 13, p. 451–462, 2023.

NOGUEIRA FILHO, S.L.G.; NOGUEIRA, SS. C.; TAKECHI, S.A. Estrutura social de pecaris (Mammalia, Tayassuidade) em cativeiro. **Revista de Etologia**, v. 1, p. 89–98, 1999.

NOGUEIRA-FILHO, S.; NOGUEIRA, S.; MENDES, A.; JORI, F. A large-scale commercial farming of collared peccary (*Tayassu tajacu*) in north-eastern Brazil. **Game and Wildlife Science**. v. 21, p. 413–420, 2014.

NUNES, A.V.; GUARIENTO, R.D.; SANTOS, B.A.; FISCHER, E. Wild meat sharing among non-indigenous people in the southwestern Amazon. **Behavioral Ecology and Sociobiology**, v. 73, p. 26, 2019.

OBERLENDER, G.; LÓPEZ, S.R.; SÁNCHEZ, A.D.; VIEIRA, L.A.; PEREIRA, M.B.; SILVA, L.D.F.; MURGAS, L.D. *In vitro* fertilization of porcine oocytes is affected by spermatic coincubation time. **Pesquisa Veterinária Brasileira**, v. 36, p. 58–64, 2016.

OHLWEILER, L.U.; MEZZALIRA, J.C.; MEZZALIRA, A. Porcine IVF embryo development and estrogen receptors are influenced by the concentration of percoll gradients during sperm selection. **Molecular Reproduction and Development**, v. 87, p. 135–141, 2020.

OLIVEIRA, A.P.M.; LIMA C.D.; SARMENTO, A.C. Epididymal morphophysiology of adult collared peccaries (*Pecari tajacu*). **Brazilian Journal of Veterinary Research and Animal Science**, v. 53, p. 251–259, 2016.

PEIXOTO, G.C.; LIMA, G.L.; MAIA, K.M.; SOUZA, A.L.P.; CASTELO, T.S.; PAIVA, A. L.; PAULA, V.V.; OLIVEIRA, M.F.; BRITO, A.B.; DOMINGUES, S.F.S.; VIANA, A.C.N. Single injection of eCG/hCG leads to successful estrous synchronization in the collared peccary (*Pecari tajacu* Linnaeus, 1758). **Animal Reproduction Science**, v. 208, p. 1061–1012, 2019.

PEIXOTO, G.C.X.; SILVA, M.A.; CASTELO, T.S.; SILVA, A.M.; BEZERRA, J.A.B.; SOUZA, A.L.P.; OLIVEIRA, M.F.; SILVA, A.R. Individual variation related to testicular biometry and semen characteristics in collared peccaries (*Tayassu Tajacu* Linnaeus, 1758). **Animal Reproduction Science**, v. 134, p.191–196, 2012.

PEIXOTO, G.C.X.; SILVA, M.A.; LIMA, G.L.; CAMPOS, L.B.; PAIVA, A.L.C.; FREITAS, V.A.; SILVA, A.R. Use of non-invasive methods for evaluating the testicular biometry in collared peccaries (*Pecari tajacu* Linnaeus, 1758). **Anatomia, Histologia, Embryologia**, v. 45, p. 60–66, 2016.

PÉREZ-IRINEO, G.; SANTOS-MORENO, A. Abundance, herd size, activity pattern and occupancy of ungulates in Southeastern Mexico. **Animal Biology**, v. 66, p. 97–109, 2016.

ROMAR, R.; CÁNOVAS, S.; MATÁS, C.; GADEA, J.; COY, P. Pig *in vitro* fertilization: where are we and where do we go? **Theriogenology**, v. 137, 113–121, 2019.

ROMERO, A.; O'NEILL, B. J.; TIMM, RM.; GEROW, K.G.; MCCLEARN, D. Group dynamics, behavior, and current and historical abundance of peccaries in Costa Rica's Caribbean lowlands. **Journal of Mammalogy**, v. 94, p. 771–791, 2013.

SALICIONI, A.M.; PLATT, M.D.; WERTHEIMER, E.V.; ARCELAY, E.; ALLAIRE, A.; SOSNIK, J.; VISCONTI, P.E. Signalling pathways involved in sperm capacitation. **Society of Reproduction and Fertility**, v. 65, p. 245, 2007.

SANTOS, C.S.; CAMPOS, L.B.; PRAXEDES, É.C.G.; MOREIRA, S.S.J.; SOUZA-JÚNIOR, J.B.F.; COMIZZOLI, P.; FEIJÓ, F.M.C.; SILVA, A.R. Influence of antibiotics on bacterial load and sperm parameters during short-term preservation of collared peccary

semen. **Animal Reproduction**, v. 18, p. e20210021, 2021.

SANTOS, D.O.; MENDES, A.; NOGUEIRA, S.S.D.C.; NOGUEIRA FILHO, S.L.G. Criação comercial de caititus (*Pecari tajacu*): uma alternativa para o agronegócio. **Revista Brasileira de Sanidade e Produção Animal**, v.10, p.1–10, 2009.

SANTOS, E.A.; SOUSA, P.C.; MARTINS, J.A.M.; MOREIRA, R.A.; MONTEIRO-MOREIRA, A.C.O.; MORENO, F.B.M.B.; OLIVEIRA, M.F.; MOURA, A.A.; SILVA, A.R. Protein profile of the seminal plasma of collared peccaries (*Pecari tajacu* Linnaeus, 1758). **Reproduction**, v. 147, p. 753–764, 2014.

SANTOS, E.A.A.; SOUSA, P.C.; PEIXOTO, G.C.X.; SIMÃO, B.R.; OLIVEIRA, M.F.; SILVA, A.R. Establishing the hypoosmotic swelling test for sperm analysis in collared peccaries (*Pecari tajacu*). **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v.65, p.1257–1260, 2013.

SCOGNAMILLO, D.; MAXIT, I. E.; SUNQUIST, M.; POLISAR, J. Coexistence of jaguar (*Panthera onca*) and puma (*Puma concolor*) in a mosaic landscape in the Venezuelan llanos. **Journal of Zoology**, v. 259, p. 269–279, 2003.

SILVA, A.M.; CAMPOS, L.B.; PRAXEDES, E.C.G.; MAIA, K.M.; MOREIRA, S.S.J.; BEZERRA, L.G.P.; APOLINARIO, C.A.C.; OLIVEIRA, M.F.; SILVA, A.R. Avaliação da viabilidade de tecido testicular fresco ou vitrificado de catetos (*Pecari tajacu*) utilizando diferentes métodos de dissociação celular. **Revista Brasileira de Reprodução Animal**, v. 41, p. 362, 2017.

SILVA, A.M.; PEREIRA, A.G.; BRASIL, A.V.; MACEDO, L.B.; SOUZA-JUNIOR, J.; MOURA, C.E.B.; PEREIRA, A.F.; OLIVEIRA, M.F.; COMIZZOLI, P.; SILVA, A.R. Influence of freezing techniques and glycerol-based cryoprotectant combinations on the survival of testicular tissues from adult collared peccaries. **Theriogenology**, v. 167, p.111–119, 2021.

SILVA, A.M.; PEIXOTO, G.C.X.; BEZERRA, J.A.B.; CASTELO, T.S.; SANTOS, E.A.A.; SILVA, A.R. Relationship between Neubauer counting chamber and spectrophotometer used for the sperm concentration determination in collared peccaries (*Pecari tajacu*). **Ciência Rural**, v. 44, p. 1494–1498, 2014.

SILVA, S.D.S.B.; LE PENDU, Y.; OHASHI, O.M.; OBA, E.; ALBUQUERQUE, N.I.; GARCIA, A.R.; MAYOR, P.; GUIMARÃES, D.A.A. Sexual behavior of *Pecari tajacu* (Cetartiodactyla: Tayassuidae) during periovulatory and early gestation periods. **Behavioural Processes**, v. 131, p. 68–73, 2016.

SILVEIRA, A.L.; PACHECO, S. A. Ocorrência de queixada (*Tayassu pecari*), caititu (*Pecari tajacu*) e javaporco (*Sus scrofa*)(Mammalia, Cetartiodactyla) em João Pinheiro, Cerrado de

Minas Gerais, Brasil. **Revista Brasileira de Zoociências**, v. 19, p. 91–136, 2018.

SONNER, J.B.; MIGLINO, M.A.; SANTOS, T.C.; CARVALHAL, R.; NETO, A.C.A.; MOURA, C.E.B.; OLIVEIRA, M.F. Macroscopic and morphometric features of testes in collared peccary and lipped peccary. **Biota Neotropica**, v. 4, p. 1–12, 2004.

SOUSA, P.C.; SANTOS, E.A.A.; SOUZA, A.L.P.; LIMA, G.L.; BARROS, F.F.P.C.; OLIVEIRA, M.F.; SILVA. A.R. Sperm morphological and morphometric evaluation in captive collared peccaries (*Pecari tajacu*). **Pesquisa Veterinária Brasileira**, v. 33, p. 924–930, 2013.

SOUZA, A.L.P.; CASTELO, T.S.; QUEIROZ, J.P.A.F.; BARROS, I.O.; PAULA, V.V. Evaluation of anesthetic protocol for the collection of semen from captive collared peccaries (*Tayassu tajacu)* by eletroejaculation. **Animal Reproduction Science**, v.116, p.370–375, 2009.

SOUZA, A.L.P.; LIMA, G.L.; PEIXOTO, G.C.X.; CASTELO, T.S; OLIVEIRA, M.G.C.; PAULA, V.V.; SILVA, A.R. Sperm characteristics following freezing in extenders supplemented with whole egg yolk and different concentrations of low-density lipoproteins in the collared peccary (*Pecari tajacu*). **Reproductive Biology**, v. 15, p. 223–228, 2015.

SOUZA, A.L.P.; LIMA, G.L.; PEIXOTO, G.C.X.; SILVA, A.M.; OLIVEIRA, M.F.; SILVA, A.R. Use of *Aloe vera*-based extender for chilling and freezing collared peccary (*Pecari tajacu*) semen. **Theriogenology**, v. 85, p. 1432–1438, 2016.

TABER A.; BECK, H.; ALTRICHTER, M.; GONGORA, J.; DON W.E.; RUSSELL A.M. Handbook of the mammals of the world. Edição 2. Barcelona, Spain: Lynx Ediciones, 2011.

VIANA, A.C.N.; FREITAS, J.L.; MAGALHÃES, L.C.; CAMPOS, L.B.; SILVA, A.R.; OLIVEIRA, M.F.; FREITAS, V.J.; MELO, L.M. Ovarian gene expression in collared peccary (*Pecari tajacu* Linnaeus, 1758) subjected to gonadotropin stimulation protocols. **Reproduction in Domestic Animals**, v. 56, p.351–359, 2021

WU, Z. W.; GAO, Z. R.; LIANG, H.; FANG, T.; WANG, Y.; DU, Z. Q.; YANG, C. X. Network analysis reveals different hub genes and molecular pathways for pig in vitro fertilized early embryos and parthenogenotes. **Reproduction in Domestic Animals**, v. 57, p. 1544–1553, 2022.

CAPÍTULO 2 – EMBRYO PRODUCTION BY *IN VITRO* FERTILIZATION IN WILD UNGULATES – PROGRESS AND PERSPECTIVES

Artigo de revisão: Embryo production by *in vitro* fertilization in wild ungulates – progress and perspectives

Periódico de submissão: Annals of animal science

Fator de impacto: 2,667

Data de submissão: 23 de agosto de 2021

Data de aceite: 03 de janeiro de 2022

DOI: https://doi.org/10.2478/aoas-2022-0013

Embryo production by *in vitro* fertilization in wild ungulates – progress and perspectives*

Maria Valéria de Oliveira Santos[1], Alexandre Rodrigues Silva[2], Alexsandra Fernandes Pereira[1]♦

[1]Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoró, RN, Brazil

[2]Laboratory of Animal Germplasm Conservation, Federal Rural University of Semi-Arid, Mossoró, RN, Brazil

♦Corresponding author: Alexsandra Fernandes Pereira
Laboratory of Animal Biotechnology
Universidade Federal Rural do Semi-Árido
Av. Francisco Mota, 572, Mossoró, RN, 59625-900, Brazil
Phone: +55 84 3317 8361
E-mail address: alexsandra.pereira@ufersa.edu.br

Abbreviated title: Wild ungulates embryo *in vitro* production

---

*Financed by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES, Financial Code 001). AF Pereira and AR Silva are recipients of CNPq grants.

Abstract

Wild ungulates are mammals with hooves of fundamental importance for the balance of ecosystems, as well as interesting species of economic source. In the last decades, an accelerated population reduction of these species has resulted in the development of Assisted Reproduction Techniques (ARTs), such as *in vitro* fertilization (IVF), as a tool for conservation and multiplication. In this scenario, IVF protocols have been developed based on methodologies used in domestic ungulates. Nevertheless, due to the physiological and reproductive differences among the species, many factors associated to IVF and its relationship with the characteristics of the species of interest require clarification. *In vitro* conditions for the collection and selection of female and male gametes, oocyte maturation, sperm capacitation,

co-incubation of gametes and embryonic development, can influence the result of IVF. Thus, the present review considers the main advances received in the methodologies already used in wild ungulates, emphasizing the strategies for improving the protocols to obtain better efficiency rates. Additionally, we discuss the conditions of each IVF stage, with emphasis on aspects related to *in vitro* manipulation and making a parallel with the protocols of domestic ungulates.

Key words: wildlife conservation, assisted reproduction, *in vitro* fertilization, gametes, embryos.

Introduction

The ungulates are mammals with hooves that constitute a large and diverse group, divided into artiodactyls as animals with even-toed and perissodactyls as species with odd-toed. In the first case, we have as some representatives the bovids, cervids, giraffe, pig, and camelids, while in the second order we have horses, rhinoceros, and tapir. These animals play a key role in balancing diverse ecosystems through grazing, trampling, defecation, seed dispersal and nutrient cycling (Mohr et al., 2005; Velamazan et al., 2020). Additionally, wild ungulates represent an important economic resource for many local communities due to the appreciation of meat and leather, as well as wildlife tourism (Gordon et al., 2004).

According to the International Union for Conservation of Nature (IUCN, 2021), approximately 140 species of more than 250 wild ungulates are on the extinction pathways, being classified as near threatened (32), vulnerable (52), endangered (42), critically endangered (15) and extinct in the wild (2). Moreover, currently, the population of more than 160 species is decreasing. These animals are constantly affected by habitat loss, forest fragmentation, illegal hunting, among other consequences of anthropogenic activities (Malakoutikhah et al., 2020). Therefore, the use of Assisted Reproduction Techniques (ARTs) has been applied to wild ungulates to conserve biodiversity, increase genetic variability, and rapidly multiply genotypes (reviewed by: Mastromonaco and Songsasen, 2020). ARTs are attractive tools for the reproduction and maintenance of wild species in zoos and reserves (reviewed by: Comizzoli et al., 2000; Herrick, 2019), as well as for the economic activity efficiency associated with farm-raised non-domestic ungulates, such as the red deer (Berg and Asher, 2003).

An interesting ART that has been developed in wild ungulates is *in vitro* fertilization (IVF). IVF can be divided into a few stages, such as collection, selection and maturation of oocytes, sperm collection, selection and capacitation, gamete co-incubation, *in vitro* embryo culture and transfer of embryos. In general, the use of IVF associated with embryo transfer in

wild ungulates has allowed the rescue of genetic material from males and females dead and disabled for natural reproduction (Mahesh et al., 2011), the utilization of low quality and quantity gametes (Hermes et al., 2009), and gametes transported for long periods (García-Álvarez et al., 2011).

Despite many application possibilities and the success in domestic ungulates, there are few reports of offspring born from IVF in wild ungulates. Overall, carrying out IVF in wild ungulates is a great challenge mainly due to the shortage number of available male and female gametes as well as the lack of expert knowledge to handle animals in safety. These facts hamper developing continuous studies and the establishment of this ART (Hildebrandt et al., 2018).

There are several species of domestic or domesticated ungulates that can be used as experimental models for wild species. Nevertheless, it is important to note that the protocols of model species are a starting point to start studies on phylogenetically close wild species (Figure 1). Therefore, as the physiology of the species of interest is known, it is important to adapt and test different conditions to meet intraspecific needs and achieve success in the IVF. In red deer (*Cervus elaphus*), many advances have been achieved at IVF based on the usual protocols of domestic ruminants (Berg et al., 2002). However, Berg and Asher (2003) observed that the composition of the oviduct fluid of this species could be different from cattle and sheep. Thus, the authors confirmed this difference and formulated specific culture media for red deer, achieving a significantly higher percentage of cleavage (68.0 *vs*. 49.3%) and blastocyst (12.9 *vs*. 4.5%).

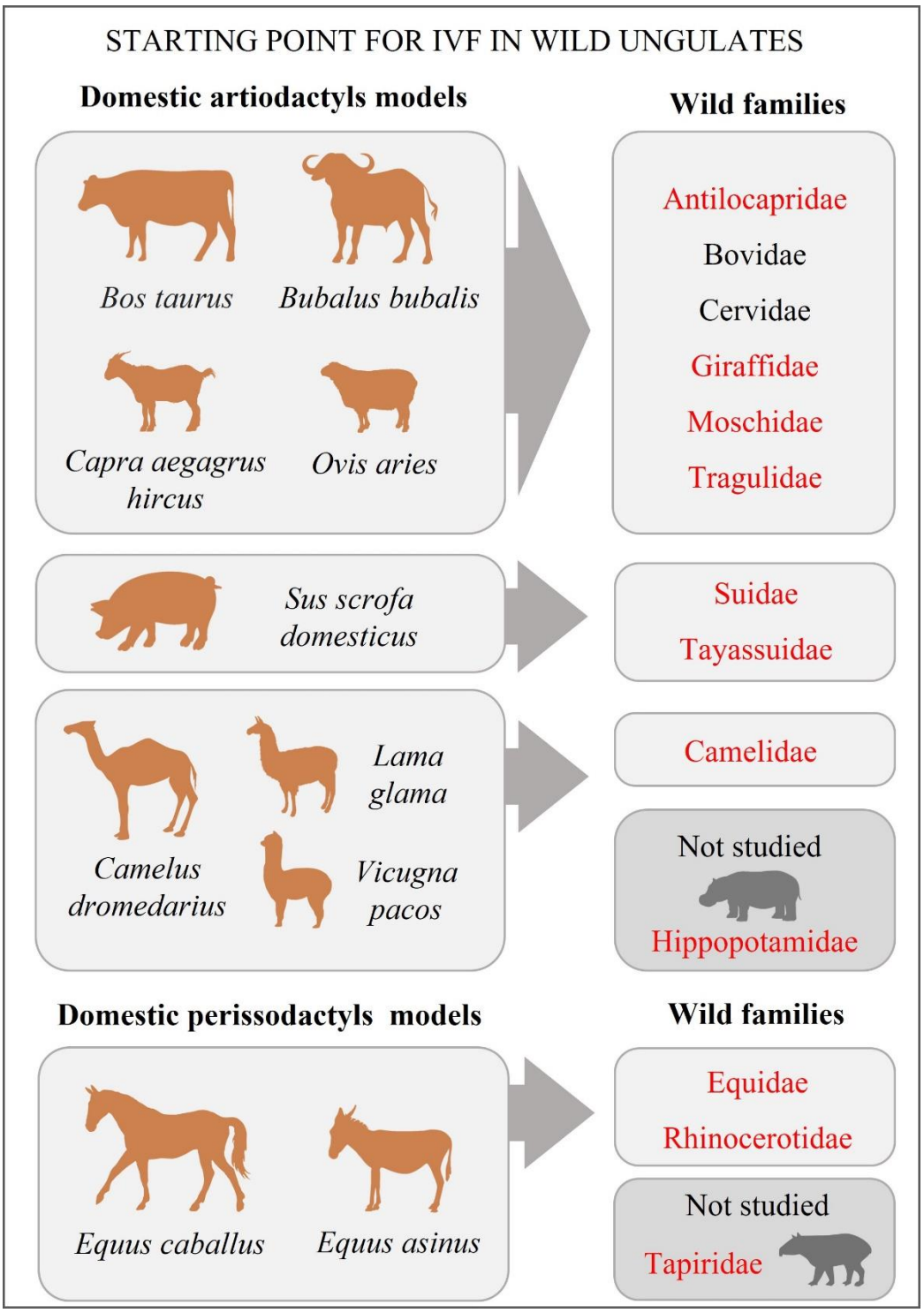

Figure 1. Domestic or domesticated ungulate species that due to phylogenetic proximity could be used as an experimental model for species of wild ungulate families, aiming *in vitro* fertilization (IVF). Family names written in red indicate that there are few or no studies.

The low efficiency of IVF in wild ungulates is the result of technical limitations such as: insufficient knowledge about basic reproduction and physiology of gametes and embryos, difficulty in dealing with animals safely and without stress, availability of biological material and lack of studies establishing ideal conditions for the *in vitro* manipulation of gametes and

embryos for different species (Andrabi and Maxwell, 2007; Cervantes et al., 2016; Mastromonaco and Songsasen, 2020; Thongphakdee et al., 2017). Although these factors are associated, this review will focus primarily on aspects related to *in vitro* manipulation.

The quality and competence of gametes are fundamental to the success of fertilization and embryonic development (Berlinguer et al., 2008; Thongphakdee et al., 2017). These characteristics can be affected by several inherent factors of the technique, as well as the culture conditions at each stage of the IVF determine the maintenance of this quality and stimulate the development of the gametes and embryos.

To be successful in IVF, it is particularly important to imitate the conditions found in the female reproductive tract during fertilization and pre-implantation embryonic development. Once this scenario is not replicated properly, the quality and competence of gametes and embryos are negatively affected (Berg and Asher, 2003). Thus, we will review the main advances obtained in the stages of IVF carried out in wild ungulates, emphasizing the strategies for improving the protocols to obtain better efficiency rates. Additionally, we discuss the conditions for collection and preparation of gametes, as well as factors related to the co-incubation of these gametes and embryo culture making a parallel with the protocols of domestic ungulates.

Oocyte preparation

Collection and selection of oocytes

The availability of competent oocytes is a marked limitation of IVF in wild ungulates. This fact makes difficult to establish ideal protocols for the manipulation of oocytes without jeopardizing their quality, which, in other species of ungulates, has been recognized as the main factor affecting blastocyst yields (Rizos et al., 2002). In this sense, the quality of oocytes for IVF can be influenced by the oocyte collection conditions performed *in vivo* or *post-mortem*.

In an ideal situation, the best strategy for obtaining good quality oocytes is to collect them *in vivo*. To accomplish this, the follicular aspiration method by laparoscopy (Thongphakdee et al., 2017), laparotomy (Berlinguer et al., 2008) and transvaginal ultrasound (Cervantes et al., 2017) techniques can be used (Table 1). Nevertheless, the reality is that few species have been studied thoroughly enough for this type of manipulation to be safe and efficient (Hildebrandt et al., 2018). This requires reliable and efficient methods for monitoring reproductive activity, which is a very challenging task in wild ungulates, as they are very temperamental and sensitive animals (Sontakke, 2018).

In domesticated camelids, such as llama (*Lama glama*, Trasorras et al., 2009) and alpaca (*Vicugna pacos*, Ratto et al., 2007), the efficiency of the laparotomy method reaches more than

80%; nevertheless, in vicunas (*Vicugna vicugna*) the recovery rate was only 55.4% (46 oocytes from the 83 follicles; Chaves et al., 2004). This shows that adaptations to the collection protocol are necessary to achieve greater success in the species of interest.

An interesting work was carried out in white rhinoceros (*Ceratotherium simum cottoni*) to allow safe and efficient collection oocytes *in vivo*. Hildebrandt et al. (2018) developed a new protocol for ovarian stimulation, anesthesia and transrectal oocyte recovery, as well as a specific collection instrument for rhinoceros. In this case, only the procedure to remove the oocyte from inside the follicle was like that done in horses, a phylogenetically close domestic species. Thus, the authors achieved a promising recovery rate of 26.4% (83 oocytes from 314 follicles).

Regarding to wild ungulates *post-mortem* female, it is possible to obtain gametes from a timely manner when individuals are euthanized, ovariectomized (in the case of hunted or slaughtered animals) or experience occasional death (Table 1). In these situations, the collection efficiency may vary, as the reproductive stage and conditions of death are often unknown, and euthanasia or ovariectomies may be performed for health reasons (Mahesh et al., 2011). Nevertheless, this can provide a unique opportunity to obtain genetic material and study the ideal conditions for *in vitro* manipulation of oocytes from wild females. Mahesh et al. (2011) had the opportunity to obtain oocytes from a female nilgai (*Boselaphus tragocamelus*) that died in an outbreak of foot and mouth disease. Despite the presence of disease and the length of time before the ovaries recovered after the death (7–24 h), it was possible to obtain of 61.9% quality oocytes, 12% of which reached the 4–8 cell stage after *in vitro* co-incubation with sperm.

From *post-mortem* females, the ovary collection and transportation conditions can also influence oocyte quality, especially when the place of death is far from laboratories, a common occurrence for wild ungulates. In Iberian red deer (*Cervus elaphus hispanicus*), ovaries can be stored for 12 h at different temperatures (5–8°C or 20–25°C) without affecting blastocyst rates ranging from 9.6 to 17.7% (García-Álvarez et al., 2011). On the other hand, oocytes recovered from Hokkaido sika deer (*Cervus nippon yesoensis*) transported at 20–25°C for 12 h had a higher maturation rate (71.0%) compared to those transported for 24 h (31.0%, Tulake et al., 2014). Although it is possible to carry ovaries securely for a few hours, it is beneficial to reduce transportation time to obtain better results.

Traditionally, follicular aspiration and ovarian slicing are the methods used to collect oocytes *in vitro* (*post-mortem*). The choice of these methods depends on the laboratory routine, ovary anatomy and oocyte attachment to the follicular wall, with great variation in recovery rates (number of oocytes recovered per female) (Table 1). In general, it is important that the method used permits recovery of good quality oocytes. For domestic ungulates, normally oocytes with many layers of *cumulus* cells and homogeneous cytoplasm are considered of

quality for maturation. In white-tailed deer (*Odocoileus virginianus*; Siriaroonrat et al., 2010) and Indian blackbuck (Rao et al., 2010), high quality oocytes had an improved maturation rate (77.6 and 68.0%) compared to those with poor morphological characteristics (17.9 and 48.0%), such as minimal layers of *cumulus* cells and heterogeneous cytoplasm.

In vitro **maturation conditions**

In domestic ungulates, as bovine, goat, sheep, swine, equine and donkey, the base medium for *in vitro* maturation (IVM) is composed of TCM-199 with 5–10% fetal bovine serum or follicular fluid and supplements that vary among species (Table 1). Normally in wild ungulates the same base medium has been used, as in wood bison (Cervantes et al., 2016), gazelle (Berlinguer et al., 2008), mouflon (Ptak et al., 2002), deer species (Berg et al., 2002; Locatelli et al., 2012; Thongphakdee et al., 2017), collared peccary (*Pecari tajacu*, Borges et al., 2020), and zebra (Meintjes et al., 1997). Interestingly, for black rhinoceros oocytes IVM, Stoops et al. (2011) used TCM-199 with 10% foetal bovine serum and obtained only 3.6% (3/83) oocytes in metaphase II (MII). Years later, Hildebrandt et al. (2018) used DMEM-F12 medium with 10% rhinoceros estrum serum for the needs of the species, reaching a maturation rate of 38.6% in white rhinoceros.

The compounds most often used as supplement to stimulate *in vitro* oocyte maturation are FSH, LH, and estradiol, as well as epidermal growth factor (EGF). Considering this, Siriaroonrat et al. (2010) evaluated the effect of 1 μg/mL of estradiol during IVM in white-tailed deer oocytes and observed a higher maturation rate in the presence of this hormone (77.6 vs. 51.1%). An improved maturation rate was also obtained by FSH and LH supplementation compared to hormone-free medium (68.0 vs. 39.6%) in blackbuck (Rao et al., 2010). These positive results can be attributed to similarities to the *in vivo* hormonal conditions in these animals.

1 Table 2. Efficiency of oocyte collection and *in vitro* maturation in wild ungulates.

Species	IUCN	Source of collection	Collection method	Oocytes/ female (%)	IVM medium*	IVM duration	IVM (%)	BL (%)	References
Collared peccary (*Pecari tajacu*)	LC	Slaughter	Follicular aspiration	16.2 (333/20)	TCM-199, 0.2 mM sodium pyruvate, 10% FBS, 20 µg/mL FSH/LH, 100 µM cysteamine, 10 ng/mL EGF	44 h	68.4	23.5	Borges et al. (2020)
Southern White Rhinoceros (*Ceratotherium simum simum*)	NT	Transrectal ultrasound guided	Follicular aspiration	4.6 (83/18)	DMEM-F12, 10% rhinoceros estrum serum	36–48 h	38.6	8.4	Hildebrandt et al. (2018)
Eld's deer (*Rucervus eldii thamin*)	EN	Ovariectomy/ laparoscopy	Slicing; Follicular aspiration	44.3 (133/3)	TCM-199, 25 mM $NaHCO_3$, 0.2 mM pyruvate, 10% FBS, 100 µg/mL FSH, 100 µg/mL LH, 1 µg/mL estradiol, 0.1 mM cysteamine	24 h	60.0	7.8	Thongphakdee et al. (2017)
Iberian red deer (*Cervus elaphus hispanicus*)	LC	Hunting	Follicular aspiration	3.4 (301/88)	TCM-199, 10% FBS, 10 mU/mL FSH, 10 mU/mL LH, 50 ng/mL EGF	24 h	50.0	ND	Macías-García et al. (2018)
Wood bison (*Bison bison athabascae*)	NT	Transvaginal ultrasound guided	Follicular aspiration	7.1 (106/15)	TCM-199, 5% FBS, 5 µg/mL LH, 0.5 µg/mL FSH	24 h	71.0	ND	Cervantes et al. (2016)
Nilgai (*Boselaphus tragocamelus*)	LC	Death by disease	Slicing	73.9 (517/7)	TCM-199, 10% FBS, 0.22 mM sodium pyruvate, 10 µg/mL FSH, 6 IU/mL LH, 1.0 µg/mL 17-β estradiol,	24 h	63.4	0.0	Mahesh et al. (2011)
African black rhinoceros (*Diceros bicornis*)	CR	Occasional death	Follicular aspiration and slicing	14.8 (74/5)	TCM-199, 0.1 g/L L-glutamine, 0.1% BSA, 2 mM/L pyruvate, 10% FBS, 1 µg/mL FSH, 5 µg/mL LH, 1 µg/mL estradiol	36 h	3.6	0.0	Stoops et al. (2011)
White-tailed deer (*Odocoileus*	LC	Slaughter	Slicing	14.2 (624/44)	TCM-199, 0.33 mM pyruvate, 2 mM glutamine, 2.2 mg/L $NaHCO_3$, 10% FBS, 5 µg/mL FSH, 5 µg/mL	24 h	77.6	ND	Siriaroonrat et al. (2010)

virginianus)					LH, 1 µg/mL estradiol				
Indian blackbuck (*Antilope cervicapra*)	LC	Occasional death	Slicing	21.8 (131/6)	TCM-199, 10% FBS, 0.22 mM pyruvate, 10 µg/mL FSH, 6 UI/mL LH	24 h	68.0	13.0	Rao et al. (2010)
Dama gazelle (*Nanger dama*)	CR	Semi-laparotomy	Follicular aspiration	5.8 (35/6)	TCM-199, 10% estrus sheep serum, 10 µg/mL FSH/LH, 1 µg/mL estradiol, 0.1 mg/mL glutamine	24 h	68.9	0.0	Berlinguer et al. (2008)
Burchell's zebra (*Equus quagga*)	NT	Slaughter	Slicing and scraping	10.2 (61/6)	TCM-199, 10% FBS, 2.5 µg/mL FSH, 2.5 µg/mL LH, 1 µg/mL estradiol	27-34 h	ND	1.0	Meintjes et al. (1997)
Hartmann's zebra (*Equus zebra hartmannae*)	VU			13.8 (83/6)					

BL: blastocyst. LC: least concern. NT: near threatened. VU: vulnerable. CR: critically endangered. EN: endangered (IUCN, 2021). ND: not determined. FBS: Fetal bovine serum. *Antibiotics and antimycotics have been omitted from the composition of the medium.

Regarding the effect of adding EGF, in Iberian red deer, an increase in oocyte maturation rate was observed in medium supplemented with FSH and LH and 50 ng/mL EGF (46.3%) compared to medium without EGF (19.5%, Macías-García et al., 2018). In collared peccary, we observed that the presence of 10 ng/mL of EGF during IVM did not improve meiotic development; nevertheless, improved oocyte morphometry (Borges et al., 2020). Thus, different oocyte responses to this growth factor are observed in these ungulates and are likely due to the physiological characteristics of each species.

Curiously, in sika deer (*Cervus nippon hortulorum*), there are differences in IVM media requirements due to seasonality. In this species, it was observed that during the estrous season, the best IVM medium was that which was supplemented with 10 µg/mL of FSH, 1 µg/mL of LH, 0.2 mM of cysteamine, and 50 ng/mL of EGF, while in the anoestrous season, twice these concentrations was more efficient (Yin et al., 2012). Additionally, based on the findings observed in studies with other deer species, Rola et al. (2021) observed that the IVM medium containing 5.0 µg/mL of FSH, 100 µg/mL of hCG and 1.0 of µg/mL estradiol was efficient for maturation of brown brocket deer (*Mazama gouazoubira*) oocytes (64.5%).

The ideal times for oocyte maturation in domestic ungulates can be quite variable, ranging from 22–24 h for cattle, sheep, and goats, 42–44 h for pigs, and 36 h for horses. Considering this, oocyte maturation times (24 and 30 h) were compared in wood bison, and it was observed that 24 h was enough for 71% of the oocytes to reach MII, like cattle (Cervantes et al., 2016). On the other hand, 27 h was necessary for 75% of red deer (*Cervus elaphus*) oocytes to reach MII using the standard medium for domestic ruminants (Berg et al., 2002). Likewise, in collared peccary, 48 h IVM was more efficient compared to 24 h for the nuclear stage, like the swine time (76.2% vs. 19.0%; Borges et al., 2018). In vicunas, 41% of oocytes were matured *in vitro* for 27 h, which is a shorter time compared to other domesticated camelids (Chaves et al., 2004). Finally, in rhinoceros, the IVM time of 36–44 h has been reported (Hildebrandt et al., 2018), which is the approximate time of horses and donkeys (Li et al., 2021). Therefore, establishing the IVM time is an initial step to obtain oocytes suitable for fertilization.

Sperm collection, selection, and capacitation

After collection, the main factors influencing sperm quality for IVF are selection and capacitation conditions. Historically, semen has been collected from wild ungulates mainly by electroejaculation (Pukazhenthi, 2016). Nevertheless, in species without electroejaculation protocols established, it is possible to obtain sperm from the epididymis in case of occasional death, castration, and euthanasia (Table 2).

In general, fresh, or preserved sperm can be used for IVF in wild ungulates. IVF has

been consistently combined with sperm refrigeration (Hermes et al., 2009) and cryopreservation (García-Álvarez et al., 2011; Locatelli et al., 2012) for practicality and due to germplasm banks (Benham et al., 2021). Nevertheless, semen conservation protocols can impair sperm quality, as already reviewed by Pukazhenthi (2016). Therefore, it is important to note that sperm storage protocols have not been defined for many wild ungulates´s species. Thus, it is necessary to develop efficient methods to maintain the viability and fertility of these gametes.

The two most common sperm selection methods in wild ungulates are the Percoll® (colloidal silica coated with polyvinylpyrrolidone) gradient and swim-up methods as shown in the Table 2. When using the appropriate method for the species, the selection increases the percentage of motility, viability, normal morphology, and fertility (Liu et al., 2013; Santiago-Moreno et al., 2014). In addition, this step is essential to remove seminal plasma (fresh semen), extender and cryoprotectants (frozen/thawed sperm) that may negatively influence the fertilization (Liu et al., 2013). For frozen-thawed scimitar-horned oryx (*Oryx dammah*) spermatozoa, better quality sperm were obtained using Percoll and swim-up compared to the washing method (centrifugation); nevertheless, the success of heterologous fertilization was similar among treatments (65–72.7% cleavage; O'Brien and Roth, 2000). For gazelle (*Gazella dama mhorr*), motility increased from 53.1 to 77.5% after the selection of frozen/thawed sperm using the swim-up (Berlinguer et al., 2008).

1 Table 2. Sperm collection, selection and co-incubation conditions in wild ungulates associated with the development of embryos produced by *in vitro*
2 fertilization.

Species	IUCN	Sperm collection	Sperm types	Selection method	Motile (%)	Concentration (x 10^6)	IVF medium	Efficiency (%)	References
Wood bison (*Bison bison athabascae*)	EN	ND	Frozen/ thawed	Percoll® (45/90%)	60–75	5.0	Brackett-Oliphant	53.8 BL	Palomino et al. (2019)
Eld's deer (*Rucervus eldii thamin*)	EN	ND	Frozen/ thawed	Percoll® (45/90%)	ND	0.05	Deer-SOF	7.8 BL	Thongphakdee et al. (2017)
Vietnamese sika deer (*Cervus Nippon pseudaxis*)	LC	Electroejaculation	Frozen/ thawed	Percoll® (45/90%)	ND	3.0	SOFaa, 20% ESS, 5 µg/mL heparin	29 BL	Locatelli et al. (2012)
Iberian red deer (*Cervus elaphus hispanicus*)	LC	Epididymis	Frozen/ thawed	Percoll® (45/90%)	ND	1.0	Deer-SOF, 20% ESS	17.7 BL	García-Álvarez et al. (2011)
Nilgai (*Boselaphus tragocamelus*)	LC	Epididymis	Fresh	Swim-up	50–60	0.01	TALP, 100 µg⁄mL heparin	42 CL	Mahesh et al. (2011)
Black rhinoceroses (*Diceros bicornis minor*)	CR	ND	Cooling (4ºC)	Swim-up	70	0.5	SOF, 20 µg/mL heparin, 5% ERS	50 CL	Hermes et al. (2009)
Gazelle (*Nanger dama*)	CR	Electroejaculation	Frozen/ thawed	Swim-up	77.5	1.0	SOF, 2% ESS, 1 µg⁄ml hypotaurin, 10 µg⁄ml heparin	30 CL	Berlinguer et al. (2008)
European Mouflon (*Ovis*	VU	ND	Frozen/	Swim-up	ND	1.0	SOF, 20% ESS,	37 BL	Ptak et al.

orientalis musimon)			thawed				2.9 mM calcium lactate, 16 mM isoproterenol		(2002)
Burchell's zebra (*Equus quagga*)	NT	Epididymis	Cooling (4ºC)	Not selected	ND	9.0	Hsm's F10, 1 µM calcium ionophore	38 CL	Meintjes et al. (1997)

1 EN: endangered. LC: least concern. VU: vulnerable. CR: critically endangered (IUCN, 2021). ND: not determined. ESS: estrus sheep serum. ERS:
2 estrous (white) rhinoceros serum. *Only centrifugation. CL: cleavage rate. BL: blastocyst rate.

The methods of sperm selection in Iberian ibex (*Capra pyrenaica*) and European mouflon (*Ovis orientalis*) have been compared. Capripure® (colloidal silica particles coated with silane) density-gradient centrifugation and dextran swim-up methods showed recovery rates of quality spermatozoa of 60.6 vs. 11.3% (*C. pyrenaica*) and 47.8 vs. 27.8% (*O. orientalis*), respectively. Therefore, density-gradient centrifugation was recommended for both species, highlighting the similarities among the different genera (Santiago-Moreno et al., 2014). Also, the Percoll gradient improved the linearity (64.5 *vs*. 28.5%) and viability (76.0 *vs*. 40.9%) of post-thaw sperm in Iberian red deer (García-Álvarez et al., 2016).

After sperm selection, proper oocyte-sperm interaction requires sperm capacitation, which is usually induced by different capacitating agents present in the IVF culture medium. Heparin has been widely used for sperm capacitation in domestic ruminants. However, although heparin for 1–4 h increases sperm capacitation in cattle, in the scimitar-horned oryx (*Oryx dammah*) that belongs to the same family, the rate of acrosome reaction did not increase in 6 h of incubation, suggesting that this species may require a longer incubation time (Roth et al., 1998). On the other hand, the effect of heparin (60 µg/mL) as a capacitating agent was evaluated in fallow deer (*Dama dama*) and led to an improved rate of acrosome reaction compared to the control medium (Fernández et al., 2013).

In general, equine sperm has difficulties to fertilize oocytes *in vitro*, probably due to deficient capacitation (Leemans et al., 2019). Thus, studies to improve the capacitation conditions for sperm in domestic and wild horses are fundamental for the success of IVF in these animals. In Persian Onager (*Equus hemionus onager*), the acrosome integrity was evaluated after sperm thawing, being observed good resistance of this species that kept 63% of intact acrosomes after 24 h of incubation at 22ºC (Schook et al., 2013). Although capacitation allows fertilization to occur, studies on sperm capacitation of wild ungulates are still restricted.

***In vitro* fertilization and embryo culture**

During gamete co-incubation, sperm concentration and culture medium may influence the IVF result. In the gaur (*Bos gaurus*), a higher sperm concentration (5.0×10^6 sperm/mL) increased polyspermy by 40% when compared to a lower concentration

(1.0 × 10^6 sperm/mL, Johnston et al., 1994). In red deer, a rate of 9% blastocyst formation after IVF with 2.0 × 10^6 sperm/mL was observed, while a sperm concentration of 1.0 × 10^6 sperm/mL was not able to generate blastocysts (Comizzoli et al., 2001a). On the other hand, under optimized conditions in the same species, Berg et al. (2002) evaluated sperm concentrations ranging from 0.001 to 1.0 × 10^6 sperm/mL, and the best monospermic penetration rate was obtained using 0.4 × 10^6 sperm/mL (77.0%). These controversial data show the importance of continuous studies in different conditions to achieve more satisfactory results, allowing efficient fertilization without causing polyspermy.

The composition of the IVF medium should favor the maintenance of the quality of the gametes and stimulate fertilization. Among the most common medium of IVF in ungulates are synthetic oviduct fluid (SOF, Tervit et al., 1972), Tyrode's albumin lactate pyruvate (Parrish et al., 1988), FERT (Rath et al., 1999) and Brackett-Oliphant (Brackett and Oliphant, 1975), which are supplemented with capacitation agents. Nevertheless, these conditions have been little studied in wild ungulates. In red deer, the addition of the capacitation agents: sheep serum (20%), heparin (20 µg/mL) and/or bovine serum albumin (BSA; 6 mg/mL) in the IVF medium was evaluated. At work, only the medium containing sheep serum allowed an acceptable rate of monospermic fertilization (56.2%), like is observed in small domestic ruminants (Berg et al., 2002).

In horses, the success of IVF is very limited due to the difficulty for sperm to penetrate the zona pellucida *in vitro* (Hinrichs et al., 2002). Compared to other domestic mammals, conventional culture conditions have not been shown to be effective in activating the sperm capacitance triggers that allow efficient fertilization (Leemans et al., 2019). For this reason, alternatives have been sought to overcome this barrier. Meintjes et al. (1997) used a drilling technique with acid solution of the zona pellucida before co-incubation with Burchell's Zebra sperm. In this case, only 41% (31/75) of the oocytes cleaved and only 2% (1/75) developed to the blastocyst stage. In this sense, advances achieved in the IVF stage about the use of intracytoplasmic sperm injection (ICSI) can strongly benefit horses. Recently, a 6% rate of blastocysts was observed performing the ICSI of zebra sperm (*Equus quagga burchelli*) in horse oocytes (Gambini et al., 2020).

Additionally, ICSI methodology can increase the success of IVF in wild ungulates with low quality gametes. In the rhinoceros (*Ceratotherium simum* and *Diceros bicornis*), the use of IVF and ICSI for embryo production have been described; nevertheless, it was

only possible to obtain a 4-cell embryo after IVF in Black rhinoceros, suggesting further reproductive studies are required (Hermes et al., 2009). Subsequently, ICSI was performed on Southern White rhinoceros (*Ceratotherium simum simum*) and a rate of 14.5% of cleaved embryos and 8.4% of blastocysts was obtained (Hildebrandt et al., 2018).

The time of *in vitro* development to the blastocyst stage varies among species of domestic ungulates, being 7–8 days in cattle and sheep, as well as 6–7 days in pigs and horses (Piliszek and Madeja, 2018). Several *in vitro* culture protocols of domestic and wild ungulate embryos use the SOF medium, although in domestic swine the medium for porcine embryos (PZM) is widely used. Nevertheless, interestingly, collared peccary embryos showed good development *in vitro* using SOF medium (Borges et al., 2020).

Few species of wild ungulates have been studied until the stage of embryo culture in the IVF, and consequently only some species had embryos transferred and births (Figure 2). In many cases embryonic development does not reach the blastocyst phase, or the rate of blastocyst formation was low (Table 2). This is likely due to the conditions of the previous steps being unsuitable to promote efficient embryonic development. Moreover, the *in vitro* embryo culture (IVC) medium must also be carefully chosen to ensure successive cleavage of the embryos. Red sheep (*Ovis orientalis gmelini*) zygotes were cultured in Charles Rosenkrans 2 medium (CR2) or modified Brinster's ovum culture medium with bovine oviductal epithelial cells. These culture conditions did not lead to different cleavage rates, which ranged from 23.0 to 29.0%, and both medium allowed the birth of offspring after embryo transfer (Flores-Foxworth et al., 1995). In red deer, the presence of ovine oviduct epithelial cells in SOF medium during IVC increases the rate of blastocyst formation compared to the medium alone (39.0 vs. 5.0%; Locatelli et al., 2005).

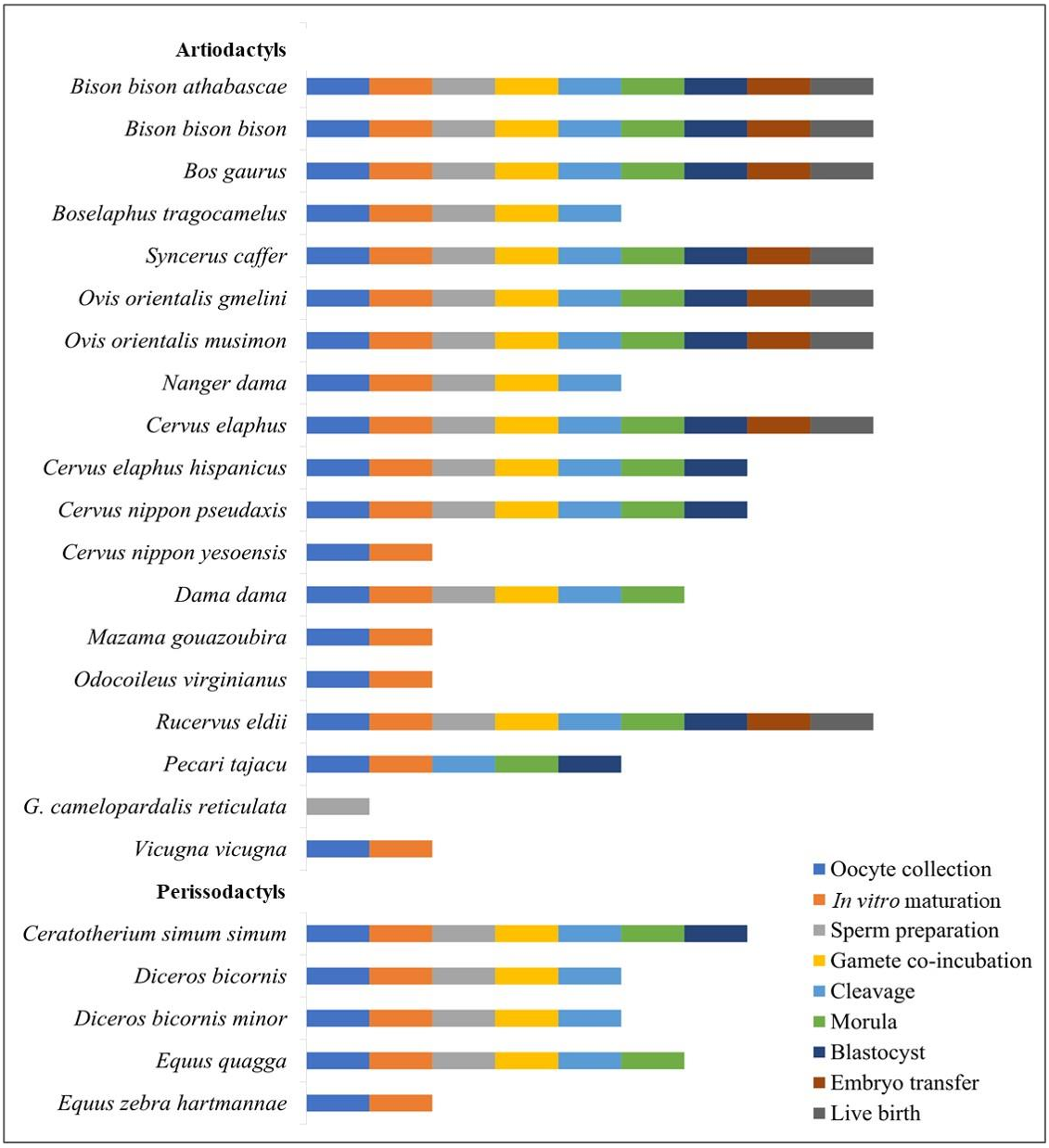

Figure 2. Development of *in vitro* fertilization (IVF) steps in several wild ungulate's species.

Techniques that assist in the establishment of IVF protocols

Parthenogenetic activation

Parthenogenetic or artificial oocyte activation strategy has been used as a model for evaluating experimental conditions that can influence embryonic development after IVF and ICSI in wild species (Yin et al., 2012). This technique can be used to assess oocyte competence and establish embryo culture media (Borges et al., 2020). Moreover, most mammals subjected to ICSI require an activation protocol to induce embryonic

development. Thus, artificial oocyte activation can help establish these protocols. Rao et al. (2010) evaluated the competence of matured oocytes from Indian blackbuck for embryonic development using parthenogenetic activation and obtained 58.0% of cleavage and 13.0% of blastocysts. In wild deer species, artificial activation was used to assess the competence of oocytes collected immediately or 12 h after the animal death, being no significant effect observed on embryonic development (Brahmasani et al., 2013).

For this type of application, it is important to establish the ideal activation protocol in different species. Yin et al. (2012) evaluated two chemical activation protocols for sika deer oocytes and obtained a higher rate of blastocysts (32.4%) using a combination of ionomycin and 6-dimethylaminopurine (6-DMAP). The authors compared the quality of activated embryos with embryos produced *in vivo* and observed no difference, demonstrating the efficiency of artificial oocyte activation. Similarly, in collared peccary, we tested ionomycin and four secondary activator combinations (6-DMAP, 6-DMAP and cytochalasin B, cycloheximide, and cycloheximide and cytochalasin B), and ionomycin and 6-DMAP was superior in producing blastocysts (23.5%) and could be used to establish culture conditions for ICSI and cloning by somatic cell nuclear transfer (Borges et al., 2020).

Heterologous *in vitro* fertilization

Studies on IVF conditions in wild ungulates are scarce, likely due to the challenge in obtaining enough mature oocytes for the evaluation of different experimental groups. To this end, heterologous IVF could be used, as it has already been applied as an alternative tool to evaluate sperm fertility and the response of spermatozoa to different cryopreservation and processing protocols. In this sense, this technique was useful to assess the longevity of frozen–thawed oryx sperm pre-incubated for 12 and 24 h before co-incubation with oocytes, obtaining 56.2 and 19.4% of embryos with ≥8-cell stage (O'Brien and Roth, 2000). Pradieé et al. (2018) evaluated the effect of different methods of cryopreserving Iberian ibex epididymal sperm on fertilizing capacity using bovine oocytes and observed similar cleavage rates between freezing (31.3%) and vitrification (45.1%), demonstrating that both methods can be used.

Heterologous IVF was used for sika deer sperm and zona-free bovine oocytes to assess IVF media and characterize sperm-oocyte interactions, being highlighted that this

technique can be a tool for sperm selection that confers high development potential before homologous IVF or to understand unsuccessful homologous IVF (Comizzoli et al., 2001b). In this sense, the male reproductive seasonality of the North American bison (*Bison bison*) was also evaluated using heterologous IVF with bovine oocytes. Sperm recovered in the reproductive season led to a higher rate of blastocyst formation (30.5 *vs.* 13.7%, Krishnakumar et al., 2015).

Another interesting work was carried out with heterologous IVF aiming to evaluate the effectiveness of the cattle IVF protocol for African antelope species: antelope (*Antidorcas marsupialis*), impala (*Aepyceros melampus*) and blesbok (*Damaliscus dorcus phillipsi*). The authors observed that the protocol enabled rates above 50% penetration and cleavage, but improvements are needed to obtain embryos in the blastocyst stage in these antelope species (Chatiza et al., 2013). This reinforces those protocols of domestic species can be used as a starting point for studies in wild species. From the results obtained new hypotheses are generated and improvements can be made.

Furthermore, it is known that many closely related species can produce viable hybrid individuals in nature, such as hybrids of sika deer (*Cervus nippon*; 2n=68) and red deer (*Cervus elaphus*; 2n=68); as well as hybrids for genetic improvement purposes, such as American bison (*Bison bison*; n=60) with domestic cattle (2n=60) (Gabryś et al., 2021) can be produced. This knowledge is also useful for studies with IVF, as observed by Meintjes et al. (1997) who produced zebroid embryos using Burchell's Zebra (*Equus burchelli*) sperm and Hartmann's Zebra (*Equus zebra hartmannae*) oocytes for evaluation of culture conditions. On the other hand, Owiny et al. (2009) evaluated the hybridization potential of African buffalo (*Syncerus caffer caffer*) sperm and cattle oocytes for genetic improvement purposes, but was not successful in producing blastocysts, probably due to chromosomal disparity.

Heterologous ICSI can also provide important information to improve embryo *in vitro* production. This technique has been applied to assess the fertility of giraffe (*Giraffa camelopardalis reticulata*) sperm preserved by the freeze-drying method using mouse oocytes, with 100% pronucleus formation observed (Kaneko et al., 2014). Gambini et al. (2020) showed valuable information regarding the manipulation of Zebra sperm and used porcine oocytes to estimate its ability to induce activation after ICSI, that showed 30% pronucleus formation. After this evaluation, the sperm were injected into oocytes of

horses and were able to develop until the blastocyst stage. This result highlights that horses can be models for wild equine, as oocytes supported hybrid development without compromising the expression and localization of cell differentiation markers and blastocyst cell number (Gambini et al., 2020).

Heterologous ICSI also played a key role in the development of a protocol for rhinocero homologous gametes. Initially, Hildebrandt et al. (2018) inject rhinocero sperm into pig oocytes to assess fertility and the need for artificial activation. There is an increase of 30 to 90% in the fertilization rate and the need to activate oocytes after ICSI of rhinoceros with low performance was noted.

Final considerations

IVF can be a valuable tool for the conservation of wild ungulates. In these animals, the greatest advances in IVF in terms of obtaining embryos and births occurred in species of artiodactyl from the Bovidae and Cervidae families. Among the reasons for this, we highlight the diversity of well-studied domestic models and the availability of individuals of the wild species for studies. In the other families of wild ungulates, the results are initial, often with low efficiency and reproducibility.

Many factors must be considered to achieve successful IVF in these animals. Initially, it is necessary to promote research to understand fundamental characteristics of reproduction and physiology of gametes and embryos from different families of wild ungulates. It is important to use advances made in domestic species in favor of acquiring more information about wild species. For this, techniques such as heterologous IVF, ICSI and artificial activation can be more and better explored. Furthermore, copying protocols from domestic models to wild species is not an effective strategy. Reproductive information for the species of interest should be combined with information from the available domestic model(s). Thus, it is possible to create appropriate experimental designs to make the best use of the availability of gametes to establish the ideal culture and manipulation conditions for different species.

It is unlikely that IVF/embryo transfer will become a routine technique for wild ungulates management, but it is undoubtedly an alternative that presents numerous advantages over other reproductive techniques. Recent efforts to establish IVF techniques are encouraging and will be central to the conservation of endangered ungulates and the

management of abundant species with economic value.

References

Andrabi S.M.H., Maxwell W.M.C. (2007). A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim. Reprod. Sci., 99: 223–243.

Benham H.M., McCollum M.P., Nol P., Frey R.K., Clarke P.R., Rhyan J.C., Barfield J.P. (2021). Production of embryos and a live offspring using *post-mortem* reproductive material from bison (*Bison bison bison*) originating in Yellowstone National Park, USA. Theriogenology, 160: 33–39.

Berg D.K., Thompson J.G., Asher G.W. (2002). Development of in vitro embryo production systems for red deer (*Cervus elaphus*): part 2. The timing of in vitro nuclear oocyte maturation. Anim. Reprod. Sci., 70: 77–84.

Berg D.K., Asher, G.W. (2003). New developments reproductive technologies in deer. Theriogenology, 59: 189–205.

Berlinguer F., González R., Succu S., Del Olmo A., Garde J.J., Espeso G., Gomendio M., Ledda S., Roldan E.R. (2008). *In vitro* oocyte maturation, fertilization and culture after ovum pick–up in an endangered gazelle (*Gazella dama mhorr*). Theriogenology, 69(3): 349–359.

Borges A.A., Santos, M.V.O., Nascimento L.E., Lira G.P.O., Praxedes É.A., Oliveira M.F., Silva A.R., Pereira A.F. (2020). Production of collared peccary (*Pecari tajacu* Linnaeus, 1758) parthenogenic embryos following different oocyte chemical activation and *in vitro* maturation conditions. Theriogenology, 142: 320–327.

Borges A.A., Santos M.V.O., Queiroz Neta L.B., Oliveira M.F., Silva A.R., Pereira A.F. (2018). *In vitro* maturation of collared peccary (*Pecari tajacu*) oocytes after different incubation times. Pesq. Vet. Bras., 38(9): 1863–1868.

Brackett B.G., Oliphant G. (1975). Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod., 12: 260–274.

Brahmasani S.R., Yelisetti U.M., Katari V., Komjeti S., Lakshmikantan U., Pawar R.M., Sisinthy S. (2013). Developmental ability after parthenogenetic activation of *in vitro* matured oocytes collected postmortem from deers. Small Rumin. Res., 113(1): 128–135.

Cervantes M.P., Palomino J.M., Anzar M., Mapletoft R.J., Adams G.P. (2016). *In vivo*

and *in vitro* maturation of oocytes collected from superstimulated wood bison (*Bison bison athabascae*) during the anovulatory and ovulatory seasons. Anim. Reprod. Sci., 173: 87–96.

Cervantes M.P., Palomino J.M., Anzar M., Mapletoft R.J., Mastromonaco G.F., Adams G.P. (2017). *In vitro*–production of embryos using immature oocytes collected transvaginally from superstimulated wood bison (*Bison bison athabascae*). Theriogenology, 92: 103–110.

Chatiza F.P., Bartels P., Nedambale T.L., Wagenaar G.M. (2013). Sperm–egg interaction and functional assessment of springbok, impala and blesbok cauda epididymal spermatozoa using a domestic cattle *in vitro* fertilization system. Anim. Reprod. Sci., 143(1–4): 8–18.

Chaves M.G., Miragaya M.H., Capdevielle E.F., Rutter B., Giuliano S.M., Agüero A. (2004). *In vitro* maturation of vicuna oocytes recovered by surgical aspiration of follicles from superstimulated ovaries. Biocell, 28: 545.

Comizzoli P., Mermillod P., Mauget R. (2000). Reproductive biotechnologies for endangered mammalian species. Reprod. Nutr. Dev., 40: 493–504.

Comizzoli P., Mermillod P., Cognie Y., Chai N., Legendre X., Mauget R. (2001a). Successful *in vitro* production of embryos in the red deer (*Cervus elaphus*) and the sika deer (*Cervus nippon*). Theriogenology, 55(2): 649–659.

Comizzoli P., Mauget R., Mermillod P. (2001b). Assessment of *in vitro* fertility of deer spermatozoa by heterologous IVF with zona-free bovine oocytes. Theriogenology, 56(2): 261–274.

Fernández S., Sestelo A., Rivolta M., Córdoba M. (2013). Capacitation and acrosome reaction induction on thawed Dama dama deer spermatozoa: glycine effect as cryopreservation diluent supplement. Zool. Sci., 30(12): 1110–1116.

Flores–Foxworth G., Coonrod S.A., Moreno J.F., Byrd S.R., Kraemer D.C., Westhusin M. (1995). Interspecific transfer of IVM IVF–derived red sheep (*Ovis orientalis gmelini*) embryos to domestic sheep (*Ovis aries*). Theriogenology, 44(5): 681–690.

Gabryś J., Kij B., Kochan J., Bugno-Poniewierska M. (2021). Interspecific hybrids of animals-in nature, breeding and science–a review. Ann. Anim. Sci., 21(2): 403–415.

Gambini A., Duque Rodríguez M., Rodríguez M.B., Briski O., Flores Bragulat A.P., Demergassi N., Losinno L., Salamone D.F. (2020). Horse ooplasm supports *in vitro*

preimplantation development of zebra ICSI and SCNT embryos without compromising YAP1 and SOX2 expression pattern. Plos one, 15(9): e0238948.

García–Álvarez O., Maroto–Morales A., Berlinguer F., Fernández–Santos M.D.R., Esteso M.C., Mermillod P., Ortiz J.A., Ramon M., Pérez-Guzmán M.D., Garde J.J., Soler A.J. (2011). Effect of storage temperature during transport of ovaries on *in vitro* embryo production in Iberian red deer (*Cervus elaphus hispanicus*). Theriogenology, 75(1): 65–72.

García–Álvarez O., Soler A., Maulen Z., Maroto–Morales A., Iniesta–Cuerda M., Martín–Maestro A., Fernández-Santos M.R., Garde J. (2016). Selection of red deer spermatozoa with different cryoresistance using density gradients. Reprod. Domest. Anim., 51: 895–900.

Gordon I.J., Hester A.J., Festa-Bianchet M. (2004). The management of wild large herbivores to meet economic, conservation and environmental objectives. Reprod. Domest. Anim., 41(6): 1021–1031.

Hermes R., Göritz F., Portas T.J., Bryant B.R., Kelly J.M., Maclellan L.J., Keeley T., Schwarzenberger F., Walzer C., Schnorrenberg A., Spindler R.E., Saragusty J., Kaandorp S., Hildebrandt T.B. (2009). Ovarian superstimulation, transrectal ultrasound–guided oocyte recovery, and IVF in rhinoceros. Theriogenology, 72(7): 959–968.

Herrick J.R. (2019). Assisted reproductive technologies for endangered species conservation: developing sophisticated protocols with limited access to animals with unique reproductive mechanisms. Biol. Reprod., 100: 1158–1170.

Hildebrandt T.B., Hermes R., Colleoni S., Diecke S., Holtze S., Renfree M.B., Stejskal J., Hayashi K., Drukker M., Loi P., Göritz F., Lazzari G., Galli C. (2018). Embryos and embryonic stem cells from the white rhinoceros. Nat. Commun., 9: 1–9.

Hinrichs K., Love C.C., Brinsko S.P., Choi Y.H., Varner D.D. (2002). *In vitro* fertilization of *in vitro*-matured equine oocytes: effect of maturation medium, duration of maturation, and sperm calcium ionophore treatment, and comparison with rates of fertilization *in vivo* after oviductal transfer. Biol. Reprod., 67(1): 256–262.

IUCN – International Union for Conservation of Nature and Natural Resources [homepage on the Internet]. Red List of Threatened Species [cited 2021 March 01]. Available from: http://www.iucnredlist.org/details/41777/0.

Johnston L.A., Parrish J.J., Monson R., Leibfried–Rutledge L., Susko–Parrish J.L., Northey D.L., Rutledge J.J., Simmons L.G. (1994). Oocyte maturation, fertilization and embryo development *in vitro* and *in vivo* in the gaur (*Bos gaurus*). Reproduction, 100(1): 131–136.

Kaneko T., Ito H., Sakamoto H., Onuma M., Inoue–Murayama M. (2014). Sperm preservation by freeze–drying for the conservation of wild animals. PloS one, 9(11): e113381.

Krishnakumar S., Whiteside D.P., Elkin B., Thundathil J.C. (2015). Effect of reproductive seasonality on gamete quality in the North American bison (*Bison bison bison*). Reprod. Domest. Anim., 50(2): 206–213.

Leemans B., Stout T.A., Schauwer C., Heras S., Nelis H., Hoogewijs M., Soom A.V., Gadella B.M. (2019). Update on mammalian sperm capacitation: how much does the horse differ from other species? Reproduction, 157(5): 181–197.

Li Z., Song X., Yin S., Yan J., Lv P., Shan H., Cui K., Liu H., Liu Q. (2021). Single-Cell RNA-seq revealed the gene expression pattern during the *in vitro* maturation of donkey oocytes. Genes, 12(10): 1–15.

Liu B., Cui Y., Yu S. (2013). Effect of swim–up and percoll treatment on sperm quality and *in vitro* embryo development in yak. J. Integr. Agric., 12: 2235–2242.

Locatelli Y., Cognié Y., Vallet J.C., Baril G., Verdier M., Poulin N., Legendre X., Mermillod P. (2005). Successful use of oviduct epithelial cell coculture for *in vitro* production of viable red deer (*Cervus elaphus*) embryos. Theriogenology, 64(8): 1729–1739.

Locatelli Y., Hendriks A., Vallet J.C., Baril G., Duffard N., Bon N., Ortiz K., Scala C., Maurel M-C., Mermillod P., Legendre, X. (2012). Assessment LOPU–IVF in Japanese sika deer (*Cervus nippon nippon*) and application to Vietnamese sika deer (*Cervus nippon pseudaxis*) a related subspecies threatened with extinction. Theriogenology, 78(9): 2039–2049.

Macías-García B., González-Fernández L., Matilla E., Hernández N., Mijares J., Sánchez-Margallo F.M. (2018). Oocyte holding in the Iberian red deer (*Cervus elaphus hispanicus*): Effect of initial oocyte quality and epidermal growth factor addition on *in vitro* maturation. Reprod. Domest. Anim., 53(1): 243–248.

Mahesh Y.U., Rao B.S., Suman K., Lakshmikantan U., Charan K.V., Gibence H.R.W.,

Shivaji S. (2011). *In vitro* maturation and fertilization in the nilgai (*Boselaphus tragocamelus*) using oocytes and spermatozoa recovered post-mortem from animals that had died because of foot and mouth disease outbreak. Reprod. Domest. Anim., 46(5): 832–839.

Malakoutikhah S., Fakheran S., Hemami M.R., Tarkesh M., Senn J. (2020). Assessing future distribution, suitability of corridors and efficiency of protected areas to conserve vulnerable ungulates under climate change. Divers. Distrib., 26(10): 1383–1396.

Mastromonaco G.F., Songsasen N. (2020). Reproductive technologies for the conservation of wildlife and endangered species. In: Reproductive Technologies in Animals, Presicce G.A. (ed). Academic Press, Rome, Italy, pp. 99–117.

Meintjes M., Bezuidenhout C., Bartels P., Visser D.S., Meintjes J., Loskutoff N.M., Fourie F. L., Barry D.M., Godke R.A. (1997). *In vitro* maturation and fertilization of oocytes recovered from free–ranging Burchell's zebra (*Equus burchelli*) and Hartmann's zebra (*Equus zebra hartmannae*). J. Zoo Wildl. Med., 251–259.

Mohr D., Cohnstaedt L.W., Topp W. (2005). Wild boar and red deer affect soil nutrients and soil biota in steep oak stands of the Eifel. Soil Biol. Biochem., 37(4): 693–700.

O'Brien J.K., Roth T.L. (2000). Functional capacity and fertilizing longevity of frozen–thawed scimitar–horned oryx (*Oryx dammah*) spermatozoa in a heterologous *in vitro* fertilization system. Reprod. Fertil. Dev., 12(8): 413–421.

Owiny O.D., Barry D.M., Agaba M., Godke R.A. (2009). *In vitro* production of cattle×buffalo hybrid embryos using cattle oocytes and African buffalo (*Syncerus caffer caffer*) epididymal sperm. Theriogenology, 71(6): 884–894.

Palomino J.M., Mastromonaco G.F., Cervantes M.P., Mapletoft R.J., Anzar M., Adams G.P. (2020). Effect of season and superstimulatory treatment on *in vivo* and *in vitro* embryo production in wood bison (*Bison bison athabascae*). Reprod. Domest. Anim., 55: 54–63.

Parrish J.J., Susko–Parrish J., Winer M.A., First N.L. (1988). Capacitation of bovine sperm by heparin. Biol. Reprod., 38: 1171–1180.

Piliszek A., Madeja Z.E. (2018). Pre-implantation development of domestic animals. Curr. Top. Dev. Biol., 128: 267–294.

Pradieé J., Sánchez–Calabuig M.J., Castaño C., O'Brien E., Esteso M.C., Beltrán–Breña P., Maillo V., Santiago-Moreno J., Rizos D. (2018). Fertilizing capacity of vitrified

epididymal sperm from Iberian ibex (*Capra pyrenaica*). Theriogenology, 108, 314–320.

Ptak G., Clinton M., Barboni B., Muzzeddu M., Cappai P., Tischner M., Loi P. (2002). Preservation of the wild European mouflon: the first example of genetic management using a complete program of reproductive biotechnologies. Biol. Reprod., 66(3): 796–801.

Pukazhenthi B.S. (2016). Saving wild ungulate diversity through enhanced management and sperm cryopreservation. Reprod. Fertil. Dev., 28(8): 1133–1144.

Rao B.S., Mahesh,Y.U., Lakshmikantan U.R., Suman K., Charan K.V., Shivaji S. (2010). Developmental competence of oocytes recovered from *postmortem* ovaries of the endangered Indian blackbuck (*Antilope cervicapra*). J. Reprod. Dev., 56: 623–629.

Rath D., Long C.R., Dobrinsky J.R., Welch G.R., Schreier L.L., Johnson L.A. (1999). *In vitro* production of sexed embryos for gender preselection: high–speed sorting of X–chromosome–bearing sperm to produce pigs after embryo transfer. J. Anim. Sci., 77: 3346–3352.

Ratto M., Gomez C., Berland M., Adams G.P. (2007). Effect of ovarian superstimulation on COC collection and maturation in alpacas. Anim. Reprod. Sci., 97(3–4): 246–256.

Rizos D., Ward F., Duffy P.A.T., Boland M.P., Lonergan P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. Mol. Reprod. Dev., 61: 234–248.

Rola L.D., Zanetti E.S., Del Collado M., Peroni E.D.F.C. Duarte J.M.B. (2021). Collection and *in vitro* maturation of *Mazama gouazoubira* (brown brocket deer) oocytes obtained after ovarian stimulation. Zygote, 29(3): 216–222.

Roth T.L., Weiss R.B., Buff JL., Bush L.M., Wildt D.E., Bush M. (1998). Heterologous *in vitro* fertilization and sperm capacitation in an endangered African antelope, the scimitar–horned oryx (*Oryx dammah*). Biol. Reprod., 58(2), 475–482.

Santiago–Moreno J., Esteso M.C., Castaño C., Toledano–Díaz A., Rodríguez E., López–Sebastián A. (2014). Sperm selection by Capripure® density–gradient centrifugation versus the dextran swim–up procedure in wild mountain ruminants. Anim. Reprod. Sci., 149(3–4): 178–186.

Schook M.W., Wildt D.E., Weiss R.B., Wolfe B.A., Archibald K.E., Pukazhenthi B.S.

(2013). Fundamental studies of the reproductive biology of the endangered Persian onager (*Equus hemionus onager*) result in first wild equid offspring from artificial insemination. Biol. Reprod., 89(2): 41–51.

Siriaroonrat B., Comizzoli P., Songsasen N., Monfort S.L., Wildt D.E., Pukazhenthi B.S. (2010). Oocyte quality and estradiol supplementation affect *in vitro* maturation success in the white–tailed deer (*Odocoileus virginianus*). Theriogenology, 73(1): 112–119.

Sontakke S.D. (2018). Monitoring and controlling ovarian activities in wild ungulates. Theriogenology, 109: 31–41.

Stoops M.A., O'brien J.K., Roth T.L. (2011). Gamete rescue in the African black rhinoceros (*Diceros bicornis*). Theriogenology, 76(7): 1258–1265.

Tervit H.R., Whittingham D.G., Rowson L.E.A. (1972). Successful culture in vitro of sheep and cattle ova. Reproduction, 30: 493–497.

Thongphakdee A., Berg D.K., Tharasanit T., Thongtip N., Tipkantha W., Punkong C., Tongthainan D., Noimoon S., Maikeaw U., Kajornklin N., Siriaroonrat B., Comizzoli P., Kamolnorranath S. (2017). The impact of ovarian stimulation protocol on oocyte quality, subsequent *in vitro* embryo development, and pregnancy after transfer to recipients in Eld's deer (*Rucervus eldii thamin*). Theriogenology, 91: 134–144.

Trasorras V.L., Chaves M.G., Miragaya M.H., Pinto M., Rutter B., Flores M., Agüero A. (2009). Effect of eCG superstimulation and buserelin on *cumulus*–oocyte complexes recovery and maturation in llamas (*Lama glama*). Reprod. Domest. Anim., 44(3): 359–364.

Tulake K., Yanagawa Y., Takahashi Y., Katagiri S., Higaki S., Koyama K., Wang X., Li H. (2014). Effects of ovarian storage condition on *in vitro* maturation of Hokkaido sika deer (*Cervus nippon yesoensis*) oocytes. Jpn. J. Vet., 62(4): 187–192.

Velamazan M., Perea R., Bugalho M.N. (2020). Ungulates and ecosystem services in Mediterranean woody systems: A semi–quantitative review. J. Nat. Conserv., 55: 125837.

Yin Y., Tang L., Zhang P., Kong D., Wang Z., Guan J., Song G., Tan, B., Li Z. (2013). Optimizing the conditions for *in vitro* maturation and artificial activation of sika deer (*Cervus nippon hortulorum*) oocytes. Reprod. Domest. Anim., 48(1), 27–32.

CAPÍTULO 3 – EFFECTS OF DIFFERENT SELECTION TECHNIQUES ON PHYSIOLOGICAL AND FUNCTIONAL QUALITY OF SPERM COLLECTED FROM COLLARED PECCARIES

Artigo experimental: Effects of different selection techniques on physiological and functional quality of sperm collected from collared peccaries

Periódico de submissão: Reproductive Biology

Fator de impacto: 2,089

Data de submissão: 8 de fevereiro de 2023

Effects of different selection techniques on physiological and functional quality of sperm collected from collared peccaries

Maria Valéria de Oliveira Santos[a], Andréia Maria da Silva[b], Leonardo Vitorino Costa de Aquino[a], Lhara Ricarliany Medeiros de Oliveira[a], Samara Sandy Jeronimo Moreira[b], Moacir Franco de Oliveira[c], Alexandre Rodrigues Silva[b], Alexsandra Fernandes Pereira[a*]

[a]Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoro, RN, Brazil. [b]Laboratory of Animal Germplasm Conservation, Federal Rural University of Semi-Arid, Mossoro, RN, Brazil. [c]Laboratory of Applied Animal Morphophysiology, Federal Rural University of Semi-Arid, Mossoro, RN, Brazil.

*Corresponding author: Alexsandra Fernandes Pereira
Laboratory of Animal Biotechnology
Federal Rural University of Semi-Arid
Av. Francisco Mota, 572, Mossoró, RN, 59625-900, Brazil
Phone: +55 84 3317 8361
E-mail address: alexsandra.pereira@ufersa.edu.br

Acknowledgment and funding

The authors thank the slaughterhouse and industrial refrigerator of Mossoro (RN, Brazil) for providing ovaries. This study was supported by the Brazilian Council of Scientific Development – CNPq (Process no. 309078/2021-0) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES, Financial Code 001). MF Oliveira, AR Silva, and AF Pereira received CNPq grants.

Abstract

This study aimed to evaluate sperm quality and heterologous embryo production using collared peccary sperm, which were selected using different techniques. We collected nineteen ejaculates and used for three experiments. In the first experiment, we evaluated the effects of centrifugation to remove seminal plasma before sperm swim-up. In the second experiment, Percoll® gradient (PG) densities (PG45–90% and PG35–70%) were compared; sperm quality was evaluated on kinetic patterns, morphology, membrane functional integrity, viability, reactive oxygen species (ROS), glutathione (GSH), and DNA integrity. In the third experiment, we analyzed PG and washing by centrifugation (WC) on embryo production using swine oocytes and motility patterns. Non-selected sperm was used as the control. Swim-up decreased motility patterns and enhanced ROS compared to those of the control. Swim-up with centrifugation reduced the membrane functional integrity and viability compared to the control, while this process enhanced GSH. Therefore, swim-up was not used for embryo production. PG45–90% improved velocity average pathway (VAP), velocity straight line, and linearity (LIN) of sperm compared with that in the control and PG35–70%. PG35–70% negatively affected motility, membrane functional integrity, viability, morphology, and GSH. Motility patterns were similar in the control, PG45–90%, and WC. PG45–90% improved VAP, amplitude of lateral head displacement, straightness, and LIN compared with that of the control and WC. Cleavage and morula frequencies did not differ between the groups. In summary, PG45–90% and WC were efficient in isolating peccary motile sperm capable of fertilizing swine oocytes and promoting embryo development.

Keywords: Wild ungulates; Density gradient Centrifugation; Swim-up; Washing by centrifugation.

1. Introduction

Collared peccaries are a group of wild ungulates, internationally categorized as a least-concern species in terms of the risk of extinction [1]. However, its reduced population has been in the Caatinga and Atlantic Forest biomes owing to lost habitat and predatory hunting [2]. The population loss affects the ecological role of the collared peccary involved in seed dispersal [3] and the food chain balance that can immediately affect large

carnivores [4]. On the other hand, collared peccaries adapt well to captive breeding, which is suitable for the Assisted Reproduction Techniques (ARTs) performed in the view of scientific and commercial development [5].

Different strategies have been reported for developing semen technologies and physiological characterization of semen of collared peccaries. We have highlighted advances in semen collection [6], evaluation [7], refrigeration [8], cryopreservation [9], protein [10] and biochemical [11] characterization of semen, and artificial insemination [12]. In general, the selection and isolation of motile spermatozoa from semen samples can benefit ARTs, including cryopreservation followed by *in vitro* embryo production (IVEP) [13]. Specifically, to perform IVEP using *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), the isolation of sperm from seminal plasma or extenders is crucial in any species, including collared peccaries [14]; moreover, this step allows an increase in kinetic patterns as well as the removal of unwanted cells and microorganisms [15].

An effective sperm separation method has not yet been established for collared peccaries. There are many studies on pigs, which are the domestic animal phylogenetically close to collared peccaries. However, ART protocols used in domestic species cannot be applied to wild animals without prior evaluation [16]. Sperm samples collected from collared peccaries, similar to swine samples, show sensitivity to stressful situations, such as centrifugation and cryopreservation [6, 14, 17]; nevertheless, ruminant-specific semen storage protocols are more effective in collared peccaries [6, 18]. Moreover, the calcium and magnesium contents of seminal plasma obtained from collared peccaries are similar to that of bulls and rams [11]. Protein profiles of seminal plasma were compared among rams, swine, and collared peccaries; a distinctly high expression of clusterin isoforms and a lower amount of spermadhesins were observed in collared peccaries [10]. Hence, the differences and similarities with domestic species must be considered to establish an ideal sperm-separation technique for the collared peccary.

The simple and economical swim-up method of sperm separation is suitable for sensitive sperm, where sperm swim above the surface of the medium [19]. The sample used for

selection may contain seminal plasma (SP), which can be removed using centrifugation. Notably, SP contains decapacitating factors and cell debris that can be harmful for sperm quality, and its removal by centrifugation can also sensitize the sperm before they swim-up [20].

The Percoll® gradient (PG) technique has been used for sperm separation to improve motility and fertility-associated features in several species, such as pigs [21], goats [22], and cattle [15]. However, Percoll® has an endotoxic effect, hence its use has been banned in human ARTs since 1996 [23]. In this technique, sperm reach the bottom of a tube containing different densities of Percoll®; 45–90% of Percoll® is used in this method more frequently, followed by 35–70% Percoll® used for both swine and ruminants. The density of Percoll® influences the sperm quality and the recovery rate; hence, the best conditions for collared peccary need to be optimized and the tolerance of the species to Percoll® should be assessed [24, 25].

In contrast, centrifugation washing (WC), a simple and fast method that recovers a high sperm concentration and removes SP, is widely used [22, 26]. Furthermore, simple centrifugation is previously used for sperm processing in collared peccaries [6].

Therefore, this study aimed to evaluate different systems for sperm selection in collared peccaries. We (i) compared the effect of centrifugation to remove SP before swimming, (ii) evaluated two densities of PG for sperm separation, and (iii) analyzed the fertility of sperm separated by the established methods or WC using heterologous IVF with porcine oocytes. Sperm quality was evaluated based on their kinetic patterns, morphology, membrane functional integrity, and viability. To assess cellular stress levels, we evaluated the reactive oxygen species (ROS) and glutathione (GSH) contents, as well as DNA integrity. Heterologous IVF and embryonic development were performed to verify sperm functionality.

2. Material and methods

2.1. Bioethics and chemicals

The experiments were performed following the rules of the Animal Ethics Committee of

the Federal Rural University of Semi-Arid (no. 30/2019) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 71834-1). Unless otherwise stated, the reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Semen collection

Nineteen ejaculates, obtained from sexually mature 25–30 months old, collared peccaries, were used for semen collection. Animals were obtained from the Center of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil; 5°10'S, 37°10'W) and registered at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, no. 1478912). Collared peccaries were kept in outdoor paddocks (20 × 3 m^2) covering an area of (3 × 3 m^2), with access to water *ad libitum,* and feed and fruits were provided.

The animals were fasted for 12 h before semen collection, physically restrained using a hand net, and anesthetized using intravenously administered propofol (Propovan, Cristalia, Fortaleza, Brazil) given as a bolus (5 mg/kg) [27]. Subsequently, they were placed in the lateral decubitus position to collect semen using an electroejaculation protocol, previously optimized for collared peccaries [6]. Fresh ejaculates were immediately evaluated for appearance, color, pH, and concentration using a Neubauer chamber.

2.3. Experimental design

The study was divided into three experiments, and in the control group (not separated), fresh semen was evaluated immediately after collection. Sperm were evaluated for motility patterns, morphology, membrane functional integrity, viability, recovery rate (concentration after selection with a Neubauer chamber), ROS and GSH levels, and DNA integrity. Moreover, heterologous IVF was performed using swine oocytes.

2.3.1. Experiment I: swim-up

Sperm separation was evaluated using the swim-up method after applying various sample preparation conditions, such as using centrifugation or without centrifugation to remove SP. Fresh semen was divided into two portions; one portion was diluted up to 100 × 10^6

sperm/mL (non-centrifuged group) and subjected to swim-up, whereas, the other portion was diluted in the ratio of 1:1 and centrifuged at 300×*g* for 3 min, the pellet was resuspended, the concentration was adjusted to 100 × 10^6 sperm/mL, and subjected to swim-up. Throughout the procedure, the sperm tyrode lactate (SPTL) medium [100 mM NaCl, 3.1 mM KCl, 0.4 mM NaH_2PO_4, 21.6 mM Na lactate, 25 mM $NaHCO_3$, 0.5 mM caffeine, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 0.6% bovine serum albumin (BSA), 1 mM sodium pyruvate, 1% antibiotic–antimycotic solution] was used. The separation was done following the method reported by Olivares et al. [22], with modifications described below. Initially, 250 µL of the sperm suspension was placed in a 15 mL tube and more than 1.0 mL of SPTL medium was carefully added to it. The swim-up step was performed for 1 h at 38.5 ºC and 5% CO_2, using the tubes tilted at 45º. Subsequently, the supernatant (200–300 µL) was carefully removed and centrifuged (300×*g* for 3 min) to concentrate the sperm suspension. The separated sperm was resuspended in SPTL medium and evaluated.

2.3.2. Experiment II: Percoll® gradient

We compared different Percoll® gradient densities, including 45–90% [24] and 35–70% [28], for the separation of collared peccary sperm. The gradient volume and centrifugation conditions were selected following previous experiments. Briefly, we compared the total volumes of 1.0 and 2.0 mL of the Percoll® gradient and detected better recovery using a total volume of 1.0 mL (500 µL 45% and 500 µL 90% Percoll®). We evaluated the recovery after centrifugation at 900×*g* for 15 and 30 min. It was verified that a long time did not increase the recovery; therefore, 15 min was considered the optimum recovery time.

In a 1.5 mL conical microtube, 500 µL of 45% Percoll® was added to the bottom, and subsequently, 500 µL of Percoll® 90% was carefully dispensed on it avoiding mixing of both dilutions. The same procedure was performed to study the 35–70% concentration gradient. Semen was diluted to a concentration of 100 × 10^6 sperm/mL using an SPTL medium. A 250 µL sample of this sperm suspension was placed over the gradients and centrifuged at 900×*g* for 15 min (37 ºC). The supernatant was discarded, and the pellet was resuspended in SPTL medium, which was subsequently centrifuged (300×*g* for 3

min) to remove Percoll®. The selected sperm were resuspended in SPTL and evaluated.

2.3.3. Experiment III: heterologous IVF

In terms of fertilization performance, the most efficient conditions for sperm separation using PG and swim-up were compared using the washing by centrifugation (WC) method. For WC, fresh semen was diluted using SPTL medium in a ratio of 1:1 and 1.0 mL of this suspension was subjected to centrifugation steps of 100×*g* for 3 min repeated three times. Before conducting the experiments, we confirmed if the speed and time of centrifugation were sufficient to form pellets containing sperm. After washing, the pellets were resuspended, and the concentration was adjusted for evaluation and IVF.

Sperm were separated using different techniques and subjected to IVF using swine oocytes. Sperm were evaluated for motility patterns, morphology, and effectiveness for oocyte-sperm interaction and embryonic development.

2.4. Sperm evaluation

2.4.1. Motility patterns

The motility of sperm was analyzed using a computer-assisted sperm assessment system (IVOS 7.4G; Hamilton-Thorne Research, Beverly, MA, USA), with settings validated for collared peccaries [8]. Five viewed fields were randomly selected and scanned. The instrument settings were as follows: temperature, 37 °C; 60 frames/s; 45 as minimum contrast; 30% straightness threshold; 10 μ/s low-velocity average pathway (VAP) cutoff; and 30 μ/s medium VAP cutoff. The total motility (%), progressive motility (%), VAP (μm/s), velocity straight line (VSL; μm/s), curvilinear velocity (VCL; μm/s), the amplitude of the lateral head (ALH; μm), beat cross frequency (BCF; Hz), straightness (STR; %), and linearity (LIN; %) of sperm were analyzed. Sperm populations were subdivided into four categories: rapid, medium, slow, and static.

2.4.2. Morphology and functional integrity of the membrane

Sperm present in smears stained with Bengal rose (Cromato®) were viewed under the optical microscope (1000×; 100 cells per slide) [29] and their morphology was investigated. The functional integrity of the sperm membrane was reflected by the sperm

osmotic response evaluated using 100 sperm samples by a hypo-osmotic swelling test. For this purpose, an aliquot of sperm suspension was incubated in a hypo-osmotic solution (distilled water:0 mOsm/L), and cells with a swollen curled tail were considered to have a functional membrane [7].

2.4.3. Viability and mitochondrial activity

Sperm viability was analyzed based on the structural integrity of the plasma membrane and mitochondrial activity. For this evaluation, spermatozoa were incubated with 40 µg/mL Hoechst 33342 at 37 °C for 10 min and with 0.5 mg/mL propidium iodide and 500 µM chloromethyl-X-rosamine for 8 min (Invitrogen, Carlsbad, CA, USA). Next, 100 cells were analyzed using an epifluorescence microscope (400×; Olympus BX51TF). Individual spermatozoon appearing blue with a midpiece appearing red was considered to have an intact sperm membrane and mitochondrial activity [30].

2.4.4. The integrity of the DNA

To assess the DNA integrity, we used an assay based on an acid challenge that denatures DNA molecules from a susceptible chromatin structure, which included DNA strand separation, intercalation of the acridine orange probe to DNA generating red (denatured single-stranded DNA) or green (double-stranded DNA) fluorescence [31]. Initially, sperm suspensions were smeared and dried in the air. The method reported by Martins et al. [32] was followed, with the exception that 25 min was required as the time to verify chromatin integrity. The smears were fixed in Carnoy's solution (methanol and glacial acetic acid in a 3:1 ratio) for 24 h, dried again, and incubated for 25 min in a buffer solution composed of 15 mM Na_2HPO_4 and 80 mM citric acid (pH 2.5) at 75 °C to check chromatin stability. Smears were stained using acridine orange (0.2 mg/mL) for 10 s, washed with distilled water, and covered using a coverslip. A total of 100 cells were evaluated using epifluorescence microscopy. Sperm with green fluorescence on the head contained intact DNA, and those with orange or red fluorescence had denatured DNA.

2.4.5. Oxidative status

To assess the oxidative status of sperm cells, ROS levels were evaluated by tagging sperm with 2',7' dichlorodihydrofluorescein diacetate (H_2DCFDA; 10 µM; Invitrogen, Carlsbad,

CA, USA), and GSH levels were analyzed using 7-amino-4-chloromethylcoumarin (CellTracker Blue; 10 µM; Invitrogen, Carlsbad, CA, USA) [33]. Briefly, 2.5 mL of phosphate-buffered solution (PBS) was added to a 0.5 µL (0.1 M) aliquot of each fluorescent probe. Then, 50 µL of each diluted probe was added to 50 µL of the sperm suspension at 40×10^6 sperm/mL. The samples were then incubated at 37 °C for 30 min in the dark. Subsequently, the probes were removed by centrifugations (500×*g*/5 min) repeated twice, and the precipitate was resuspended in PBS. The slides were visualized using an epifluorescence microscope and microscopic images were evaluated using ImageJ software. From each sample, up to 100 sperm were selected for the quantification of fluorescence intensity. Fresh spermatozoa were used to calibrate the measurements, and the measured value of each sperm was divided by the mean of the calibrator to generate relative expression levels [arbitrary fluorescence units (AFU)].

2.5. Heterologous IVF

2.5.1. Oocyte collection and in vitro maturation

Ovaries were collected from swine females in a local slaughterhouse, stored in NaCl (0.9% at 35–37 °C), and transported to the laboratory. Follicles with 3–6 mm diameter were aspirated using a 21G needle and 5.0 mL syringe. Oocytes with more than one layer of cumulus cells and a homogeneous cytoplasm were selected. The *in vitro* maturation (IVM) was performed in drops (100 µL) covered with mineral oil in a controlled humid atmosphere at 38.5 °C and 5% CO_2 for 42–44 h. The maturation medium was composed of TCM199 supplemented with 2.2 g/L sodium bicarbonate, 25 mM HEPES, 0.3 mM sodium pyruvate, 5 µg/mL myo-inositol, 10% porcine follicular fluid, 5 ng/mL epidermal growth factor (EGF), 20 µg/mL follicle-stimulating hormone associated with luteinizing hormone (FSH/LH; Pluset®, Hertape Calier, Juatuba, MG) and 1% antibiotic-antimycotic solution [34].

2.5.2. Co-incubation and embryo development

After IVM, the oocytes were washed and pelleted a few times to remove excess *cumulus* cells. They were grouped (10–15 oocytes) and incubated at 38.5 ºC and 5% CO_2 with separated sperm in drops of 50 µL of IVF medium (114 mM NaCl, 3.2 mM KCl, 0.35 mM NaH_2PO_4, 10 mM Na lactate, 5 mM glucose, 25 mM $NaHCO_3$, 2 mM caffeine, 4.7

mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, 0.3% BSA, 0.11 mM sodium pyruvate, 1% antibiotic–antimycotic solution) [35], covered with mineral oil. The sperm concentration was 3×10^5 sperm/mL, and the IVF process lasted for 6 h [36].

After the IVF process, the structures were washed and pipetted to remove the unbound sperm and cumulus cells. The presumed heterologous zygotes were subjected to *in vitro* development in 50 µL drops of synthetic oviduct fluid (SOF) supplemented with 0.2 mM sodium pyruvate, 0.2 ml L-glutamine, 0.34 mM citrate sodium, 2.8 mM myo-inositol, 2% essential amino acid solution, 1% non-essential amino acid solution, 1% antibiotic/antimycotic, 5 mg/mL BSA and 2.5% fetal bovine serum [37]. After 48 h of culture, 50% of the medium was changed and the non-cleaved cells were removed and evaluated for oocyte-sperm interaction and fertilization using Hoechst 33342 (10 µg/mL, 15 min) under a fluorescence microscope. The cleaved embryos were quantified and classified according to the number of cells (two cells and > three cells) and cultured for six days. Embryos that reached the morula stage were quantified.

2.6. Statistical analysis

Data are expressed as mean ± standard error (one male/one repetition) and were analyzed using GraphPad software (GraphPad Software Inc., La Jolla, CA, USA). All results were verified for normality using the Shapiro-Wilk test and homoscedasticity using Levene's test. Because the data did not show a normal distribution, they were arcsine transformed and analyzed using ANOVA followed by Tukey's test. All other data were compared using the chi-squared test. Statistical significance was set at $P < 0.05$.

3. Results

All 19 ejaculates were white watery samples, with pH 7.0 and an average concentration of $450 \pm 89.1 \times 10^6$ sperm/mL. Only the samples showing motility greater than 80% were used in the analyses.

3.1. Experiment I: swim-up

The swim-up method using centrifuged or non-centrifuged samples significantly decreased the values of motility, progressive motility, VAP, VSL, VCL, ALH, and rapid

sperm frequency compared to that of the control. Moreover, swim-up increased the percentage of static sperm (Table 1).

Table 1 Motility patterns of collared peccary sperm separated by swim-up after the removal or not of seminal plasma (SP) by centrifugation.

CASA	Control (No separation)	Swim-up	
		Centrifuged (Without SP)	Non-centrifuged (With SP)
Motility (%)	90.4 ± 2.4[a]	31.4 ± 8.1[b]	51.9 ± 6.4[b]
Progressive motile (%)	68.6 ± 3.9[a]	17.7 ± 3.8[b]	28.9 ± 3.8[b]
VAP (µm/sec)	74.0 ± 4.5[a]	47.2 ± 5.1[b]	47.9 ± 1.9[b]
VSL (µm/sec)	54.2 ± 6.1[a]	37.6 ± 4.3[b]	37.6 ± 2.0[b]
VCL (µm/sec)	137.6 ± 5.3[a]	91.1 ± 9.1[b]	88.4 ± 3.5[b]
ALH (µm)	6.1 ± 0.2[a]	5.1 ± 0.2[b]	4.8 ± 0.3[b]
BCF (Hz)	36.3 ± 0.5[a]	35.3 ± 1.4[a]	35.9 ± 1.0[a]
STR (%)	74.0 ± 3.1[a]	74.7 ± 1.5[a]	73.4 ± 2.3[a]
LIN (%)	42.3 ± 3.2[a]	43.7 ± 1.8[a]	43.3 ± 2.5[a]
Rapid (%)	80.1 ± 2.9[a]	21.1 ± 5.2[b]	35.1 ± 4.2[b]
Medium (%)	9.9 ± 1.3[a]	10.3 ± 3.8[a]	17.1 ± 2.6[a]
Slow (%)	2.7 ± 0.6[a]	6.1 ± 1.4[a]	6.1 ± 1.8[a]
Static (%)	7.0 ± 2.2[a]	62.1 ± 8.6[b]	41.7 ± 8.0[b]

[a,b]: values (mean ± standard error) with different superscript letters within lines are significantly different ($P < 0.05$). Seven repetitions.

The percentage of cells with normal morphology was similar in different groups (Fig. 1A). The non-centrifuged group showed membrane functional integrity and viability similar to that of the control, whereas these parameters in the centrifuged group were decreased significantly (Fig. 1B and C). The rate of sperm recovery did not differ between centrifuged and non-centrifuged samples before separation (Fig. 1D).

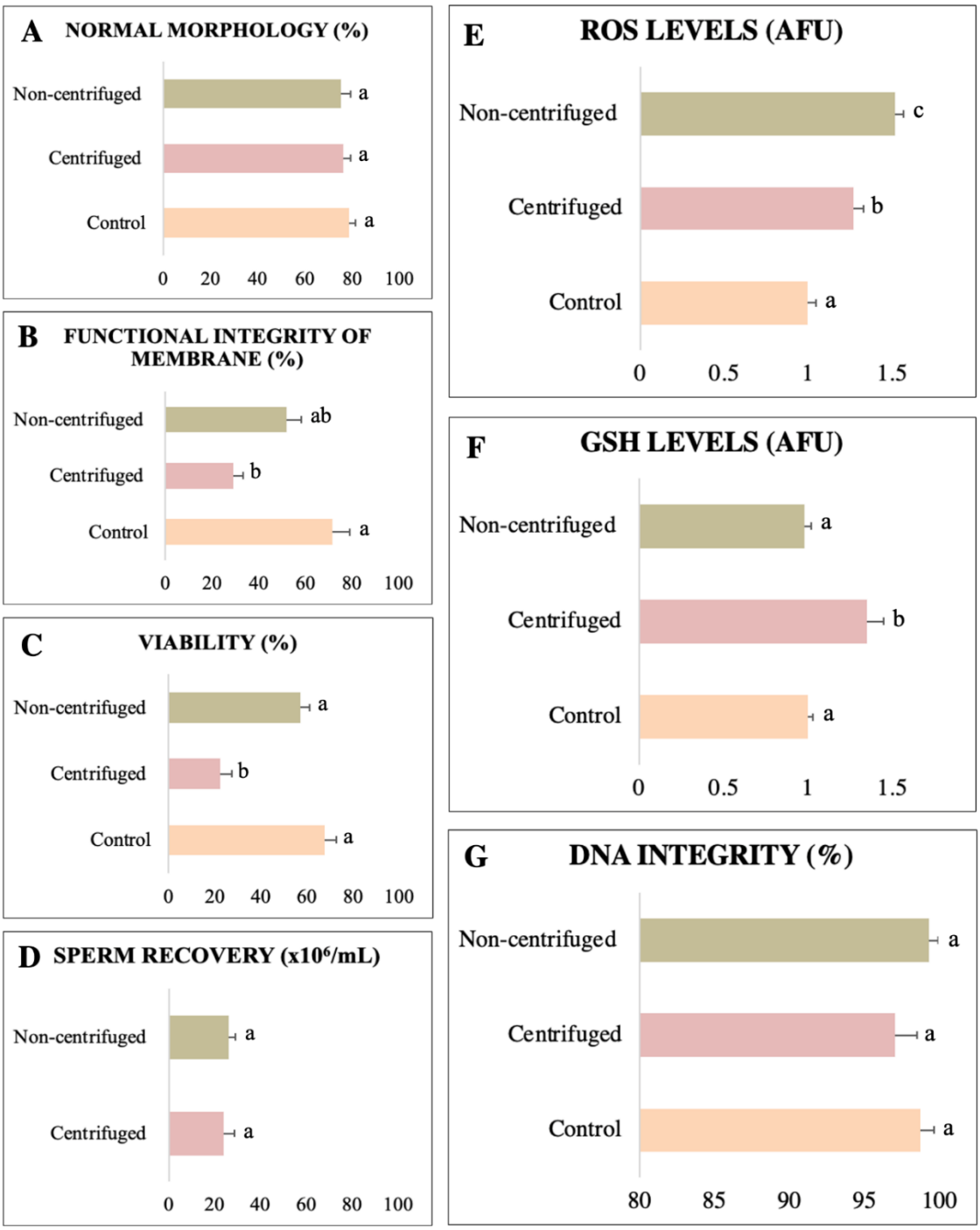

Fig. 1. Quality of collared peccary sperm separated by swim-up technique. (A) Normal morphology. (B) Functional integrity of the membrane. (C) Viability. (D) Sperm recovery rate. (E) ROS levels. (F) GSH levels. (G) DNA integrity. [a,b,c]: values with different superscript letters are significantly different ($P < 0.05$).

ROS levels increased after the swim-up compared to the control counterpart, with its highest value observed in the centrifuged samples (Fig. 1E; $P < 0.05$). GSH levels were

higher ($P < 0.05$) in the centrifuged group (1.35 ± 0.10 AFU) than in the non-centrifuged group (0.98 ± 0.04 AFU) and control (1.00 ± 0.03 AFU) (Fig.1F). The DNA integrity was similar in the sperm separated and control groups (Fig. 1G). Hence, among sample preparation methods, the non-centrifuged group caused less damage to the sperm quality. In general, the swim-up method did not improve the motility and other quality-related parameters of collared peccary sperm and was inefficient for this species under the studied conditions. Therefore, this method was not used in our third experiment.

3.2. Experiment II: Percoll® gradient

Kinetic and motility parameters are listed in Table 2. The total and progressive motilities were similar between the control and PG 45/90% groups. However, the 35–70% concentration gradient showed a significant and sharp decrease in these parameters. The separation with PG 45–90% significantly improved VAP, VSL, and LIN compared to that of the control and PG 35–70% that showed harmful effects on ALH, STR, and rapid, medium, and static subpopulations. Moreover, the 45–90% concentration gradient increased the percentage of static sperm compared to the control, which was significantly more increased by the 35–70% concentration gradient ($P < 0.05$).

Normal morphology, membrane functional integrity, and viability were superior in the PG 45–90% and control groups compared to the PG 35–70% group which impaired sperm quality (Fig. 2A, B, and C). However, sperm recovery rates at the two tested Percoll® concentration gradients were similar (Fig. 2D). The levels of ROS and DNA integrity were similar in all sperm (Fig. 2E and G). Conversely, PG 35–70% significantly decreased the levels of GSH (0.55 ± 0.02 AFU) compared with that recorded in PG 45–90% (0.85 ± 0.03 AFU) and control groups (1.00 ± 0.04 AFU) (Fig. 2F). Hence, the separation of sperm from collared peccary using PG 45–90%, which improved kinetic parameters and maintained sperm quality, was used for heterologous IVF.

1 **Table 2** Motility patterns of collared peccary sperm selected by Percoll® gradient at
2 different densities.

CASA	Control (No separation)	Percoll® gradient	
		PG 45–90%	PG 35–70%
Motility (%)	95.0 ± 1.8[a]	87.5 ± 3.6[a]	8.2 ± 2.1[b]
Progressive motile (%)	70.8 ± 6.0[a]	75.3 ± 5.2[a]	3.3 ± 1.4[b]
VAP (µm/sec)	69.7 ± 7.5[b]	96.8 ± 9.4[a]	40.7 ± 6.1[b]
VSL (µm/sec)	56.0 ± 6.6[b]	88.4 ± 8.9[a]	27.3 ± 7.0[b]
VCL (µm/sec)	121.4 ± 11.4[a]	129.6 ±7.5[a]	95.4 ± 12.0[a]
ALH (µm)	5.4 ± 0.4[a]	4.4 ± 0.3[a]	6.2 ± 0.6[b]
BCF (Hz)	36.4 ± 0.9[a]	36.0 ± 1.9[a]	38.8 ± 2.2[a]
STR (%)	76.0 ± 2.2[a]	87.2 ± 1.4[a]	64.7 ± 6.2[b]
LIN (%)	46.0 ± 3.1[b]	66.0 ± 4.7[a]	35.7 ± 6.7[b]
Rapid (%)	81.0 ± 6.2[a]	79.7 ± 5.3[a]	5.2 ± 1.6[b]
Medium (%)	14.0 ± 4.9[a]	7.7 ± 1.8[ab]	2.8 ± 0.7[b]
Slow (%)	2.2 ± 0.3[a]	2.7 ± 0.7[a]	2.3 ± 0.6[a]
Static (%)	3.0 ± 1.6[a]	10.0 ± 3.0[b]	89.8 ± 2.6[c]

3 [a,b]: values (mean ± standard error) with different superscript letters within lines are
4 significantly different ($P < 0.05$). Six repetitions.

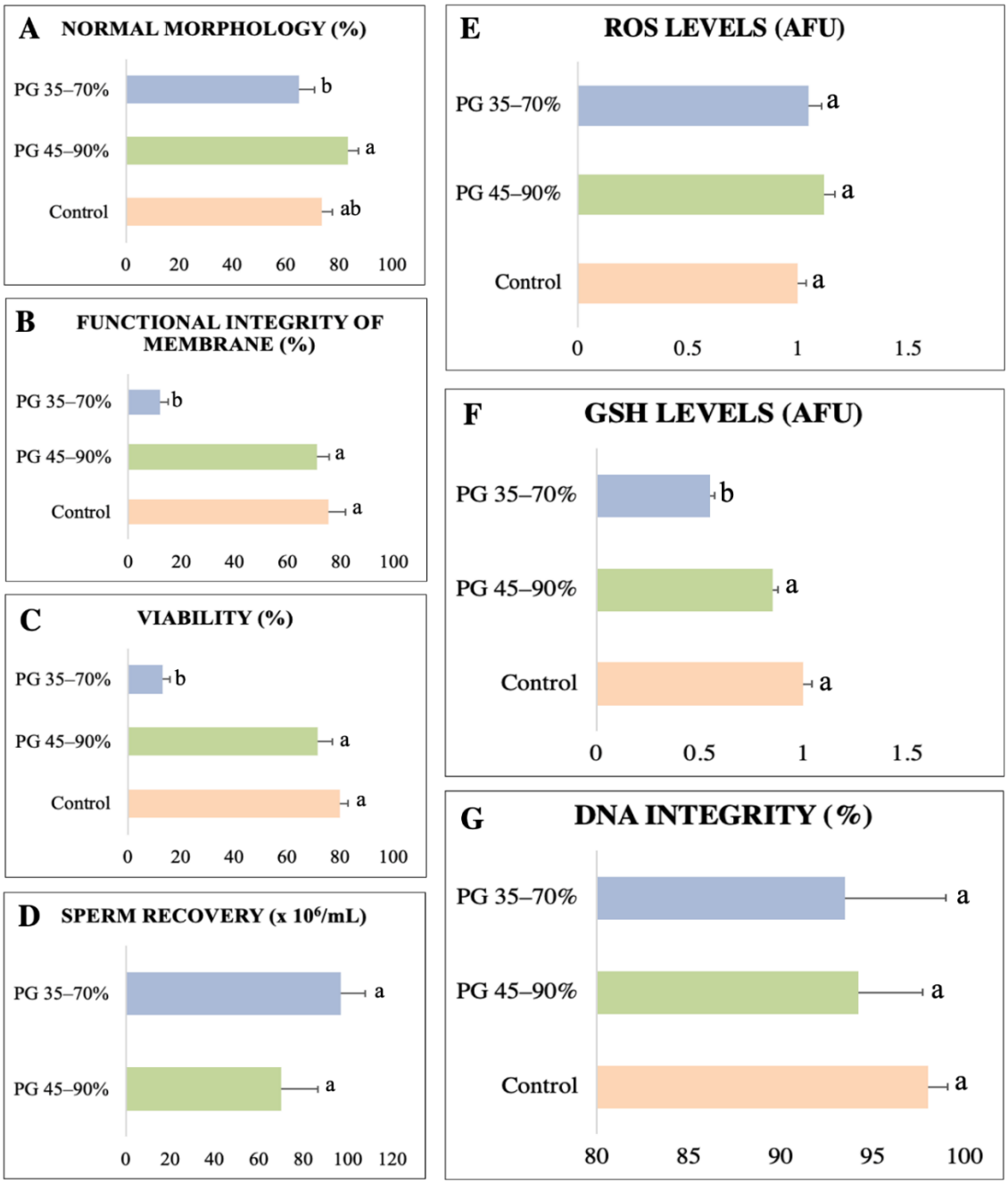

Fig. 2. Quality of collared peccary sperm separated by Percoll® gradient technique. (A) Normal morphology. (B) Functional integrity of the membrane. (C) Viability. (D) Sperm recovery rate. (E) ROS levels. (F) GSH levels. (G) DNA integrity. [a,b]: values with different superscript letters are significantly different ($P < 0.05$).

3.3. Experiment III: heterologous IVF

Sperm separated for heterologous IVF were evaluated for motility patterns and normal

morphology (Table 3). Motility patterns of all sperms were similar in the control and WC groups. PG improved VSL, ALH, STR, and LIN values compared to that in the control and WC groups. Nevertheless, PG significantly increased the number of static sperm compared with that recorded in the control and WC groups. No significant difference was detected in the percentage of morphologically normal sperm.

Table 3 Motility patterns and morphology of the collared peccary sperm separated using Percoll® gradient and washing by centrifugation for heterologous IVF.

CASA	Fresh control	Percoll® gradient	Washing by centrifugation
Motility (%)	96.8 ± 2.0[a]	66.3 ± 10.2[a]	89.0 ± 5.4[a]
Progressive motile (%)	59.5 ± 12.1[a]	47.5 ± 7.9[a]	56.0 ± 12.5[a]
VAP (µm/sec)	83.0 ± 8.7[a]	105.3 ± 7.3[a]	96.0 ± 8.8[a]
VSL (µm/sec)	61.7 ± 9.3[b]	98.7 ± 7.2[a]	72.6 ± 9.3[ab]
VCL (µm/sec)	157.3 ± 9.1[a]	139.4 ± 10.6[a]	166.7 ± 9.3[a]
ALH (µm)	7.3 ± 0.2[b]	4.2 ± 0.6[a]	7.2 ± 0.6[b]
BCF (Hz)	34.4 ± 0.7[a]	36.2 ± 2.5[a]	33.1 ± 1.7[a]
STR (%)	71.3 ± 3.7[b]	92.0 ± 3.2[a]	72.5 ± 3.6[b]
LIN (%)	40.3 ± 3.9[b]	72.3 ± 4.7[a]	46.0 ± 4.7[b]
Rapid (%)	62.5 ± 12.3[a]	48.8 ± 8.5[a]	59.5 ± 12.6[a]
Medium (%)	11.8 ± 4.0[a]	4.0 ± 4.0[a]	7.0 ± 1.5[a]
Slow (%)	22.3 ± 6.7[a]	8.5 ± 2.7[a]	22.3 ± 6.2[a]
Static (%)	3.3 ± 2.0[a]	39.0 ± 11.2[b]	11.0 ± 5.4[a]
Normal morphology (%)	79.7 ± 7.4[a]	82.0 ± 6.4[a]	82.0 ± 6.4[a]

[a,b]: values (mean ± standard error) with different superscript letters within lines are significantly different ($P < 0.05$). Four repetitions.

The number of sperm bound to mature, not mature, and total oocytes did not differ with various separation methods (Table 4). Furthermore, fertilization analyzed by the presence of the second polar body and two pro-nuclei did not differ between PG and WC, presenting fertilized matured oocytes at 22.5–25.0%.

1 **Table 4** Sperm-oocyte interaction and fertilization after heterologous IVF of collared peccary sperm separated by different techniques.

Selection technique	Matured oocytes, %	Sperm bound to matured oocytes	Sperm bound to non-matured oocytes	Sperm bound to total oocytes	Second polar body, %	Two pronucleus, %	Fertilized oocytes	
							Total, %	Matured, %
Percoll gradient	65.6 ± 1.2 (40/61)	4.9 ± 2.8 (196/40)	9.3 ± 4.3 (196/21)	3.2 ± 1.6 (196/61)	9.8 ± 3.1 (6/61)	4.9 ± 2.7 (3/61)	14.8 ± 4.9 (9/61)	22.5 ± 10.8 (9/40)
Washing by centrifugation	52.5 ± 1.5 (32/61)	3.2 ± 1.9 (103/32)	3.6 ± 4.7 (103/29)	1.7 ± 1.3 (103/61)	3.3 ± 2.4 (2/61)	9.8 ± 3.6 (6/61)	13.1 ± 4.0 (8/61)	25.0 ± 9.5 (8/32)

2 No differences were observed among treatments (mean ± standard error, $P > 0.05$). Six repetitions.

The separation techniques did not alter the cleavage rate in fertilized oocytes, which was detected to be 26.3% and 25.2% in WC and PG, respectively (Fig. 3A and B). More than 88% of the cleaved hybrid embryos had more than three cells. In total, 10.5% and 8.1% of embryos reached the morula stage in the WC and PG groups, respectively (Fig. 3C and D). Among cleaved embryos, the frequencies of morula development were 40% and 32.3% achieved with WC and PG, respectively. No difference in morula development was found between the experimental groups (WC and PG).

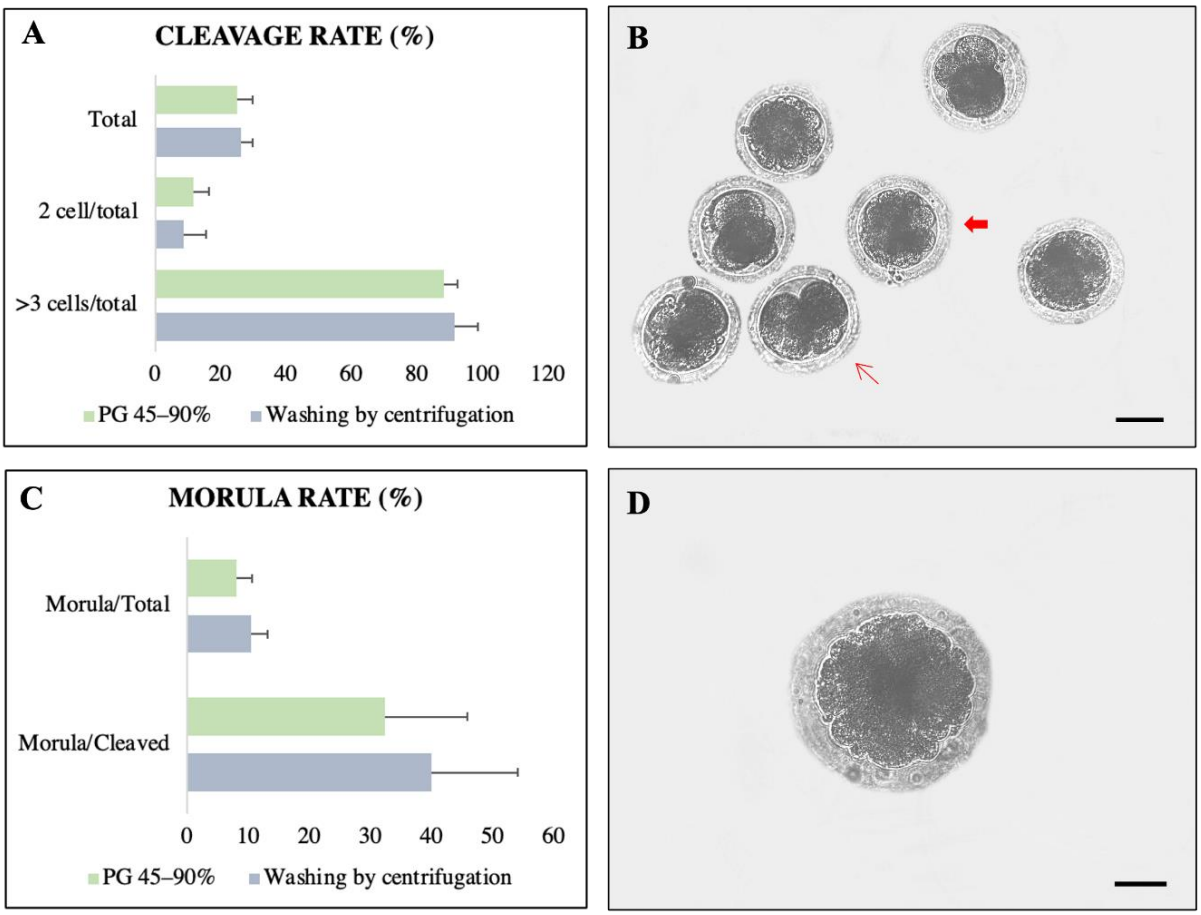

Fig. 3. Embryonic development after heterologous fertilization of collared peccary sperm separated by different techniques. (A) Percentage of embryos in D3 with 2 cells, and 3 to 7 cells. (B) Embryos two days after *in vitro* fertilization (thin arrow: embryo with three cells; wide arrow: embryo with more than three cells). (C) Percentage of embryos in D6. (D) Morula stage embryo after six days of development. No differences were observed among treatments ($P > 0.05$). Mean ± standard error. Six repetitions. Scale bars = 150 µm.

4. Discussion

This study, for the first time, evaluated different separation techniques for sperm of collared peccaries. We identified a 45–90% Percoll® density gradient to be the most effective for

selecting fresh sperm from these animals. Although the total and progressive motilities did not improve, the quality of the sperm kinetics expressed in terms of VAP, VSL, and LIN was superior when PG 45–90% was used. In swine sperm, these parameters were reported to be positively correlated with fertility [38, 39, 40].

Matás et al. [24] analyzed three density combinations of PG (45 and 60%, 60 and 75%, and 45 and 90%) for swine sperm, 45–90% recovered morphologically normal sperm with less DNA damage, and improved motility and capacitation compared to the control and other PG densities. In collared peccary, PG 45–90% maintained sperm quality noticed in the control set and did not induce oxidative stress or impaired DNA integrity. Hence, Percoll®, which is toxic to human samples, showed no toxic effect on this species. However, its use is not allowed in fertilization clinics because of the risk of contamination with endotoxins and changes in possible sperm [41].

In contrast, sperm selection with PG 35–70% had adverse effects on samples of collared peccaries. We hypothesized that the low-density gradient causes a greater impact of centrifugation on sperms, leading to their sensitivity to this process. It is possible that the pellet formed was denser and faster, causing the sperm cells to stay in contact for a longer time compared to PG45–90%. Additionally, PG 35–70% might not effectively separate cell debris from dead cells. These may have caused structural damage and oxidative stress that affected the quality and functionality of collared peccary spermatozoa.

No significant increase in ROS levels was detected, whereas GSH levels decreased after PG 35–70% assisted separation process. Decreased level of GSH, a main antioxidant defense component of sperm cells, indicates an imbalanced cellular defense that makes sperm more susceptible to damage from oxidative stress [42]. In contrast, PG 35–70% used for pig samples, induced a significant increase in the percentage of mobile sperm (82.3% and 76.4% after and before selection, respectively), and improved sperm morphological characteristics [28]. This highlights the importance of optimizing an ideal protocol for each species, as species, despite close phylogenetic relation, show different responses.

Before any processing, collared peccary semen already had excellent quality parameters. Nevertheless, it is still necessary to remove the SP for IVF, optimize the separation of the highest possible percentage of mobile spermatozoa, and improve kinetic patterns [15]. Our results indicated that PG 45–90% acted efficiently for SP removal and improved the kinetic

(motility) patterns of collared peccary spermatozoa. Additionally, sperm separation with colloid density (Porcicoll) contributes to the removal of microorganisms from swine semen samples [43]. Fresh semen from collared peccary is also contaminated with bacteria ($2.3 \pm 0.9 \times 10^6$ colony-forming units/mL), which can impair sperm quality [44]. A method that allows the separation of microorganisms from spermatozoa may benefit the use of collared peccary semen in techniques such as IVF.

Under the conditions studied in the present study, the swim-up method did not effectively support the isolation of mobile sperm from collared peccaries. Nevertheless, swim-up has been successfully used to isolate highly mobile sperm in pigs [39]. Moreover, this method, when used to remove diluent from frozen-thawed pig semen after centrifugation, increased viability (89.3% from 79.7%) and normal morphology (98.5% from 82.9%) compared with that recorded in the pre-separation semen sample [19]. Moreover, this technique enhanced sperm motility in cattle (83.8% *vs*. 69.7%) [15] and yak (76.7 *vs*. 32.4%) [45] compared with that of unselected samples. In contrast, in llama (14.5 vs. 32.2%) [46] and goat (41.1 vs. 55.5%) [22], the swim-up method rather than Percoll®-based techniques showed low recovery of mobile sperm, which is similar to the results observed in collared peccaries.

Considering the preparation of the collared peccary samples for swim-up, we inferred that these sperm would be more sensitive to physical stress caused by centrifugation than to oxidative stress caused by SP. In the centrifuged samples, the decreased functional integrity of the membrane and viability as well as increased levels of ROS and the antioxidant defense of GSH. However, SP that was retained in the sperm sample caused high ROS-stimulated oxidative stress but did not alter the sperm quality. Castelo et al. [6] observed that centrifugation (3000 rpm/3 min), performed before or after the semen underwent a freeze/thawing process, reduced sperm motility and increased morphological defects.

Since the swim-up strategy was not efficient, we performed heterologous IVF using only the PG 45–90% group. Hence, this study, for the first time, demonstrates heterologous IVF performed between collared peccary sperm and porcine oocytes to obtain embryos developed up to the cleavage and morula stages. This technique for assessing sperm fertility would be crucial during the limited availability of oocytes, which is common in wild species [47]. Campos et al. [48] evaluated the binding ability of collared peccary sperm using immature porcine oocytes. In this study, it was observed that porcine oocytes could be used to assess the functionality of collared peccary spermatozoa, but the potential for fertilization and

development of hybrid embryos has not been verified.

Before IVF, the PG group had a higher percentage of ALH, STR, and LIN than that of the WC group. Previously, in collared peccary, the number of sperm bonded to porcine oocytes was positively correlated with STR; similar correlations were detected between LIN and sperm binding to the egg perivitelline membrane [48]. However, PG rather than WC increased the subpopulation of static sperm, which imposed a negative correlation in the egg perivitelline membrane binding test [48].

Therefore, collared peccary sperm separation techniques had no significant influence on sperm-oocyte interaction, fertilization, and development of hybrid embryos. In swine species, PG allowed a better penetration rate (96.2 vs. 62.3%), cleavage (43.5 vs. 26.6%), and blastocyst (18.1 vs. 10.5%) compared to WC [49]. In cattle, a better blastocyst rate was also observed (56 vs. 38%) after sperm separation with PG compared with that recorded after washing [50].

Despite its simplicity, WC allowed the same rate of fertilization and embryo development as PG did, however, PG improved some kinetic parameters. These improvements may be related to the IVF system, where the sperm faces fewer difficulties on the way to the oocyte compared to the female reproductive tract [41]. Differences between the PG and WC methods could be more evident if fertility is evaluated *in vivo*. Moreover, it is important to further evaluate the effects of these separation methods on frozen/thawed samples that show loss of motility and quality during the process and may present unique patterns [51].

In conclusion, the 45–90% Percoll® concentration gradient was detected to be the most suitable one for collared peccaries. However, swim-up using the tested conditions did not yield satisfactory results and is not recommended for this species. According to the results of heterologous IVF, both PG 45–90% and WC can be used to select sperm from semen samples of collared peccaries. Furthermore, swine oocytes are a great alternative for assessing the fertility of these animals, as they support fertilization and early embryonic development.

Credit authorship contribution statement

Maria Valéria de Oliveira Santos: Conceptualization, methodology, writing, original draft, and investigation. **Andréia Maria da Silva**: Methodology and investigation. **Leonardo Vitorino Costa de Aquino**: Methodology and investigation. **Lhara Ricarliany Medeiros de Oliveira**: Methodology and investigation. **Samara Sandy Jeronimo Moreira**: Methodology

and investigatioin. **Moacir Franco de Oliveira**: Conceptualization, methodology, and investigation. **Alexandre Rodrigues Silva**: Conceptualization, methodology, investigation, and review. **Alexsandra Fernandes Pereira**: Conceptualization, methodology, writing, original draft, supervision, project administration, and funding acquisition.

Declaration of Competing interest

The authors declare no conflicts of interest.

References

[1] Gongora J, Reyna-Hurtado R, Beck H, Taber A, Altrichter M, Keuroghlian A. *Pecari tajacu*. The IUCN Red List of Threatened Species 2011: e.T41777A10562361. https://dx.doi.org/10.2305/IUCN.UK.2011-2.RLTS.T41777A10562361.en. 2011 [Accessed 05 January 2023].

[2] Desbiez ALJ, Keuroghlian A, Beisiegel BM, Medici EP, Gatti A, Pontes ARM, et al. Avaliação do risco de extinção do cateto *Pecari tajacu* Linnaeus, 1758, no Brasil. Bio Brasil 2012;1:74–83.

[3] Pérez-Irineo G, Santos-Moreno A. Abundance, herd size, activity pattern and occupancy of ungulates in Southeastern Mexico. Anim Biol 2016;66:97–109. https://doi.org/10.1163/15707563-00002490

[4] Scognamillo D, Maxit IE, Sunquist M, Polisar J. Coexistence of jaguar (*Panthera onca*) and puma (*Puma concolor*) in a mosaic landscape in the Venezuelan llanos. J Zool 2003;259:269–79. DOI: https://doi.org/10.1017/S0952836902003230

[5] Morais BHS, Cardoso DL, Costa JS, Mayor P, Albuquerque NI, Chisté RC, Guimarães DAA. Use of wildlife as an alternative protein source: Collared peccary meat. Meat Sci 2022;192:108895. doi: 10.1016/j.meatsci.2022.108895.

[6] Castelo TS, Bezerra FS, Lima GL, Alves HM, Oliveira IR, Santos EA, et al. Effect of centrifugation and sugar supplementation on the semen cryopreservation of captive collared peccaries (*Tayassu tajacu*). Cryobiology 2010;61:275–9. DOI: 10.1016/j.cryobiol.2010.09.005

[7] Santos EAS, Sousa PC, Peixoto GCX, Simão BR, Oliveira MF, Silva AR. Establishing the hypoosmotic swelling test for sperm analysis in collared peccaries (*Pecari tajacu*). Arq Bras Med Vet Zootec 2013;65:1257–60. https://doi.org/10.1590/S0102-09352013000400044

[8] Souza ALP, Lima GL, Peixoto GCX, Oliveira MF, Silva AR. Use of Aloe vera–based extender for chilling and freezing collared peccary (*Pecari tajacu*) sêmen. Theriogenology 2016;85:1432–8. doi: 10.1016/j.theriogenology.2016.01.007.

[9] Moreira SS, Santos CS, Castelo TS, Bezerra LGP, Praxedes ÉCG, Matos TM, et al. Investigating the need for antibiotic supplementation to the extender used for semen cryopreservation in collared peccaries. Front Vet Sci 2022;9:954921. doi: 10.3389/fvets.2022.954921.

[10] Santos EA, Sousa PC, Martins JAM, Moreira RA, Monteiro-Moreira ACO, Moreno FBMB, et al. Protein profile of the seminal plasma of collared peccaries (*Pecari tajacu* Linnaeus, 1758). Reproduction 2014;147:753–64. doi: 10.1530/REP-13-0220.

[11] Moreira SS, Silva AM, Praxedes ÉC, Campos LB, Santos CS, Souza ALP, et al. Composition of collared peccary seminal plasma and sperm motility kinetics in semen obtained during dry and rainy periods in a semiarid biome. Anim Reprod Sci 2019;211: 91–01. doi: 10.1016/j.anireprosci.2019.106229.

[12] Peixoto GC, Lima GL, Maia KM, Souza ALP, Castelo TS, Paiva AL, et al. Single injection of eCG/hCG leads to successful estrous synchronization in the collared peccary (*Pecari tajacu* Linnaeus, 1758). Anim Reprod Sci 2019;208:106112. doi: 10.1016/j.anireprosci.2019.106112.

[13] Morrell JM. Effect of colloid centrifugation on boar sperm quality during storage and function in *in vitro* fertilization. Theriogenology 2019;137:122–6. doi: 10.1016/j.theriogenology.2019.05.046.

[14] Deori S, Ntallaris T, Wallgren M, Morrell JM, Johannisson A. Comparison of Single Layer Centrifugation and magnetic activated cell sorting for selecting viable boar spermatozoa after thawing. Livest Sci 2022;257:104853. https://doi.org/10.1016/j.livsci.2022.104853

[15] Arias ME, Andara K, Briones E, Felmer R. Bovine sperm separation by Swim-up and density gradients (Percoll and BoviPure): Effect on sperm quality, function and gene expression. Reprod Biol 2017;17:126–32. doi: 10.1016/j.repbio.2017.03.002

[16] Santos MVO, Silva, AR, Pereira AF. Embryo production by *in vitro* fertilization in wild ungulates: progress and perspectives. Ann Anim Sci 2022;22:1151–62. doi: https://doi.org/10.2478/aoas-2022-0013

[17] Campos LB, Silva MA, Bezerra JAB, Castelo TS, Peixoto GCX, Silva AR. Survival of collared peccaries (*Pecari tajacu*) sperm after freezing-thawing using different extenders. Acta Sci Vet 2014;42:1–7.

[18] Souza ALP, Lima GL, Peixoto GCX, Castelo TS, Oliveira MG, Silva AR. Sperm characteristics following freezing in extenders supplemented with whole egg yolk and different concentrations of low-density lipoproteins in the collared peccary (*Pecari tajacu*). Reprod Biol 2015;15:223–8. doi: 10.1016/j.repbio.2015.10.006.

[19] Navarro-Serna S, París-Oller E, Simonik O, Romar R, Gadea J. Replacement of Albumin

by preovulatory oviductal fluid in swim-up sperm preparation method modifies boar sperm parameters and improves *in vitro* penetration of oocytes. Animals 2021;11:1202. https://doi.org/10.3390/ani11051202

[20] Morrell JM, Alsina MS, Abraham MC, Sjunnesson Y. Practical applications of sperm selection techniques for improving reproductive efficiency. Anim Reprod 2018;13:340–5. doi: 10.4061/2011/894767.

[21] Noguchi M, Yoshioka K, Hikono H, Iwagami G, Suzuki C, Kikuchi K. Centrifugation on Percoll density gradient enhances motility, membrane integrity and *in vitro* fertilizing ability of frozen–thawed boar sperm. Zygote 2013;23:68–75. doi: 10.1017/S0967199413000208.

[22] Olivares CCS, Fonseca JF, Camargo LSA, Souza-Fabjan JMG, Rodrigues ALR, Brandão FZ. Comparison of different methods of goat sperm selection and capacitation for optimization of assisted reproductive technologies. Small Rumin Res 2015;127:44–9. https://doi.org/10.1016/j.smallrumres.2015.04.009

[23] Fishel S, Jackson P, Webster J, Faratian B. Endotoxins in culture medium for human in vitro fertilization. Fertil Steril 1988;49:108–11. https://doi.org/10.1016/S0015-0282(16)59659-9

[24] Matás C, Vieira L, García-Vázquez FA, Avilés-López K, López-Úbeda R, Carvajal JA, et al. Effects of centrifugation through three different discontinuous Percoll gradients on boar sperm function. Anim Reprod Sci 2011;127:62–72. doi: 10.1016/j.anireprosci.2011.06.009.

[25] Anel-Lopez L, Ortega-Ferrusola C, Álvarez M, Borragán S, Chamorro C, Peña FJ, et al. Improving sperm banking efficiency in endangered species through the use of a sperm selection method in brown bear (*Ursus arctos*) thawed sperm. BMC Vet Res 2017;13:200. https://doi.org/10.1186/s12917-017-1124-2

[26] Mehta A, Sigman M. Identification and preparation of sperm for ART. Urol Clin 2014;41: 169–80. doi: 10.1016/j.ucl.2013.08.005.

[27] Souza ALP, Castelo TS, Queiroz JPAF, Barros IO, Paula VV, Oliveira MF, et al. Evaluation of anesthetic protocol for the collection of semen from captive collared peccaries (*Tayassu tajacu*) by electroejaculation. Anim Reprod Sci 2009;16:370–5. doi: 10.1016/j.anireprosci.2009.02.017.

[28] Waberski D, Magnus F, Ardon F, Petrunkin AM, Weitze KF, Petersen ET. Binding of boar spermatozoa to oviductal epithelium i*n vitro* in relation to sperm morphology and storage time. Reproduction 2006;131:311–8. doi: 10.1530/rep.1.00814.

[29] Sousa PC, Santos EAA, Souza ALP, Lima GL, Barros FFPC, Oliveira MF, Silva AR. Sperm morphological and morphometric evaluation in captive collared peccaries (*Pecari tajacu*). Pesqui Vet Bras 2013;33:924–30. https://doi.org/10.1590/S0100-

736X2013000700014.

[30] Celeghini ECC, Arruda RP, Andrade AFC, Nascimento J, Raphael CF. Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. Reprod Domest Anim 2007;42:479–88. doi: 10.1111/j.1439-0531.2006.00810.x.

[31] Castro LS, Assis PM, Siqueira AF, Hamilton TR, Mendes CM, Losano JD, et al. Sperm oxidative stress is detrimental to embryo development: a dose-dependent study model and a new and more sensitive oxidative status evaluation. Oxid Med Cell Longev 2016;2016:8213071. doi: 10.1155/2016/8213071

[32] Martins CF, Dode MN, Bao SN, Rumpf R. The use of the acridine orange test and the TUNEL assay to assess the integrity of freeze-dried bovine spermatozoa DNA. Gen Mol Res 2007;6:94e104.

[33] Pang YW, Sun YQ, Jiang XL, Huang ZQ, Zhao SJ, Du WH, et al. Protective effects of melatonin on bovine sperm characteristics and subsequent in vitro embryo development. Mol Reprod Dev 2016;83:993–1002. doi: 10.1002/mrd.22742.

[34] Gambini A, Stéfano A, Jarazo J, Buemo C, Karlanian F, Salamone DF. Embryo aggregation does not improve the development of interspecies somatic cell nuclear transfer embryos in the horse. Theriogenology 2016;86:1081–91. https://doi.org/10.1016/j.theriogenology.2016.03.040

[35] Rath D, Long CR, Dobrinsky JR, Welch GR, Schreier LL, Johnson LA. *In vitro* production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. J Anim Sci 1999;77:3346–52. doi: 10.2527/1999.77123346x.

[36] Weng XG, Cai MM, Zhang YT, Liu Y, Gao ZL, Song J, Liu ZH. Effect of Astragalus polysaccharide addition to thawed boar sperm on *in vitro* fertilization and embryo development. Theriogenology 2018;121:21–6. doi: 10.1016/j.theriogenology.2018.07.030.

[37] Borges AA, Santos MVO, Nascimento LE, Lira GPO, Praxedes ÉA, Oliveira MF, et al. Production of collared peccary (*Pecari tajacu* Linnaeus, 1758) parthenogenic embryos following different oocyte chemical activation and *in vitro* maturation conditions. Theriogenology 2020;142:320–7. https://doi.org/10.1016/j.theriogenology.2019.10.016

[38] Liu DY, Clarke CN, Baker HWG. Relationship between sperm motility assessed with the Hamilton-Thorn motility analyzer and fertilization rates *in vitro*. J Androl 1991;12:231–9.

[39] Holt C, Holt WV, Moore HD, Reed HC, Curnock RM. Objectively measured boar sperm motility parameters correlate with the outcomes of on-farm inseminations: results of two fertility trials. J Androl 1997;18:312–23.

[40] Broekhuijse MLWJ, Šoštarić E, Feitsma H, Gadella BM. Application of computer-assisted semen analysis to explain variations in pig fertility. J Anim Sci 2012;90:779–89. doi: 10.2527/jas.2011-4311

[41] Henkel RR, Schill WB. Sperm preparation for ART. Reprod Bioland Endocrinol 2003;1:1–22. doi: 10.1186/1477-7827-1-108.

[42] Zhu Z, Li R, Lv Y, Zeng W. Melatonin protects rabbit spermatozoa from cryo-damage via decreasing oxidative stress. Cryobiology 2019;88:1–8. doi: 10.1016/j.cryobiol.2019.04.009.

[43] Martínez-Pastor F, Lacalle E, Martínez-Martínez S, Fernández-Alegre E, Álvarez-Fernández L, Martinez-Alborcia MJ, et al. Low density Porcicoll separates spermatozoa from bacteria and retains sperm quality. Theriogenology 2021;165:28–36. https://doi.org/10.1016/j.theriogenology.2021.02.009.

[44] Santos CS, Campos LB, Praxedes ÉCG, Moreira SSJ, Souza-Júnior JBF, Comizzoli P, et al. Influence of antibiotics on bacterial load and sperm parameters during short-term preservation of collared peccary semen. Anim Reprod 2021;18:e20210021. doi: 10.1590/1984-3143-AR2021-0021.

[45] Ben L, Yan C, Yu SJ. Effect of swim-up and percoll treatment on sperm quality and *in vitro* embryo development in yak. J Integr Agric 2013;12:2235–22. https://doi.org/10.1016/S2095-3119(13)60378-0

[46] Cruz RS, Giuliano SM, Gambarotta MC, Morrell JM, Abraham MC, Miragaya MH, et al. Comparison of different methods of sperm selection of llama raw semen. *Animal* Reprod Sci 2016;173:8–12. doi: 10.1016/j.anireprosci.2016.08.001.

[47] Pradieé J, Sánchez-Calabuig MJ, Castaño C, O'Brien E, Esteso MC, Beltrán-Breña P, et al. Fertilizing capacity of vitrified epididymal sperm from Iberian ibex (*Capra pyrenaica*). Theriogenology 2017;108:314–20. doi: 10.1016/j.theriogenology.2017.11.021.

[48] Campos LB, Peixoto GCX, Silva AM, Souza ALP, Castelo TS, Maia KM, et al. Estimating the binding ability of collared peccary (*Pecari tajacu* Linnaeus, 1758) sperm using heterologous substrates. Theriogenology 2017;92:57–62. doi:10.1016/j.theriogenology.2

[49] Matás C, Coy P, Romar R, Marco M, Gadea J, Ruiz S. Effect of sperm preparation method on *in vitro* fertilization in pigs. Reproduction 2003;125:133–41. doi: 10.1530/rep.0.1250133.

[50] Cunha AT, Carvalho JO, Guimarães AL, Leme LO, Caixeta FM, Viana JH, et al. Bovine epididymal spermatozoa treatment for *in vitro* fertilization: Heparin accelerates fertilization and enables a reduction in coincubation time. Plos one 2019;14:e0209692. doi: 10.1371/journal.pone.0209692.

[51] Bezerra LGP, Souza ALP, Silva HVR, Vasconcelos FR, Moura ADAA, Pereira AF, et al. Ultrastructural description of fresh and frozen/thawed sperm derived from collared peccaries

(*Pecari tajacu* Linnaeus, 1758). Microsc Res Tech 2018;81:1301–09. https://doi.org/10.1002/jemt.23138

CAPÍTULO 4 – EFFECTS OF CAPACITATING MEDIA AND INCUBATION TIME ON COLLARED PECCARY SPERM QUALITY, ACROSOME REACTION, AND FERTILIZING ABILITY

Artigo de experimental: Effects of capacitating media and incubation time on collared peccary sperm quality, acrosome reaction, and fertilizing ability

Periódico de submissão: Animals

Fator de impacto: 3,231

Data de submissão: 7 de fevereiro de 2023

Article

Effects of Capacitating Media and Incubation Time on Collared Peccary Sperm Quality, Acrosome Reaction, and Fertilizing Ability

Maria V. O. Santos [1], Andréia M. Silva [2], Leonardo V. C. Aquino [1], Lhara R. M. Oliveira [1], João B. F. Souza-Junior [2], Moacir F. Oliveira [3], Alexandre R. Silva [2] and Alexsandra F. Pereira [1,*]

[1] Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoro, RN, Brazil; valeriasnts07@gmail.com (M.V.O.S.), leonardovt@live.com (L.V.C.A.), lharagirs@hotmail.com (L.R.M.O.), Alexsandra.pereira@ufersa.edu.br (A.F.P.)
[2] Laboratory of Animal Germplasm Conservation, Federal Rural University of Semi-Arid, Mossoro, RN, Brazil; andreia.m.silva@hotmail.com (A.M.S.), souza.jr@ufersa.edu.br (J.B.F.S.J), alexrs@ufersa.edu.br (A.R.S.)
[3] Laboratory of Applied Animal Morphophysiology, Federal Rural University of Semi-Arid, Mossoro, RN, Brazil; moacir@ufersa.edu.br (M.F.O.)
* Correspondence: alexsandra.pereira@ufersa.edu.br (A.F.P.)

Simple Summary: The collared peccary is a wild ungulate that is essential for the maintenance of ecosystems in America. The *in vitro* fertilization can contribute to the conservation and knowledge of the reproductive characteristics of this species. Several assisted reproduction techniques have been proposed; however, there is little information about the requirements for sperm capacitation in the collared peccary, an initial step in *in vitro* fertilization. For this, we compared different capacitating media (heparin, caffeine, or their combination) and incubation times (1, 3, and 6 h). We observed that heparin improved sperm motility and promoted the capacitation of collared peccary spermatozoa *in vitro*. Furthermore, the co-incubation of gametes in a medium with heparin for 3 h can be efficient for *in vitro* fertilization in collared peccaries.

Citation: Maria V. O. Santos; Andréia M Silva, A.M.; Leonardo V. C. Aquino; Lhara R. M. Oliveira; João B. F. Souza-Junior; Moacir F. Oliveira; Alexandre R. Silva; Alexsandra F. Pereira. Effects of Capacitating Media and Incubation Time on Collared Peccary Sperm Quality, Acrosome Reaction, and Fertilizing Ability. *Animals* **2023**, *12*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s):

Received: date
Accepted: date
Published: date

Abstract: Several assisted reproduction techniques have been proposed for collared peccaries; however, there is little information about the requirements for sperm capacitation, an initial step in *in vitro* fertilization. We aimed to determine the optimal conditions for collared peccary sperm capacitation by comparing Tyrode's albumin lactate pyruvate (TALP) or the same media plus heparin, caffeine, or their combination at different exposure times (1, 3, and 6 h). The samples were evaluated for kinetic parameters, membrane functionality and integrity, mitochondrial activity, morphology, DNA integrity, oxidative stress, acrosome reaction, and sperm fertilizing capacity using swine oocytes. Samples incubated with caffeine or heparin had a higher percentage of capacitated spermatozoa. The maximum percentage of capacitation was achieved after 3 h of incubation with either agent. Moreover, spermatozoa subjected to heparin capacitation showed better motility when compared to caffeine and lower oxidative stress. No differences were observed among the groups for the other parameters. In summary, collared peccary spermatozoa can be capacitated with caffeine or heparin; however, heparin better maintains sperm motility and oxidative stress. The co-incubation of gametes in a medium with heparin for 3 h can be efficient for *in vitro* fertilization in collared peccaries.

Keywords: assisted reproduction; wild ungulates; capacitation; spermatozoa; heparin

1. Introduction

Mammalian spermatozoa are not ready for fertilization after ejaculation. In addition to the maturation that occurs in the epididymis, spermatozoa must undergo capacitation in the female reproductive tract to be able to fertilize oocytes [1]. Sperm capacitation is characterized by alterations in plasma membrane permeability, biochemical changes in the membrane surface, protein phosphorylation, calcium, bicarbonate, and cyclic adenosine 3′,5′-monophosphate (cAMP) fluxes [2,3]. These structural and molecular changes result in the hyperactivation of motility and efficient acrosome reaction after binding to zona pellucida, which are related to incubation time in an appropriate environment [4]. In an *in vitro* fertilization (IVF) system, capacitation is induced in a controlled atmosphere using a defined medium that mimics the composition of fluids present in the female reproductive tract [5].

In collared peccaries, there is little information regarding the necessary requirements for sperm capacitation. This ungulate mammal plays an important ecological role in American biomes [6] and has adapted well to the captive breeding system, making it economic role relevant because of the interest in meat and leather [7,8]. Thus, establishing capacitation for IVF in collared peccaries could contribute to their conservation and management, as these animals suffer from poaching and habitat loss [9].

Nevertheless, although collared peccaries have similarities and are phylogenetically close to domestic swine, sperm cryopreservation is more effective when using protocols adapted from ruminants [10,11]. Furthermore, the composition of the seminal plasma of the collared peccary is different from that of the swine and has similarities with bulls and rams in terms of calcium and magnesium concentration [12-14]. These differences make it necessary to evaluate the most used capacitating conditions in both pigs and domestic ruminants to develop an ideal protocol for collared peccaries.

In IVF, the basic medium for sperm preparation and gamete co-incubation, such as medium Tyrode's Albumin Lactate Pyruvate (TALP), typically contains molecules involved in capacitation, such as bicarbonate, calcium, and albumin. Moreover, for ruminants and pigs, additional media supplementation has been carried out with more capacitating substances, such as heparin and caffeine [15].

The capacitating effect of heparin has been known for decades in bovine species [16]. This molecule can promote capacitation by removing capacitance-inhibiting seminal plasma proteins from the plasma membrane [17]. Heparin use is less frequent in pigs than in cattle [15]. Nevertheless, it was observed that 10 μg/mL heparin can increase the percentage of capacitation and acrosome reaction without affecting the viability of porcine spermatozoa [18,19]. In most porcine IVF protocols, caffeine is used to induce capacitation [20,15]. This molecule is a phosphodiesterase inhibitor that can elevate the concentration of cAMP in sperm stimulating capacitation and acrosome reaction [21]. In summary, many IVF protocols use a combination of caffeine and heparin to achieve a synergistic effect that can improve sperm fertilization [22].

The effect of capacitating media may also be related to the exposure time, which in turn may determine the co-incubation time of oocytes and spermatozoa for fertilization. The time must be considered important, especially for species related to pigs, which have a high incidence of polyspermy *in vitro* and a short co-incubation time between gametes [15].

Therefore, this study aimed to determine the best conditions for collared peccary sperm capacitation by comparing the medium TALP or the same medium plus heparin, caffeine, or their combination at different exposure times (1, 3, and 6 h). Moreover, we examined sperm functionality by assessing sperm binding to the zona pellucida of porcine oocytes, with co-incubation being performed under the best capacitation conditions.

2. Materials and Methods

Unless otherwise stated, reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Bioethics and Animals

All experiments were performed in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 30/2019) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 71834-1). All males used were from the Center of Multiplication of Wild Animals (CEMAS/UFERSA, Mossoró, RN, Brazil; 5°10′S, 37°10′W), registered at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, no. 1478912). Porcine ovaries were utilized from a local slaughterhouse.

Eleven sexually mature male collared peccaries were used for semen collection. Collared peccaries were housed outdoors in paddocks (20 × 3 m^2), covering an area of (3 × 3 m^2). The animals were fed pig food and fruit, and water was provided *ad libitum*.

2.2. Anesthesia, Semen Collection, and Processing

Before collection, the collared peccaries were fasted for 12 h and physically restrained using a hand net. Anesthesia was performed using an intravenous administration of propofol (Propovan, Cristalia, Fortaleza, Brazil) administered as a bolus (5 mg/kg), according to Souza et al. [23].

Semen collection was performed by electroejaculation using a previously established protocol for collared peccaries [10]. Briefly, a portable device (Autojac®, Neovet, Campinas, SP, Brazil) connected to a 12 V source was used. The stimulatory cycle comprised 10 stimuli of 3 s at each voltage with intermittent breaks of 2 s, starting from 5 V, followed by a voltage increase stepwise from 1 to 12 V. The total duration of the stimulus cycle was 10 min. The appearance, color, pH, and concentration (Neubauer chamber) of fresh ejaculates were immediately evaluated, and only samples with more than 70% motility were used.

2.3. Sperm Preparation, Capacitation, and Experimental Design

The base medium for semen preparation, capacitation, and IVF was TALP medium (114 mM NaCl, 3.2 mM KCl, 0.35 mM NaH_2PO_4, 10 mM Na lactate, 5 mM glucose, 25 mM $NaHCO_3$, 4,7 mM $CaCl_2$, 0,5 mM $MgCl_2$, 10 mM HEPES, 0.3% bovine serum albumin (BSA), 0,11 mM sodium pyruvate, 1% antibiotic–antimycotic solution) [24]. For separation of

spermatozoa from the seminal plasma, fresh semen was diluted 1:1 with pre-warmed TALP. This suspension was subjected to three centrifugations of 100×g for 3 min in conical microtubes at 37 °C [25]. The pellet was resuspended, and the concentration was assessed using Neubauer chamber for adjusting to 20×10^6 spermatozoa/mL using TALP supplemented with capacitating additional agents described below. The samples were incubated at 38.5 °C in a controlled atmosphere with 5% CO_2 and evaluated after 1, 3, and 6 h.

Two experiments were conducted. The first experiment was designed to evaluate the efficiency of different capacitating media for collared peccary spermatozoa. Thus, we analyzed the qualitative parameters of spermatozoa incubated in TALP base medium as a control without additional supplementation or TALP supplemented with 2 mM caffeine (TALP-Caf), 10 μg/mL heparin (TALP-Hep), or 2 mM caffeine plus 10 μg/mL heparin (TALP-CH) for different incubation times (1, 3, and 6 h). These times were defined based on the most common incubation time for IVF in porcine species [26]. The parameters evaluated were described below, including motility and kinetic parameters, normal morphology, functional integrity of the membrane, viability, DNA integrity, reactive oxygen species (ROS) levels, and acrosome reaction. Six animals were used, and a sample was recovered from each animal divided among treatments, with each animal considered an experimental replicate.

The second experiment was conducted to study the ideal exposure time for collared peccary spermatozoa to fertilize porcine oocytes. Sperm and porcine oocytes were incubated in the presence of the best capacitating media defined in the first experiment, and for the time of 3 h and 6 h to evaluate sperm binding to the zona pellucida of porcine oocytes. Five animals were used, and a sample was recovered from each animal divided between treatments, being each animal considered an experimental replicate.

2.4. Sperm Evaluations

2.4.1. Motility and Kinetic Parameters

Motility and kinetic parameters were analyzed using a computer-assisted sperm assessment (IVOS 7.4G; Hamilton-Thorne Research, Beverly, MA), with settings validated for the species [27]. The parameters analyzed were total motility (%), progressive motility (%), average pathway velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat/cross-frequency (BCF, Hz), straightness (STR, %), linearity (LIN, %), and rapid sperm population (%).

2.4.2. Sperm Morphology and Membrane Functional Integrity

Morphological evaluation of spermatozoa was performed with Bengal rose dye (Chromato®), and the samples were visualized by optical microscopy (1000×) from smears, where 100 cells per sample were counted [28]. To assess the functional integrity of the sperm membrane, the sperm suspension was incubated in a hypoosmotic solution (distilled water: 0 mOsm/L) to obtain an osmotic response under a hypoosmotic swelling test. Cells with a swollen curled tail were considered to have a functional membrane and 100 spermatozoa were examined per sample [29].

2.4.3. Sperm Viability

The structural integrity of the plasma membrane and mitochondrial activity were used to verify sperm viability, as described by Celeghini et al. [30]. For this, spermatozoa in TALP were incubated with Hoechst 33342 (40 μg/mL) for 10 min, followed by propidium iodide (0.5 mg/mL), and chloromethyl-X-rosamine (500 μM; Invitrogen, Carlsbad, CA, USA) for 8 min at the same time. Afterward, 100 cells were analyzed using an epifluorescence microscope (400×; Olympus BX51TF). Spermatozoa marked in blue and with a midpiece marked in red were classified as having intact sperm membranes and mitochondrial activity.

2.4.4. Sperm DNA Integrity

DNA integrity is an essential parameter for proper fertilization, then DNA integrity was assessed using acridine orange [31]. Briefly, smears of sperm suspensions were mounted, air-dried, and fixed in Carnoy's solution (methanol and glacial acetic acid in a 3:1 ratio) for 24 h. To verify chromatin integrity, the smears were dried again and incubated for 25 min in a buffer solution (15 mM Na2HPO4 and 80 mM citric acid, pH 2.5) at 75 °C. Finally, the spermatozoa were stained with acridine orange (0.2 mg/mL for 10 s) and washed with distilled water. The slides were covered with a coverslip, and 100 cells for sample were evaluated under an epifluorescence microscope. Green fluorescence on the head indicates intact DNA, and yellow, orange, or red fluorescence indicates denatured DNA.

2.4.5. ROS Levels

The appropriate balance of intracellular ROS levels is necessary to avoid oxidative stress, but physiological levels need to be maintained to allow cellular functions, such as sperm capacitation [32]. ROS levels were quantified to assess oxidative stress. Spermatozoa were stained with 2',7'-dichlorodihydrofluorescein diacetate (10 μM; Invitrogen, Carlsbad, CA, USA) according to Pang et al. [32]. An aliquot of 0.5 μL (0.1 M) was diluted with 2.5 mL of phosphate-buffered solution (PBS), and 50 μL of this solution was added to 50 μL of sperm suspension (40 × 10^6 spermatozoa/mL). The spermatozoa were then incubated at 37 °C for 30 min in the dark. Two washes were carried out by centrifugation (500×g for 5 min) and the pellet was resuspended in PBS. The samples were visualized on cover-slipped slides using epifluorescence microscopy to obtain images. This was analyzed using ImageJ software (National Institutes of Health), where 100 spermatozoa for sample were selected for quantification of fluorescence intensity. Fresh spermatozoa were used as calibrators, and the measured value of each sperm was divided by the mean of the calibrator to generate relative expression levels (arbitrary fluorescence units – AFU) [33].

2.5. Evaluation of Sperm Capacitation and Acrosome Reaction

Sperm capacitation was evaluated using chlortetracycline (CTC) staining, according to the method described by Kumar et al. [34] and Ward and Storey [35], with minor modifications. Initially, 40 μL of the sperm suspension was diluted (1:1) with 40 μL of CTC staining solution and incubated for 10 min. The CTC solution was composed of 750 mM CTC in 130 mM NaCl, 5 mM cysteine, and 20 mM Tris-HCl (pH 7.8). Excess dye was removed by centrifugation in TALP medium (100×g for 3 min). The pellet was placed on a slide to make a smear, which was covered with a

coverslip. The samples were examined using fluorescence microscopy at 1000× magnification (100 spermatozoa were analyzed per sample). The CTC assay is based on the ability of capacitated spermatozoa to exhibit a characteristic pattern by fluorescence detection. CTC forms complexes with Ca^{2+} bound to membrane phospholipids, changing the fluorescence pattern according to changes that occur in the plasma membrane [36]. Thus, intact spermatozoa had a uniform fluorescence throughout the head (pattern F), capacitated spermatozoa exhibited fluorescence in the acrosome region and a dark post-acrosome region (pattern B), and reacted spermatozoa had no fluorescence or fluorescence only in the post-acrosome region (AR pattern) (Figure 1).

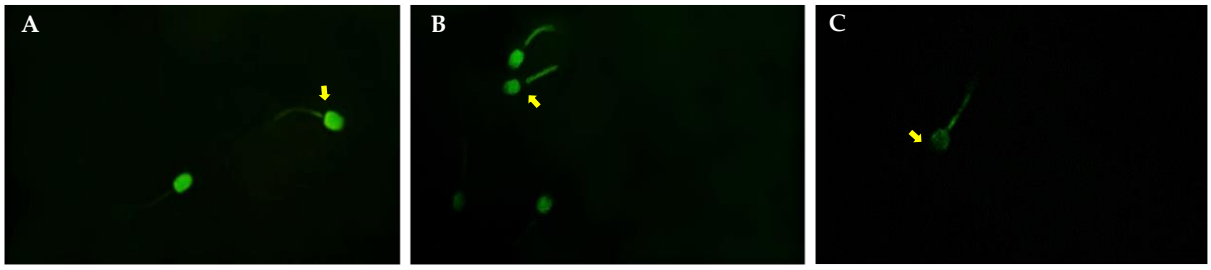

Figure 1. Capacitation and acrosome reaction of collared peccary spermatozoa incubated with different media to induce capacitation. Representative images of fluorescent staining patterns identified (1000×): (A) Intact cells with uniform fluorescence over the whole sperm head (Pattern F); (B) Capacitated cells with fluorescence-free band in the post-acrosome region (Pattern B); (C) Acrosome-reacted cells showing fluorescence only in the post-acrosome region (Pattern AR).

2.6. Assessment of Binding Ability

Oocytes were obtained from porcine ovaries collected from a slaughterhouse and transported to the laboratory in NaCl (0.9% at 35–37 °C). Follicles (3–6 mm) were aspirated using a 21G needle and 5.0 mL syringe. Only oocytes with more than one layer of *cumulus* cells and a homogeneous cytoplasm were selected. Oocytes were incubated (100 µL drops) in maturation medium [37] for 42–44 h in a humid atmosphere at 38.5 °C and 5% CO_2 until semen collection. The rate of oocyte maturation was not considered because oocytes were only used to assess sperm binding to the zona pellucida.

Spermatozoa obtained after washing by centrifugation were co-incubated with porcine oocytes. The final sperm concentration was assessed using Neubauer chamber and adjusted to 3×10^5 spermatozoa/mL; drops of 50 µL TALP medium containing capacitating agent were produced under mineral oil. The oocytes were washed, and *cumulus* cells were removed mechanically. They were grouped (10–15 oocytes) and incubated at 38.5 °C and 5% CO_2 for 3 or 6 h with spermatozoa in drops [38].

After co-incubation, the oocytes were washed and pipetted to remove the unbound spermatozoa. To assess the oocyte–sperm interaction, Hoechst 33342 (10 µg/mL, 15 min) staining was performed, and the oocytes were visualized under epifluorescence microscopy to count the number of spermatozoa bound to the zona pellucida.

2.7. Statistical Analysis

Data are expressed as mean ± standard error. All results were verified for normality using the Shapiro–Wilk test and for homoscedasticity using Levene's test. Values expressed as percentages were transformed into arcsine values. A two-way ANOVA for repeated measures was performed in the first experiment. In this analysis, we included the fixed effects of the capacitation agent, incubation time, and the interaction between these fixed effects (Equation 1). Tukey's post hoc test was used to verify the differences among treatments, incubation times, and all pairwise comparisons, which were considered significant when a $P < 0.05$. Statistical Analysis Software, version 8.0 (SAS Institute Inc., Cary, NC, USA), was used for all analyses.

$$\mathbf{Y_{ijk} = \mu + C_i + T_j + I_{ij} + e_{ijk}} \quad (1$$

where Y_{ijk} is the kth measurement of the seminal parameters at the ith capacitation agent and at the jth incubation time; μ is the overall mean; C is the fixed effect of the ith capacitation agent (i = TALP, TALP-Caf, TALP-Hep, TALP-CH); T is the fixed effect of the jth incubation time (j = 1, 3, and 6 h); I_{ij} is the effect of the interaction between the ith capacitation agent and the kth incubation time; e_{ijk} is the residual effect and includes the other sources of variation not considered in the model.

In the second experiment, the data were compared using the chi-square test. Statistical significance was set as $P < 0.05$.

3. Results

All ejaculates were milky white. To evaluate the best capacitation conditions for collared peccary spermatozoa, the volume of semen recovered was 2.4 ± 0.6 mL, and the concentration was 518.9 ± 54.6 × 10^6 spermatozoa/mL. Sperm motility immediately after collection was 94.8% ± 1.3. In the first experiment, for both total motility and ROS levels, the ANOVA for repeated measures showed a significant effect of the main effects (Treatment and Time) and the interaction (Treatment x Time). Additionally, the other variables had no interaction effect, with differences occurring either in relation to treatment or time. Then, the progressive motility and rapid speed were superior in the TALP-Hep medium compared to the TALP-Caf medium, whereas the TALP medium and the TALP-CH medium were similar to all treatments (Table 1). TALP-Hep medium had a higher percentage of STR, and LIN compared to TALP-Caf and TALP-CH medium. Except for BCF, all motility and kinetic parameters underwent significant changes over time (Table 1). All media evaluated were statistically similar in terms of normal morphology, functional integrity of membrane and viability (Table 2). DNA integrity was superior in TALP-Caf medium compared to TALP medium. Furthermore, sperm viability significantly decreased after 6 h of incubation (Table 2).

Table 1. Motility and kinetic parameters of collared peccary spermatozoa incubated with different media to induce capacitation.

Treatments	Progressive motility (%)	VAP (µm/sec)	VSL (µm/sec)	VCL (µm/sec)	ALH (µm)	BCF (Hz)	STR (%)	LIN (%)	Rapid (%)
TALP	36.8 ± 3.6[ab]	89.9 ± 4.0	62.8 ± 3.3	178.8 ± 7.3	8.2 ± 0.4	34.1 ± 0.6	67.2 ± 1.3[ab]	37.2 ± 1.4[ab]	39.5 ± 3.8[ab]
TALP-Caf	32.3 ± 2.9[b]	94.9 ± 3.1	63.3 ± 2.5	190.1 ± 5.6	8.9 ± 0.3	33.4 ± 0.4	64.6 ± 1.0[b]	35.2 ± 1.1[b]	35.6 ± 2.9[b]
TALP-Hep	48.6 ± 3.1[a]	92.9 ± 3.4	68.7 ± 2.8	174.9 ± 6.2	7.7 ± 0.3	34.3 ± 0.5	71.1 ± 1.1[a]	40.7 ± 1.2[a]	51.7 ± 3.2[a]
TALP-CH	39.8 ± 3.1[ab]	96.4 ± 3.4	64.3 ± 2.8	194.3 ± 6.2	8.9 ± 0.3	34.4 ± 0.5	63.4 ± 1.1[b]	34.3 ± 1.2[b]	44.2 ± 3.2[ab]
P-value	0.0056	0.6075	0.5002	0.1663	0.0882	0.4765	0.0009	0.0063	0.0084
Time									
1	59.2 ± 3.0[a]	100.2 ± 3.3[a]	74.6 ± 2.7[a]	184.4 ± 6.0[ab]	7.8 ± 0.3[a]	34.2 ± 0.5	71.5 ± 1.1[a]	42.5 ± 1.1[a]	62.9 ± 3.1[a]
3	43.8 ± 2.7[b]	96.9 ± 3.0[a]	63.9 ± 2.5[b]	198.4 ± 5.5[a]	9.2 ± 0.3[b]	34.1 ± 0.4	63.5 ± 1.0[b]	33.8 ± 1.0[b]	48.8 ± 2.9[b]
6	15.1 ± 2.5[c]	83.4 ± 2.8[b]	55.9 ± 2.3[b]	170.8 ± 5.1[b]	8.4 ± 0.3[ab]	33.8 ± 0.4	64.7 ± 0.9[b]	34.3 ± 1.0[b]	16.6 ± 2.6[c]
P-value	<.0001	0.0031	0.0002	0.0051	0.0197	0.7437	0.0001	<.0001	<.0001

Mean ± standard error. Six repetitions. TALP: medium without caffeine or heparin. TALP-Caf: TALP with caffeine. TALP-Hep: TALP with heparin. TALP-CH: TALP with caffeine and heparin. [a,b,c]: Lowercase letters indicate differences among treatments or incubation time.

Table 2. Quality of collared peccary spermatozoa incubated with different media to induce capacitation.

Treatments	Normal morphology (%)	Functional integrity of membrane (%)	Viability (%)	DNA integrity (%)
TALP	78.7 ± 2.8	72.7 ± 4.4	50.3 ± 5.8	92.1 ± 1.5[b]
TALP-Caf	76.9 ± 2.1	72.8 ± 3.4	46.3 ± 4.4	98.1 ± 1.2[a]
TALP-Hep	76.7 ± 2.4	68.6 ± 3.8	50.4 ± 4.9	96.8 ± 1.3[ab]
TALP-CH	78.9 ± 2.4	66.8 ± 3.8	41.0 ± 4.9	94.8 ± 1.3[ab]
P-value	0.9107	0.6511	0.5428	0.0300
Time				
1	78.3 ± 2.8	68.3 ± 3.6	57.8 ± 4.7[a]	99.1 ± 1.3
3	78.4 ± 2.1	75.9 ± 3.3	50.4 ± 4.4[a]	96.8 ± 1.2
6	76.8 ± 1.9	66.5 ± 3.1	32.9 ± 4.0[b]	95.0 ± 1.1
P-value	0.8349	0.1230	0.0025	0.2783

Mean ± standard error. Six repetitions. TALP: medium without caffeine or heparin. TALP-Caf: TALP with caffeine. TALP-Hep: TALP with heparin. TALP-CH: TALP with caffeine and heparin. [a,b]: Lowercase letters indicate differences among treatments or times.

When sperm capacitation was assessed with CTC (Table 3; Figure 1), TALP medium supplemented with heparin or caffeine increased the rate of capacitation compared to TALP medium without additional supplementation. The combination of caffeine and heparin showed similar effects to the other medium. The percentage of intact spermatozoa (non-capacitated) decreased in the TALP-Caf, TALP-Hep, and TALP-CH groups compared with that in the TALP group.

Furthermore, the capacitation rate increased significantly after 3 h of incubation and remained constant after 6 h. As a result, the number of intact spermatozoa decreased after 3 h and remained constant until 6 h. Additionally the acrosome reaction did not differ between treatments or incubation times.

In this experiment, total motility and ROS levels showed significant interaction among the treatments and the incubation times studied (Table 4). Therefore, among motility and kinetics parameters, only total motility showed interaction among treatments and incubation times. All media showed a significant decrease in the percentage of motile spermatozoa as the incubation time increased. However, when comparing different media within the same incubation times, it was observed that TALP-Hep maintains the highest motility rate after 6 h of incubation, while TALP-Caf has the lowest motility rate among the groups after 6 h (Table 1).

Table 3. Evaluation of collared peccary sperm capacitation after incubation with different media to induce capacitation.

Treatments	**Capacited spermatozoa (%)**	**Intact spermatozoa (%)**	**Acrosome-reacted spermatozoa (%)**
TALP	55.0 ± 5.2[b]	27.8 ± 3.6[a]	17.2 ± 3.1
TALP-Caf	75.9 ± 5.4[a]	14.4 ± 3.5[b]	9.7 ± 3.2
TALP-Hep	75.5 ± 5.4[a]	13.4 ± 3.5[b]	11.0 ± 3.2
TALP-CH	70.3 ± 5.2[ab]	14.3 ± 3.4[b]	15.4 ± 3.1
P-value	0.0236	0.0101	0.3473
Time			
1	53.8 ± 4.8[b]	27.8 ± 3.1[a]	18.4 ± 2.9
3	77.5 ± 4.5[a]	12.4 ± 2.9[b]	10.1 ± 2.7
6	76.2 ± 4.5[a]	12.3 ± 2.9[b]	11.5 ± 2.7
P-value	0.0009	0.0005	0.0842

Mean ± standard error. Six repetitions. TALP: medium without caffeine or heparin. TALP-Caf: TALP with caffeine. TALP-Hep: TALP with heparin. TALP-CH: TALP with caffeine and heparin. [a,b]: Lowercase letters indicate differences among treatments or times.

Regarding to ROS levels (Table 4), we observed significant interaction between media and incubation times. The TALP and TALP-Caf media had a significant decrease in ROS levels with increasing incubation time. On the other hand, TALP-CH and TALP-Hep media showed a significant increase in ROS levels with increasing incubation time. Even with these different behaviors, when comparing the media within the same incubation time, after 1 h the TALP-CH and TALP-Hep media resulted in lower ROS levels compared to TALP and TALP-Caf. After 3 h of incubation, TALP-CH medium showed lower levels of ROS, while all media were similar after 6 h.

Table 4. Results of parameters with significant interactions (total motility and ROS levels) of collared peccary spermatozoa incubated with different media to induce capacitation.

Treatments	Time	Total motility (%)	ROS levels (arbitrary fluorescence units)
TALP	1 h	88.0 ± 7.4^{a}	0.46 ± 0.02^{Ba}
	3 h	74.0 ± 4.3^{a}	0.39 ± 0.02^{Ba}
	6 h	44.5 ± 5.2^{ABb}	0.34 ± 0.02^{b}
TALP-Caf	1 h	87.7 ± 4.3^{a}	0.55 ± 0.02^{Ca}
	3 h	67.0 ± 5.2^{a}	0.35 ± 0.02^{Bb}
	6 h	18.3 ± 3.7^{Cb}	0.36 ± 0.02^{b}
TALP-Hep	1 h	90.3 ± 4.3^{a}	0.20 ± 0.02^{Aa}
	3 h	89.0 ± 5.2^{ab}	0.31 ± 0.02^{Bb}
	6 h	64.5 ± 5.2^{Ab}	0.32 ± 0.02^{b}
TALP-CH	1 h	92.5 ± 5.2^{a}	0.23 ± 0.02^{Aab}
	3 h	80.0 ± 5.2^{a}	0.21 ± 0.02^{Aa}
	6 h	32.0 ± 4.3^{BCb}	0.30 ± 0.02^{b}

Mean ± standard error. Six repetitions. TALP: medium without caffeine or heparin. TALP-Caf: TALP with caffeine. TALP-Hep: TALP with heparin. TALP-CH: TALP with caffeine and heparin. ^{ABC} Uppercase superscript letters indicate a significant difference between treatments within the same time ($P < 0.05$); ^{ab}Lowercase superscript letters indicate a significant difference within the treatments over time ($P < 0.05$).

Based on these results, we observed that TALP-Hep promoted the efficient capacitation of spermatozoa from collared peccaries and maintained better total motility, progressive motility, STR, LIN, rapid spermatozoa and ROS levels when compared to other medias. Furthermore, the 1 h incubation time was not efficient for the maximum capacitation of spermatozoa in this mammal. Thus, in the second experiment, a heterologous binding assay of peccary spermatozoa to porcine oocytes in the TALP medium supplemented with heparin (TALP-Hep) was performed, comparing the 3 and 6 h co-incubation times. A total of 81 and 73 oocytes were analyzed at 3 and 6 h, respectively. After 3 h and 6 h, 100% (81/81) and 98.6% (72/73) of the oocytes, on average, had sperm attached to the zona pellucida, respectively. There was no significant difference in the mean of bound sperm to oocytes between the incubation times (3 h: 47.0 ± 12.9 vs. 6 h: 71.1 ± 29.2) (Figure 2).

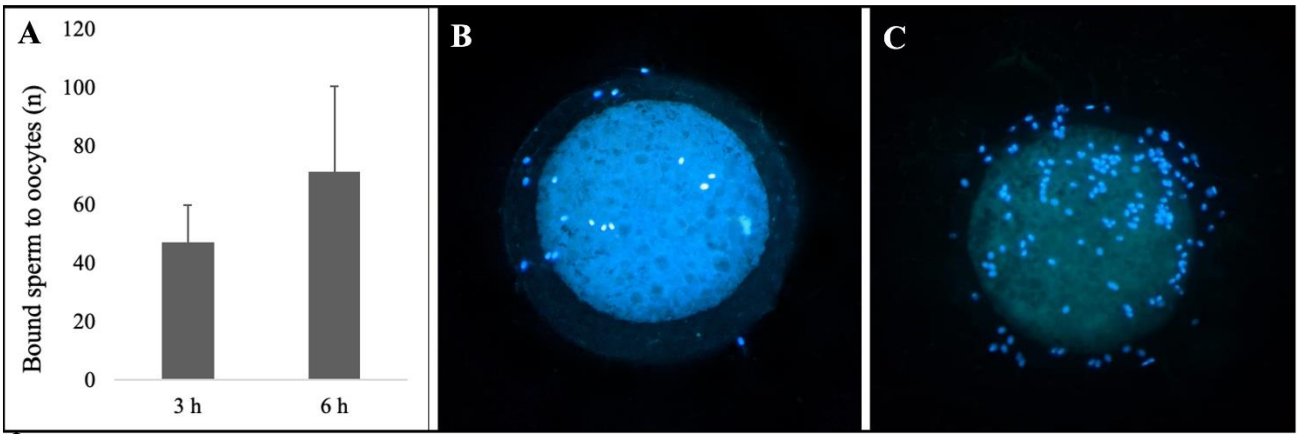

Figure 2. Binding of collared peccary spermatozoa submitted to heterologous IVF in medium containing heparin capacitating agent. (A) Mean of bound spermatozoa to oocytes. (B) Representative image of oocyte with few spermatozoa bound (400×). (C) Representative image of oocyte with many spermatozoa bound (400×).

4. Discussion

Collared peccary spermatozoa can be capacitated more efficiently in a medium containing heparin or caffeine. However, heparin promoted better progressive motility, STR, LIN and rapid spermatozoa compared to caffeine. Furthermore, using TALP-Hep the sperm total motility was better after 6 h of incubation and ROS levels were lower after 1 h if incubation in relation to caffeine. Our results provide essential insights into the development of IVF protocols for wild species. We showed that it is not feasible to apply protocols from domestic to wild species considering only phylogenetic information [39]. It is necessary to consider the unique characteristics of each species to develop the best IVF protocol for obtaining *in vitro* embryos.

In bovine species, the capacitance effect of heparin, as well as its mechanism of action, has been described for decades for use during the co-incubation of gametes for IVF [40]. Thus, the use of heparin is widespread, not only for bovine species but also for small ruminants [41]. However, for swine, the use of heparin is less common, although some studies have shown a beneficial effect of this substance on sperm capacitation. Dapino et al. [18] observed that swine spermatozoa had a higher percentage of capacitation evaluated by CTC when incubated for 2 h in the presence of 10 μg/mL heparin (46.5%) compared to the medium without heparin (28.7%).

Another point that can be highlighted is the composition of the TALP medium; it was previously observed that the culture medium must contain bicarbonate and calcium to enhance capacitation by heparin [18]. In fact, the capacitation of mammalian spermatozoa has been shown to be dependent on these components. The availability of calcium in the capacitation medium is necessary for the influx of extracellular calcium across channel proteins during incubation, which activates signaling pathways that promote hyperactivation, protein phosphorylation, and acrosome reaction [42,43]. In contrast, bicarbonate is related to increased permeability of the plasma membrane by inducing a change in the structure of the lipid membrane through adenylyl cyclase, cAMP, and protein kinase A [44].

For some species, such as cattle, the presence of calcium and bicarbonate is essential for capacitation [40,45]. For pigs, although bicarbonate is related to capacitation, the medium containing calcium and BSA, which also acts on the permeability of the plasma membrane, is sufficient to allow sperm capacitation [43]. Yeste et al. [43] concluded that bicarbonate is not an absolute requirement to achieve capacitation in pigs, in which BSA can act as the sole capacitating factor in proper conditions. In the present work, using only the TALP medium containing calcium, bicarbonate, and BSA, 55% of capacitation was observed, but this result was significantly lower compared to the medium supplemented with heparin or caffeine. Therefore, our results clearly show that TALP medium alone allows capacitation but is not sufficient to induce maximum capacitation. For collared peccary spermatozoa, the presence of heparin and caffeine promote essential stimuli to reach the highest percentage of capacitated spermatozoa. In this species, more studies are needed to determine the essential components of capacitation and their mechanisms of action. Based on these results, it would be necessary to assess whether there is a synergistic effect between heparin and calcium, bicarbonate or BSA.

One of the most crucial changes that occurs during the capacitation process is the change in the motility pattern to a hyperactivated state. The medium TALP-Hep significantly improved motility and kinetic parameters related to hyperactivated motility when compared to caffeine, indicating hyperactivated state. In addition, an interaction was observed between the times and the media studied when heparin maintained better sperm motility after 6 h of incubation compared to the other media. However, the TALP and TALP-CH media had results that can be considered middling, since they were similar to the high (TALP-Hep) and low (TALP-Caf) performance media with regard to kinetic parameters.

The other great indication of capacitation was observed when we first used fluorescent labeling CTC in collared peccaries. In this evaluation, it was also possible to observe that spermatozoa of this species required 3 h of incubation under capacitating conditions to exhibit a higher percentage of capacitation (Pattern B). This is probably the time needed for the accumulation of calcium in the acrosome (not in the cytoplasm) identified by the CTC, and with this, the spermatozoa can undergo the acrosome reaction at the time of oocyte fertilization [40]. A positive result was identified when the acrosome reaction of peccary spermatozoa remained without significant changes, regardless of the time and medium used. This result is quite satisfactory since when the spermatozoa undergo a spontaneous acrosome reaction, that is, without being faced with the appropriate stimulus that result from binding with the zona pellucida, it will enter the process of death [46]. Therefore, since acrosome reaction of collared peccary spermatozoa remained without significant changes, regardless of the time and medium used, it can be considered a positive result. Recently, in pigs was observed that the use of acrosome-reacted spermatozoa prevented blastocyst attainment after IVF [47].

Viability and morphology were not influenced by the capacitation media tested. However, the viability characterized by intact membrane and mitochondrial activity decreased after 6 h of incubation, being an expected result for spermatozoa in *in vitro* culture conditions. Additionally, DNA integrity was impaired when only TALP medium was used. However, it is not possible to state that this is related to oxidative

stress, since the TALP-Caf group showed higher levels of ROS and better DNA integrity compared to TALP.

The TALP-Hep and TALP-CH media increased ROS levels after 3 and 6 h of incubation, respectively. Despite this, it is not possible to state that the addition of heparin in the medium caused oxidative stress compared to the other groups. This can be inferred because ROS levels showed interaction between time and capacitation medium, where TALP-Hep and TALP-CH media showed lower ROS levels after 1 h of incubation, compared to TALP and TALP-Caf media, which had the highest levels of ROS. Thus, it is possible that heparin plays a positive role in oxidative balance. Furthermore, it has been observed in cattle [48] and swine [49,50] that physiological concentrations of ROS positively contribute to sperm capacitation, acrosome reaction, and protein phosphorylation. Thus, this may have contributed positively to the hyperactivation of sperm motility in collared peccaries.

Since the treatment with heparin improved sperm motility and some kinetic parameters, we assessed sperm functionality through the ability to interact with the zona pellucida of porcine oocytes in the TALP-Hep medium. Typically, IVF in porcine species is performed in periods of up to 6 h of incubation due to the high incidence of polyspermy. Since collared peccary spermatozoa already presented the highest capacitation rate after 3 h of incubation, we hypothesized that this timeframe could already be sufficient for gamete co-incubation [51]. In fact, despite the 6 h time showing a higher number of bound spermatozoa, there was no significant difference in relation to the 3 h time. Furthermore, in a short span of time, there was a large number of spermatozoa bound by oocytes (47.0 spermatozoa/oocyte), which was probably sufficient to obtain monospermic fertilization. In order to reduce the incidence of polyspermic fertilization, data on swine IVF demonstrated that spermatozoa bound to the zona pellucida after a short time of gamete co-incubation require a maximum of 2 h to penetrate the oocytes [26,52].

5. Conclusions

We can conclude that collared peccary spermatozoa can be capacitated by both caffeine and heparin; however, heparin provides better results in terms of sperm motility, kinetics, and ROS levels. The better time to induce maximum capacitation in these gametes was 3 h. Although more experiments are still required, these results indicate that the 3 h co-incubation time of gametes in the TALP medium supplemented with heparin can be efficient for use in the IVF technique for producing collared peccary embryos.

Author Contributions: Maria Valéria de Oliveira Santos: Conceptualization, methodology, writing, original draft, and investigation. Andréia Maria da Silva: Methodology and investigation. Leonardo Vitorino Costa de Aquino: Methodology, and investigation. Lhara Ricarliany Medeiros de Oliveira: Methodology, and investigation. João Batista Freire Souza-Junior: Methodology and statistical analysis. Moacir Franco de Oliveira: Conceptualization, methodology, and investigation. Alexandre Rodrigues Silva: Conceptualization, methodology, investigation, and review. Alexsandra Fernandes Pereira: Conceptualization, methodology, writing and original draft, supervision, project administration, and funding acquisition.

Funding: This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES; Financial Code –

001) and the National Council for Scientific Development (CNPq; no. 309078/2021-0).

Institutional Review Board Statement: All procedures were approved by the UFERSA Animal Ethics Committee, Opinion no. 30/2019, and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 71834-1).

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Acknowledgments: The authors thank the team of the Center for Multiplication of Wild Animals (UFERSA) and the industrial refrigerator of Mossoro for providing the animals used in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Kwon, W.S.; Rahman, M.S.; Lee, J.S.; Kim, J.; Yoon, S.J.; Park, Y.J.; You, Y.A.; Hwang, S.; Pang, M.G. A comprehensive proteomic approach to identifying capacitation related proteins in boar spermatozoa. BMC Genomics **2014**, 15, 1–13. https://doi.org/10.1186/1471-2164-15-897
2. Visconti, P.E.; Galantino-Homer, H.; Moore, G.D.; Bailey, J.L.; Ning, X.; Fornes, M.; Kopf, G.S. The molecular basis of sperm capacitation. J Androl **1998**, 19, 242–248. 10.3389/fcell.2018.00072
3. Flesch. F.M.; Gadella, B.M. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta **2000**, 1469, 197–235. Doi: 10.1016/s0304-4157(00)00018-6
4. Wada, A.; Harayama, H. Calmodulin is involved in the occurrence of extracellular Ca2+-dependent full-type hyperactivation in boar ejaculated spermatozoa incubated with cyclic AMP analogs. Anim Sci J **2021**, 92, e13552. Doi: 10.1111/asj.13552
5. Robles-Gómez, L.; González-Brusi, L.; Sáez-Espinosa, P.; Huerta-Retamal, N.; Cots-Rodríguez, P.; Avilés, M.; Gómez-Torres, M.J. Specific lectin binding sites during in vitro capacitation and acrosome reaction in boar spermatozoa. Ital J Anim Sci **2021**, 20, 372–82. https://doi.org/10.1080/1828051X.2021.1886611
6. Desbiez, A.L.J.; Keuroghlian, A.; Beisiegel, B.M.; Medici, E.P.; Gatti, A.; Pontes, A.R.M.; Campos, C.B.; Tófoli, C.F.; Júnior, E.A.M.; Azevedo, F.C.; Pinho, G.M. Avaliação do risco de extinção do cateto *Pecari tajacu* Linnaeus, 1758, no Brasil. Bio Brasil **2012**, 1, 74–83.
7. Bodmer, R.E. Responses of ungulates to seasonal inundations in the Amazon floodplain. J Trop Ecol **1990**, 6, 191–201. doi:10.1017/S0266467400004314
8. Nunes, A.V.; Guariento, R.D.; Santos, B.A.; Fischer, E. Wild meat sharing among non-indigenous people in the southwestern Amazon. Behav Ecol Sociobiol **2019**, 73, 1–10. https://doi.org/10.1007/s00265-018-2628-x
9. Gongora, J.; Reyna-Hurtado, R.; Beck, H.; Taber, A.; Altrichter, M.; Keuroghlian, A. *Pecari tajacu*. The IUCN Red List of Threatened Species (2011), Article e.T41777A10562361, 10.2305/IUCN.UK.2011-2.RLTS.T41777A10562361.en
10. Castelo, T.S.; Bezerra, F.S.; Lima, G.L.; Alves, H.M.; Oliveira, I.R., Santos, E.A.; Peixoto G.C.X.; Silva, A.R.; Effect of centrifugation and sugar supplementation on the semen cryopreservation of captive collared peccaries (*Tayassu tajacu*). Cryobiology **2010**, 61, 275–279. https://doi.org/10.1016/j.cryobiol.2010.09.005
11. Souza, A.L.P.; Lima, G.L.; Peixoto, G.C.X.; Castelo, T.S.; Oliveira, M.G.; Paula, V.V.; Silva, A.R. Sperm characteristics following freezing in extenders supplemented with whole egg yolk and different concentrations of low-density lipoproteins in the collared peccary (*Pecari tajacu*). Reprod Biol **2015**, 15, 223–228. Doi: 10.1016/j.repbio.2015.10.006
12. Mann T. 1954. The Biochemistry of Semen. London: Methuen & Co. Ltd. 1958p.
13. Pipan, M.Z.; Mrkun, J.; Strajn, B.J.; Vrtač, K.P.; Kos, J.; Pišlar, A.; Zrimšek, P. The influence of macro-and microelements in seminal plasma on diluted boar sperm quality. Acta Vet Scand **2017**, 59, 2–9. https://doi.org/10.1186/s13028-017-0279-y
14. Moreira, S.S.; Silva, A.M.; Praxedes, É.C.; Campos, L.B.; Santos, C.S.; Souza, A.L.; Pereira, A.F.; Souza-Júnior, J.B.; Costa, L.L.; Silva, A.R. Composition of collared peccary seminal plasma and sperm motility kinetics in semen obtained during dry and rainy periods in a semiarid biome. Ani reprod science **2019**, 211, 91–101. Doi: 10.1016/j.anireprosci.2019.106229
15. Romar, R.; Cánovas, S.; Matás, C.; Gadea, J.; Coy, P. Pig *in vitro* fertilization: Where are we and where do we go? Theriogenology **2019**, 137, 113–121. DOI: 10.1016/j.anireprosci.2019.106229
16. Parrish, J.; Susko-Parrish, J, Winer M, First N. Capacitation of bovine sperm by heparin. Biol Reprod **1988**, 38, 1171–1180. Doi: 10.1095/biolreprod41.4.683

17. Visconti, P.; Kopf, G. Regulation of protein phosphorylation during sperm capacitation. Biol Reprod **1998**, 59, 1–6. Doi: 10.1095/biolreprod59.1.1
18. Dapino, D.G.; Marini, P.E.; Cabada, M.O. Effect of heparin on *in vitro* capacitation of boar sperm. Biol Res **2006**, 39, 631-639. Doi: 10.4067/s0716-97602006000500006
19. Li, Y.; Li, R.H.; Ran, M.X.; Zhang, Y.; Liang, K.; Ren, Y.; He, W.C.; Zhang, M.; Zhou, G.B.; Qazi, I.H.; Zeng, C.J. High throughput small RNA and transcriptome sequencing reveal capacitation-related microRNAs and mRNA in boar sperm. BMC Genomics **2018**, 19, 1–12. Doi: 10.1186/s12864-018-5132-9
20. Abeydeera, L. R.; Day, B. N. *In vitro* penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. Theriogenology **1997**, 48, 537–544. Doi: 10.1016/s0093-691x(97)00270-7.
21. Nagai, T.; Miura, K.; Kikuchi, K.; Okamura, N. Effects of caffeine on *in-vitro* fertilization of pig follicular oocytes. J Reprod Dev **1993**, 39, 347–352. Doi: 10.31817/vjas.2018.1.2.08
22. Zhang, R.; Chu, M.; Chen, Y.; Yan, P. Heparin-induced and caffeine or ouabain supplemented capacitation of frozen-thawed yak (*Bos grunniens*) spermatozoa. *Reprod Dom Ani*. **2022**, 57, 587–597. Doi: 10.1111/rda.14098.
23. Souza, A.L.P.; Castelo, T.S.; Queiroz, J.P.A.F.; Barros, I.O.; Paula V.V.; Oliveira, M.F.; Silva, A.R. Evaluation of anesthetic protocol for the collection of semen from captive collared peccaries (*Tayassu tajacu*) by electroejaculation. Anim Reprod Sci **2009**, 16, 370–375. Doi: 10.1016/j.anireprosci.2009.02.017.
24. Rath, D.; Long, C.R.; Dobrinsky, J.R.; Welch, G.R.; Schreier, L.L.; Johnson, L. A. In vitro production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. J Anim Sci **1999**, 77, 3346–3352. Doi: 10.2527/1999.77123346x.
25. Mun, S-E.; Sim, B-W.; Yoon, S-B.; Jeong, P-S.; Yang, H-J.; Choi, S-A. et al. Dual effect of fetal bovine serum on early development depends on stage-specific reactive oxygen species demands in pigs. Plos One **2017**, 12, e0175427. https://doi.org/10.1371/journal.pone.0175427
26. Almiñana, C.; Gil, MA.; Cuello, C.; Parrilla, I.; Roca, J.; Vazquez, J.M. et al. Effects of ultrashort gamete co-incubation time on porcine *in vitro* fertilization. Anim Reprod Sci **2008**, 106, 393–401. https://doi.org/10.1016/j.anireprosci.2007.05.017
27. Souza, A.L.P.; Lima, G.L.; Peixoto, G.C.X.; Oliveira, M.F.; Silva, A.R. Use of *Aloe vera*–based extender for chilling and freezing collared peccary (*Pecari tajacu*) semen. Theriogenology **2016**, 85, 1432–1438. doi: 10.1016/j.theriogenology.2016.01.007.
28. Sousa, P.C.; Santos, E.A.A.; Souza, A.L.P.; Lima, G.L.; Barros, F.F.P.C.; Oliveira, M.F.; Silva, A.R. Sperm morphological and morphometric evaluation in captive collared peccaries (*Pecari tajacu*). Pesq Vet Bras **2013**, 33, 924–930. https://doi.org/10.1590/S0100-736X2013000700014
29. Santos, E.A.S.; Sousa, P.C.; Peixoto, G.C.X.; Simão, B.R.; Oliveira, M.F.; Silva, A.R. Establishing the hypoosmotic swelling test for sperm analysis in collared peccaries (*Pecari tajacu*). Arq Bras Med Vet Zootec **2013**, 65, 1257–1260. https://doi.org/10.1590/S0102-09352013000400044
30. Celeghini, E.C.C.; Arruda, R.P.; Andrade, A.F.C.; Nascimento, J.; Raphael, C.F. Practical techniques for bovine sperm simulta- neous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. Reprod Domest Anim **2007**, 42, 479–488. doi: 10.1111/j.1439-0531.2006.00810.x.
31. Martins, C.F.; Dode, M.N.; Bao, S.N.; Rumpf, R. The use of the acridine orange test and the TUNEL assay to assess the integrity of freeze-dried bovine spermatozoa DNA. Genet Mol Res **2007**, 6, 94e104.
32. Pang, Y.W.; Sun, Y.Q.; Jiang, X.L.; Huang, Z.Q.; Zhao, S.J.; Du, W.H., et al. Protective effects of melatonin on bovine sperm characteristics and subsequent *in vitro* embryo development. Mol Reprod Dev **2016**; 83: 993–1002. Doi: 10.1002/mrd.22742.
33. Santos, M.V.O.; Silva, A.M.; Praxedes, É.A.; Borges, A.A.; Teles Filho, A.C.D.A.; Souza-Junior, J.B.F. et al. Antioxidant effects of the essential oil of *Syzygium aromaticum* on bovine epididymal spermatozoa. Andrologia **2019**, 51, e13448. Doi: 10.1111/and.13448
34. Kumar, P.; Mohanty, T.K.; Kumaresan, A.; Nag, P.; Saraf, K.K.; Kumar, V.; Lathika, S.; Nayak, S.; Bhakat, M. Incubation of spermatozoa with Anandamide prior to cryopreservation reduces cryocapacitation and improves post-thaw sperm quality in the water buffalo (*Bubalus bubalis*). Anim Reprod Sci **2018**, 189, 77–83. doi: 10.1016/j.anireprosci.2017.12.010.
35. Ward, C.R.; Storey, B.T. Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev Biol **1984**, 104, 287–296. Doi: 10.1016/0012-1606(84)90084-8.
36. Osycka-Salut, C.E.; Martínez-León, E.; Gervasi, M.G.; Castellano, L.; Davio, C.; Chiarante, N.; Franchi, A.M.; Ribeiro, M.L.; Díaz, E.S.; Perez-Martinez, S. Fibronectin induces capacitation-associated events through the endocannabinoid system in bull sperm. Theriogenology **2020**, 153, 91–101. Doi: 10.1016/j.theriogenology.2020.04.031.
37. Gambini, A.; Stéfano, A.; Jarazo, J.; Buemo, C.; Karlanian, F.; Salamone, D.F. Embryo aggregation does not improve the development of interspecies somatic cell nuclear transfer embryos in the horse. Theriogenology **2016**, 86, 1081–1091. Doi: 10.1016/j.theriogenology.2016.03.040.

38. Weng, X.G.; Cai, M.M.; Zhang, Y.T.; Liu, Y.; Gao, Z.L.; Song, J.; Liu, Z. Effect of Astragalus polysaccharide addition to thawed boar sperm on in vitro fertilization and embryo development. Theriogenology **2018**, 121, 21–26. Doi: 10.1016/j.theriogenology.2018.07.030.
39. Santos, M.V.O.; Silva, A.R.; Pereira, A.F. Embryo production by fertilization in wild ungulates: progress and perspectives. An Anim Sci **2022**, 22, 1151–1162. Doi: https://doi.org/10.2478/aoas-2022-0013
40. Parrish, J.J. Bovine *in vitro* fertilization: *in vitro* oocyte maturation and sperm capacitation with heparin. Theriogenology **2014**, 81, 67–73. doi: 10.1016/j.theriogenology.2013.08.005.
41. Paramio, M.T.; Izquierdo, D. Recent advances in *in vitro* embryo production in small ruminants. Theriogenology **2016**, 86, 152–59. Doi: 10.1016/j.theriogenology.2016.04.027.
42. Vadnais, M.L.; Galantino-Homer, H.L.; Althouse, G.C. Current concepts of molecular events during bovine and porcine spermatozoa capacitation. Arch Androl **2007**, 53, 109–123. Doi: 10.1080/01485010701329386.
43. Yeste, M.; Fernández-Novell, J.M.; Ramió-Lluch, L.; Estrada, E.; Rocha, L.G.; Cebrián-Pérez, J.A.; Muiño-Blanco, T.; Concha, I.I.; Ramírez, A.; Rodríguez-Gil J.E. Intracellular calcium movements of boar spermatozoa during 'in vitro' capacitation and subsequent acrosome exocytosis follow a multiple-storage place, extracellular calcium-dependent model. Andrology **2015**, 3, 729–747. Doi: 10.1111/andr.12054.
44. Harrison, R.A.; Gadella, B.M. Bicarbonate-induced membrane processing in sperm capacitation. Theriogenology **2005**, 63, 342–351. Doi: 10.1016/j.theriogenology.2004.09.016.
45. Handrow, R.R.; First, N.L.; Parrish, J.J. Calcium requirement and increased association with bovine sperm during capacitation by heparin. J Exp Zool **1989**, 252, 174–182. Doi: 10.1002/jez.1402520209.
46. Florman, H.M.; First, N.L. The regulation of acrosomal exocytosis. I. Sperm capacitation is required for the induction of acrosome reactions by the bovine zona pellucida *in vitro*. Dev Biol **1988**, 12, 453–463. Doi: 10.1016/0012-1606(88)90307-7.
47. Lee, S.; Kim, Y.; Kang, B.H.; Yun, Y.S.; Park, C. The relationship between acrosome reaction and polyunsaturated fatty acid composition in boar sperm. Reprod Dom Anim **2020**, 55, 624–631. Doi: 10.1111/rda.13661.
48. Rivlin, J.; Mendel, J.; Rubinstein, S.; Ektovitz, N.; Breitbart, H. Role of hydrogen peroxide in sperm capacitation and acrosome reaction. Biol Reprod **2004**, 70, 518–522. Doi: 10.1095/biolreprod.103.020487.
49. Awda, B.J.; Mackenzie-Bell, M.; Buhr, M.M. Reactive oxygen species and boar sperm function. Biol Reprod **2009**, 81, 553–61. Doi: 10.1095/biolreprod.109.076471.
50. Chen, Y.; Wang, K.; Zhang, D.; Zhao, Z.; Huang, J.; Zhou, L.; Feng, M.; Shi, J.; Wei, H.; Li, L.; Wu, Z. GPx6 is involved in the in vitro induced capacitation and acrosome reaction in porcine sperm. Theriogenology **2020**, 156, 107–115. Doi: 10.1016/j.theriogenology.2020.06.020.
51. Funahashi, H.; Nagai. T. Regulation of *in vitro* penetration of frozen-thawed boar spermatozoa by caffeine and adenosine. Mol Reprod Dev **2001**, 58, 424–431. Doi: 10.1002/1098-2795(20010401)58:4<424::AID-MRD10>3.0.CO;2-1.
52. Gil, M.A.; Almiñana, C.; Cuello, C.; Parrilla, I.; Roca, J.; Vazquez, J.M.; Martinez, E.A. Brief coincubation of gametes in porcine *in vitro* fertilization: role of sperm: oocyte ratio and post-coincubation medium. Theriogenology **2007**, 67, 620–626. https://doi.org/10.1016/j.theriogenology.2006.09.022

6 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

As condições de seleção e capacitação espermática estabelecidas no presente trabalho representam um avanço significativo para o desenvolvimento da fertilização *in vitro* em catetos, permitindo utilização dessa técnica para a conservação e fins comerciais. Neste estudo, o gradiente de Percoll® na concentração de 45–90% mostraram melhores resultados de cinética espermática e manteve a qualidade dos espermatozoides. Por outro lado, os resultados usando o método de swim-up não foram satisfatórios, e esta técnica não foi recomendada para catetos nas condições estudadas. Quando o PG 45–90% e a lavagem por centrifugação foram comparados, o PG 45–90% melhoraram alguns parâmetros cinéticos. De acordo com os resultados da FIV heteróloga utilizando oócitos suínos, o PG 45–90% e a lavagem por centrifugação podem ser usados para separação de espermatozoides de catetos capazes de fertilizar oócitos *in vitro*.

No que diz respeito às condições de capacitação espermática de catetos, a cafeína e a heparina podem ser usadas para induzir a capacitação de forma eficiente, alcançando o valor máximo após 3 h de incubação. Contudo, a heparina melhora os resultados de motilidade espermática e níveis de espécies reativas de oxigênio. Esses resultados indicam que o tempo de co-incubação de gametas por 3 h no meio TALP suplementado com heparina pode ser eficiente para a FIV em catetos, pois permite bons resultados de ligação de espermatozoides de catetos a zona pelúcida de oócitos suínos.

Apesar dos oócitos suínos serem uma boa alternativa para avaliar a funcionalidade de espermatozoides de catetos, o desenvolvimento de embriões não ultrapassou o estágio de mórula. Portanto, o próximo passo para o estabelecimento da produção *in vitro* de embriões de catetos é aplicar os conhecimentos adquiridos neste trabalho para realização da FIV homóloga da espécie. Finalmente, esses resultados mostram importantes aspectos a respeito dos espermatozoides de catetos e reforçam as similaridades dessa espécie com suínos e ruminantes domésticos. Futuramente, será possível alcançar melhor eficiência com a utilização da FIV e de TRAs para a conservação e desenvolvimento comercial desta importante espécie silvestre.

ANEXO A –ARTIGO PUBLICADO NO PERIÓDICO ANNALS OF ANIMAL SCIENCE

Artigo de revisão: Embryo production by *in vitro* fertilization in wild ungulates – progress and perspectives

Periódico de submissão: Annals of animal science

Fator de impacto: 2,667

DOI: https://doi.org/10.2478/aoas-2022-0013

Ann. Anim. Sci., Vol. 22, No. 4 (2022) 1151–1162 *DOI: 10.2478/aoas-2022-0013*

EMBRYO PRODUCTION BY *IN VITRO* FERTILIZATION IN WILD UNGULATES: PROGRESS AND PERSPECTIVES – A REVIEW*

Maria Valéria de Oliveira Santos[1], Alexandre Rodrigues Silva[2], Alexsandra Fernandes Pereira[1]♦

[1]Laboratory of Animal Biotechnology, Federal Rural University of the Semi-arid Region, Av. Francisco Mota, 572, Mossoró, RN 59625-900, Brazil
[2]Laboratory of Animal Germplasm Conservation, Federal Rural University of the Semi-arid Region, Av. Francisco Mota, 572, Mossoró, RN 59625-900, Brazil
♦Corresponding author: alexsandra.pereira@ufersa.edu.br

Abstract

Wild ungulates are of fundamental importance for balancing ecosystems, as well as being the species of economic interest. Increasing concern over the accelerated population reduction of these species has resulted in the development of assisted reproduction techniques, such as *in vitro* fertilization (IVF), as a tool for conservation and multiplication. In the present scenario, IVF protocols were developed based on the methodologies used for domestic ungulates. Nevertheless, owing to the physiological and reproductive differences among the species, several factors associated with IVF and its relationship with the characteristics of the species of interest require clarification. *In vitro* conditions for the collection and selection of female and male gametes, oocyte maturation, sperm capacitation, co-incubation of gametes, and embryonic development can influence IVF results. Therefore, the present review considers the main advances in the methodologies already used for wild ungulates, emphasizing the strategies for improving the protocols to obtain better efficiency rates. Additionally, we discuss the conditions of each IVF stage, with emphasis on aspects related to *in vitro* manipulation and comparability with the protocols for domestic ungulates.

Key words: wildlife conservation, assisted reproduction, *in vitro* fertilization, gametes, embryos

The ungulates constitute a large and diverse group of mammals with hooves that are divided into artiodactyls or even-toed species and perissodactyls or odd-toed species. The first group includes the bovids, cervids, giraffes, pigs, peccaries, and camelids, whereas the second includes horses, rhinoceros, and tapirs. These animals play a key role in balancing diverse ecosystems through grazing, trampling, defecation, seed dispersal, and nutrient cycling (Mohr et al., 2005; Velamazan et al., 2020). Additionally, wild ungulates represent an important economic resource for many local communities because of their valuable meat and leather, as well as wildlife tourism (Gordon et al., 2004).

According to the International Union for Conservation of Nature (IUCN, 2021), approximately 140 species of more than 250 wild ungulates are on pathways to extinction and are classified as near threatened (32), vulnerable (52), endangered (42), critically endangered (15), and extinct in the wild (2). Moreover, the population of more than 160 species is currently decreasing. These animals are constantly affected by habitat loss, forest fragmentation, illegal hunting, and other consequences of anthropogenic activities (Malakoutikhah et al., 2020). Therefore, to conserve biodiversity, increase genetic variability, and rapidly multiply genotypes of wild ungulates, assisted reproduction techniques (ARTs) have been applied (reviewed by: Mastromonaco and Songsasen, 2020). ARTs are attractive tools for the reproduction and maintenance of wild species in zoos and reserves (reviewed by: Comizzoli et al., 2000; Herrick, 2019), as well as for efficiency of the economic activity associated with farm-raised non-domestic ungulates, such as red deer (Berg and Asher, 2003).

An interesting ART that has been developed in wild ungulates is *in vitro* fertilization (IVF). IVF can be divided into several stages, such as collection, selection, and maturation of oocytes, sperm collection, selection and capacitation, gamete co-incubation, *in vitro* embryo culture, and transfer of embryos. In general, the use of IVF associated with embryo transfer in wild ungulates has allowed the rescue of genetic material from male and female dead animals and those disabled for natural reproduction (Mahesh et al., 2011), the utilization of gametes of low quality and in low quantities (Hermes et

*Financed by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES, Financial Code 001). A.F. Pereira and A.R. Silva are recipients of CNPq grants.

al., 2009), and of gametes transported for long periods (García-Álvarez et al., 2011).

Despite many application possibilities and success in domestic ungulates, there are few reports of offspring of wild ungulates born from IVF. Overall, carrying out IVF in wild ungulates is a great challenge, primarily owing to the shortage of available male and female gametes, as well as the lack of expert knowledge to handle animals safely. These facts have hampered the development of continuous studies and the establishment of this ART (Hildebrandt et al., 2018).

There are several species of domestic or domesticated ungulates that can be used as experimental models for wild species. Nevertheless, notably, the protocols for model species are only a starting point for studies on phylogenetically close wild species (Figure 1). Therefore, as the physiology of the species of interest becomes known, it is important to adapt and test different conditions to meet intraspecific needs and achieve success in IVF. Many advances have been made in the IVF of red deer (*Cervus elaphus*) based on the usual protocols of domestic ruminants (Berg et al., 2002). However, Berg and Asher (2003) observed that the composition of the oviduct fluid of this species could be different from that of cattle and sheep. Therefore, these authors confirmed this difference and formulated culture media that were specific for red deer, achieving a significantly higher percentage of cleavage (68.0% *vs*. 49.3%) and blastocyst (12.9% *vs*. 4.5%).

The low efficiency of IVF in wild ungulates is due to technical limitations, such as insufficient knowledge about the basic reproduction and physiology of gametes and embryos, difficulty in handling animals safely and without stress, unavailability of biological material, and lack of studies establishing ideal conditions for the *in vitro* manipulation of gametes and embryos for different species (Andrabi and Maxwell, 2007; Cervantes et al., 2016; Mastromonaco and Songsasen, 2020; Thongphakdee et al., 2017). Although these factors are associated, this review focuses primarily on the aspects related to *in vitro* manipulation.

The quality and competence of gametes are fundamental to the success of fertilization and embryonic development (Berlinguer et al., 2008; Thongphakdee et al., 2017). These characteristics can be affected by several inherent factors of the technique, as well as the culture conditions at each stage of the IVF, which determine the maintenance of the quality and stimulate the development of gametes and embryos.

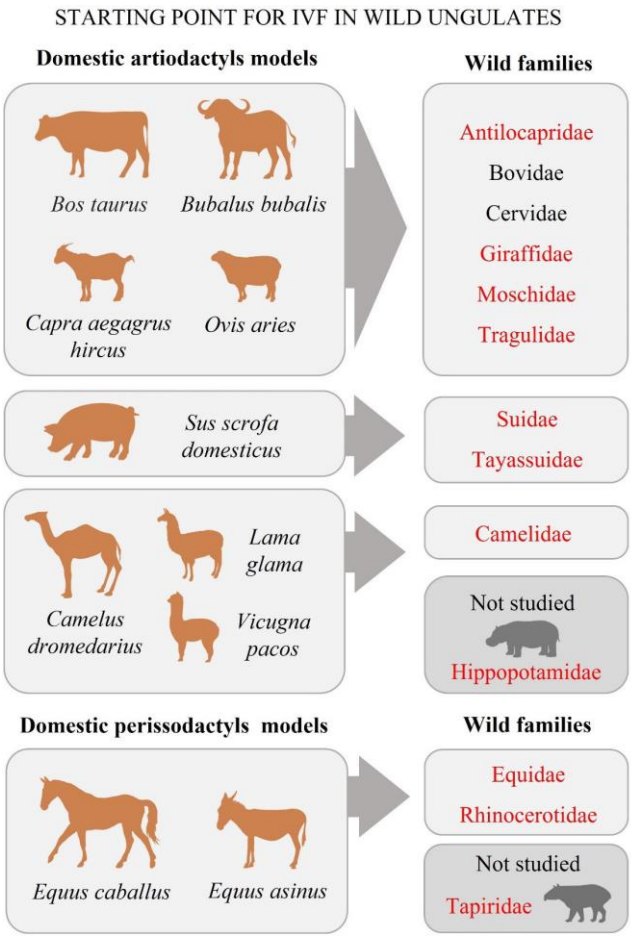

Figure 1. Domestic or domesticated ungulate species that due to phylogenetic proximity could be used as an experimental model for species of wild ungulate families, aiming *in vitro* fertilization (IVF). Family names written in red indicate that there are few or no studies

To be successful in IVF, it is particularly important to imitate the conditions found in the female reproductive tract during fertilization and pre-implantation embryonic development. If this scenario is not replicated properly, the quality and competence of gametes and embryos are negatively affected (Berg and Asher, 2003). Therefore, we will review the main advances obtained in the stages of IVF carried out in wild ungulates, emphasizing strategies for improving proto- cols to obtain better efficiency rates. Additionally, in parallel with the protocols of domestic ungulates wediscuss the conditions for collection and preparation of gametes in wild ungulates, as well as factors relatedto the co-incubation of these gametes and embryo cul- ture.

Oocyte preparation

Collection and selection of oocytes

The availability of competent oocytes, which, in other species of ungulates, has been considered as the main factor affecting blastocyst yields (Rizos et al., 2002), is a marked limitation of IVF in wild ungulates. This makes it difficult to establish ideal protocols for the manipulation of oocytes without jeopardizing their quality. Therefore, the quality of oocytes for IVF can be influenced by the conditions in which oocytes are collected *in vivo* or *post-mortem*.

In an ideal situation, the best strategy for obtaining good-quality oocytes is to collect them *in vivo*. To accomplish this, the follicular aspiration method using laparoscopy (Thongphakdee et al., 2017), laparotomy (Berlinguer et al., 2008) and transvaginal ultrasound (Cervantes et al., 2017) techniques can be used (Table 1). Nevertheless, few species have been sufficiently studied for this type of manipulation to be safe and efficient (Hildebrandt et al., 2018). This requires reli- able and efficient methods for monitoring reproductive activity, which is a very challenging task in wild un- gulates, as they are very temperamental and sensitive animals (Sontakke, 2018).

In domesticated camelids, such as llama (*Lama glama*, Trasorras et al., 2009) and alpaca (*Vicugna pacos*, Ratto et al., 2007), the efficiency of the laparotomy method exceeds 80%. However, in vicunas (*Vicugna vicugna*), the recovery rate was only 55.4% (46 oocytes from 83 follicles; Chaves et al., 2004). This shows that adaptation to the collection protocol is necessary to achieve greater success in the species of interest.

An interesting study was carried out on southernwhite rhinoceros (*Ceratotherium simum simum*) to al- low safe and efficient collection of oocytes *in vivo*, to apply to the last two remaining female northern white rhinoceros (*Ceratotherium simum cottoni*) to rescue them from extinction (Hildebrandt et al., 2018). These authors developed a new protocol for ovarian stimu- lation, anesthesia, and transrectal oocyte recovery, as well as a specific collection instrument for rhinoceros. In this case, only the procedure to remove the oocyte from inside the follicle was similar to that performed in horses, a phylogenetically close domestic species. From these procedures, the authors achieved a promising recovery rate of 26.4% (83 oocytes from 314 follicles).

In wild ungulates, it is possible to obtain gametes from *post-mortem* females in a timely manner when individuals are euthanized, ovariectomized (in the case of hunted or slaughtered animals) or experience accidental death (Table 1). In these situations, the collection efficiency may vary, as the reproductive stage and conditions of death are typically unknown, and euthanasia or ovariectomies may be performed for health reasons (Mahesh et al., 2011). Nevertheless, this can providea unique opportunity to obtain genetic material and study the ideal conditions for *in vitro* manipulation of oocytes from wild females. Mahesh et al. (2011) hadthe opportunity to obtain oocytes from a female nilgai (*Boselaphus tragocamelus*) that died during an out- break of foot and mouth disease. Despite the presenceof disease and the length of time before the ovarieswere recovered after the death (7–24 h), it was pos-sible to obtain 61.9% quality oocytes, 12% of which reached the 4–8 cell stage after *in vitro* co-incubation with sperm.

From *post-mortem* females, ovary collection and transportation conditions can also influence oocyte quality, particularly when the place of death is distant from laboratories, a common occurrence for wild ungulates. In Iberian red deer (*Cervus elaphus hispanicus*), ovaries could be stored for 12 h at different temperatures (5–8°C or 20–25°C) without affecting blastocyst rates, which ranged from 9.6 to 17.7% (García-Álvarez et al., 2011). In contrast, oocytes recovered from Hokkaido sika deer (*Cervus nippon yesoensis*) transported at 20–25°C for 12 h had a higher maturation rate (71.0%) than those transported for 24 h (31.0%, Tulake et al., 2014). Although it is possible to carry ovaries securely for a few hours, it is beneficial to reduce the transportation time to obtain better results.

Traditionally, follicular aspiration and ovarian slicing are methods used to collect oocytes *in vitro* (*post-mortem*). The choice of these methods depends on the laboratory routine, ovary anatomy, and oocyte attachment to the follicular wall, with great variation in recovery rates (number of oocytes recovered per female) (Table 1). In general, it is important that the method used permits the recovery of good-quality oocytes. For domestic ungulates, oocytes with many layers of cumulus cells and homogeneous cytoplasm are normally considered to be of good quality for maturation. In white-tailed deer (*Odocoileus virginianus*; Siriaroonrat et al., 2010) and Indian blackbuck (Rao et al., 2010), high-quality oocytes had an improved maturation rate (77.6% and 68.0%) compared to those with poor morphological characteristics (17.9% and 48.0%), such as minimal layers of cumulus cells and heterogeneous cytoplasm.

Table 1. Efficiency of oocyte collection and *in vitro* maturation in wild ungulates

Species	IUCN	Source of collection	Collection method	Oocytes/ female (%)	IVM medium*	IVM duration	IVM (%)	BL (%)	References
Collared peccary (*Pecari tajacu*)	LC	Slaughter	Follicular aspiration	16.2 (333/20)	TCM-199, 0.2 mM sodium pyruvate, 10% FBS, 20 µg/mL FSH/LH, 100 µM cysteamine, 10 ng/mL EGF	44 h	68.4	23.5	Borges et al. (2020)
Southern White Rhinoceros (*Ceratotherium simum simum*)	NT	Transrectal ultrasound guided	Follicular aspiration	4.6 (83/18)	DMEM-F12, 10% rhinoceros estrum serum	36–48 h	38.6	8.4	Hildebrandt et al. (2018)
Eld's deer (*Rucervus eldii thamin*)	EN	Ovariectomy/laparos-copy	Slicing; Follicular aspiration	44.3 (133/3)	TCM-199, 25 mM $NaHCO_3$, 0.2 mM pyruvate, 10% FBS, 100 µg/mL FSH, 100 µg/mL LH, 1 µg/mL estradiol, 0.1 mM cysteamine	24 h	60.0	7.8	Thongphakdee et al. (2017)
Iberian red deer (*Cervus elaphus hispanicus*)	LC	Hunting	Follicular aspiration	3.4 (301/88)	TCM-199, 10% FBS, 10 mU/mL FSH, 10 mU/mL LH, 50 ng/mL EGF	24 h	50.0	ND	Macías-García et al. (2018)
Wood bison (*Bison bison athabascae*)	NT	Transvaginal ultra-sound guided	Follicular aspiration	7.1 (106/15)	TCM-199, 5% FBS, 5 µg/mL LH, 0.5 µg/mL FSH	24 h	71.0	ND	Cervantes et al. (2016)
Nilgai (*Boselaphus tragocamelus*)	LC	Death by disease	Slicing	73.9 (517/7)	TCM-199, 10% FBS, 0.22 mM sodium pyruvate, 10 µg/mL FSH, 6 IU/mL LH, 1.0 µg/mL 17-β estradiol	24 h	63.4	0.0	Mahesh et al. (2011)
African black rhinoceros (*Diceros bicornis*)	CR	Occasional death	Follicular aspiration and slicing	14.8 (74/5)	TCM-199, 0.1 g/L L-glutamine, 0.1% BSA, 2 mM/L pyruvate, 10% FBS, 1 µg/mL FSH, 5 µg/mL LH, 1 µg/mL estradiol	36 h	3.6	0.0	Stoops et al. (2011)
White-tailed deer (*Odocoi-leus virginianus*)	LC	Slaughter	Slicing	14.2 (624/44)	TCM-199, 0.33 mM pyruvate, 2 mM glutamine, 2.2 mg/L $NaHCO_3$, 10% FBS, 5 µg/mL FSH, 5 µg/mL LH, 1 µg/mL estradiol	24 h	77.6	ND	Siriaroonrat et al. (2010)
Indian blackbuck (*Antilope cervicapra*)	LC	Occasional death	Slicing	21.8 (131/6)	TCM-199, 10% FBS, 0.22 mM pyruvate, 10 µg/mL FSH, 6 IU/mL LH	24 h	68.0	13.0	Rao et al. (2010)
Dama gazelle (*Nanger dama*)	CR	Semi-laparotomy	Follicular aspiration	5.8 (35/6)	TCM-199, 10% estrus sheep serum, 10 µg/mL FSH/LH, 1 µg/mL estradiol, 0.1 mg/mL glutamine	24 h	68.9	0.0	Berlinguer et al. (2008)
Burchell's zebra (*Equus quagga burchellii*)	NT	Slaughter	Slicing and scraping	10.2 (61/6)	TCM-199, 10% FBS, 2.5 µg/mL FSH, 2.5 µg/mL LH, 1 µg/mL estradiol	27–34 h	ND	1.0	Meintjes et al. (1997)
Hartmann's zebra (*Equus zebra hartmannae*)	VU			13.8 (83/6)					

BL: blastocyst. LC: least concern. NT: near threatened. VU: vulnerable. CR: critically endangered. EN: endangered (IUCN, 2021). ND: not determined. FBS: Fetal bovine serum. *Antibiotics and antimycotics have been omitted from the composition of the medium.

In vitro maturation conditions

In domestic ungulates, such as bovines, goats, sheep, swine, and equines (including donkeys), the base medium for *in vitro* maturation (IVM) is composed of TCM-199 with 5%–10% fetal bovine serum or follicu- lar fluid and supplements that vary among species (Ta- ble 1). Normally, the same base medium has been used in wild ungulates, such as wood bison (Cervantes et al., 2016), gazelles (Berlinguer et al., 2008), mouflon (Ptak et al., 2002), deer species (Berg et al., 2002; Locatelli et al., 2012; Thongphakdee et al., 2017), collared peccary (*Pecari tajacu*, Borges et al., 2020), and zebra (Meintjes et al., 1997). Interestingly, for black rhinoceros oocytes IVM, Stoops et al. (2011) used TCM-199 with 10% fetal bovine serum and obtained only 3.6% (3/83) oocytes in metaphase II (MII). In contrast, Hildebrandt et al. (2018) used DMEM-F12 medium with 10% rhinoceros estradiol serum, reaching a maturation rate of 38.6% in white rhinoceros.

The compounds typically used as supplements to stimulate *in vitro* oocyte maturation are FSH, LH, and estradiol, as well as epidermal growth factor (EGF). Siriaroonrat et al. (2010) evaluated the effect of 1 µg/mL estradiol during IVM in white-tailed deer oocytes and observed a higher maturation rate in the presence of this hormone (77.6% *vs.* 51.1%). An improved maturation rate was also obtained by FSH, and LH supplementation compared to hormone-free medium (68.0% *vs.* 39.6%) in blackbuck (Rao et al., 2010). These positive results can be attributed to similarities in the *in vivo* hormonal conditions in these animals.

Regarding the effect of adding EGF, in Iberian red deer, an increase in oocyte maturation rate was observed in a medium supplemented with FSH and LH and 50 ng/mL EGF (46.3%) than in the medium without EGF (19.5%, Macías-García et al., 2018). In collared peccary, we observed that the presence of 10 ng/mL of EGF during IVM did not improve meiotic development, but improved oocyte morphometry (Borges et al., 2020). Thus, different oocyte responses to this growth factor are observed in these ungulates and are likely owing to the physiological characteristics of each species.

Curiously, in sika deer (*Cervus nippon hortulorum*), there are differences in IVM media requirements owing to seasonality. In this species, it was observed that during the estrous season, the best IVM medium was supplemented with 10 µg/mL FSH, 1 µg/mL LH, 0.2 mM cysteamine, and 50 ng/mL EGF, while in the anestrous season, double these concentrations were more efficient (Yin et al., 2013). Additionally, based on the findings observed in studies with other deer species, Rola et al. (2021) observed that the IVM medium containing 5.0 µg/mL FSH, 100 µg/mL hCG and 1.0 µg/mL estradiol was efficient for maturation of brown brocket deer (*Mazama gouazoubira*) oocytes (64.5%).

The ideal time for oocyte maturation in domes- tic ungulates can be quite variable, ranging from 22 to24 h for cattle, sheep, and goats, 42–44 h for pigs, and 36 h for horses. Considering this, oocyte maturation times (24 and 30 h) were compared in wood bison, and it was observed that 24 h was sufficient for 71% of the oocytes to reach MII, similar to cattle (Cervantes et al., 2016). In contrast, 27 h was necessary for 75% of red deer (*Cervus elaphus*) oocytes to reach MII using the standard medium for domestic ruminants (Berg et al., 2002). Likewise, in collared peccary, 48 h IVM was more efficient than 24 h for the nuclear stage, similar to the time for swine (76.2% *vs*. 19.0%; Borges et al., 2018). In vicunas, 41% of oocytes matured *in vitro* after 27 h, which is a shorter time than for other domesticated camelids (Chaves et al., 2004). Finally, in rhinoceros, an IVM time of 36–44 h has been reported (Hildebrandt et al., 2018), which isthe approximate time for horses and donkeys (Li et al., 2021). Therefore, establishing the IVM time is an initial step in obtaining oocytes suitable for fertilization.

Sperm collection, selection, and capacitation

After collection, the main factors that influence IVF sperm quality were selection and capacitation conditions. Semen has been collected from wild ungulates primar- ily via electroejaculation till date (Pukazhenthi, 2016). Nevertheless, in species without established electroejaculation protocols, it is possible to obtain sperm from the epididymis in cases of occasional death, castration, and euthanasia (Table 2).

Fresh or preserved sperm can be used for IVF in wild ungulates. IVF has been consistently combined with sperm refrigeration (Hermes et al., 2009) and cryopreservation (García-Álvarez et al., 2011; Locatelli et al., 2012) for practicality and germplasm banks (Benham et al., 2021). Nevertheless, semen conservation protocols can impair sperm quality, as reviewed by Pukazhenthi (2016). Notably, sperm storage protocols have not been defined for many wild ungulate species. Thus, it is necessary to develop efficient methods to maintain the viability and fertility of these gametes.

The two most common sperm selection methods in wild ungulates are the Percoll® (colloidal silica coated with polyvinylpyrrolidone) gradient and swim-up methods, as presented in Table 2. When using the appropriate method for the species, selection increases the percentage of motility, viability, normal morphology, and fertility (Liu et al., 2013; Santiago-Moreno et al., 2014). In addition, this step is essential to remove seminal plasma (fresh semen), extender, and cryoprotectants (frozen–thawed sperm), which may negatively influence fertilization (Liu et al., 2013). For frozen–thawed spermatozoa of scimitar-horned oryx (*Oryx dammah*), better quality sperm were obtained using Percoll and swim-up compared to the washing method (centrifugation); however, the success of heterologous fertilization was similar among treatments (65–72.7% cleavage; O'Brien and Roth, 2000). For gazelles (*Gazella dama mhorr*), motility increased from 53.1% to 77.5% after the selection of frozen–thawed sperm using the swim-up (Berlinguer et al., 2008).

Table 2. Sperm collection, selection and co-incubation conditions in wild ungulates associated with the development of embryos produced by *in vitro* fertilization

Species	IUCN	Sperm collection	Sperm types	Selection method	Motile (%)	Concentration (x 10^6)	IVF medium	Efficiency (%)	References
Wood bison (*Bison bison athabascae*)	EN	ND	Frozen/thawed	Percoll® (45/90%)	60–75	5.0	Brackett-Oliphant	53.8 BL	Palomino et al. (2020)
Eld's deer (*Rucervus eldii thamin*)	EN	ND	Frozen/thawed	Percoll® (45/90%)	ND	0.05	Deer-SOF	7.8 BL	Thongphakdee et al. (2017)
Vietnamese sika deer (*Cervus nippon pseudaxis*)	LC	Electroejaculation	Frozen/thawed	Percoll® (45/90%)	ND	3.0	SOFaa, 20% ESS, 5 µg/mL heparin	29 BL	Locatelli et al. (2012)
Iberian red deer (*Cervus elaphus hispanicus*)	LC	Epididymis	Frozen/thawed	Percoll® (45/90%)	ND	1.0	Deer-SOF, 20% ESS	17.7 BL	García-Álvarez et al. (2011)
Nilgai (*Boselaphus tragocamelus*)	LC	Epididymis	Fresh	Swim-up	50–60	0.01	TALP, 100 µg⁄mL heparin	42 CL	Mahesh et al. (2011)
Black rhinoceroses (*Diceros bicornis minor*)	CR	ND	Cooling (4ºC)	Swim-up	70	0.5	SOF, 20 µg/mL heparin, 5% ERS	50 CL	Hermes et al. (2009)
Gazelle (*Nanger dama*)	CR	Electroejaculation	Frozen/thawed	Swim-up	77.5	1.0	SOF, 2% ESS, 1 µg⁄ml hypotaurin, 10 µg⁄ml heparin	30 CL	Berlinguer et al. (2008)
European mouflon (*Ovis orientalis musimon*)	VU	ND	Frozen/thawed	Swim-up	ND	1.0	SOF, 20% ESS, 2.9 mM calcium lactate, 16 mM isoproterenol	37 BL	Ptak et al. (2002)
Burchell's zebra (*Equus quagga*)	NT	Epididymis	Cooling (4 ºC)	Not selected	ND	9.0	Hsm's F10, 1 µM calcium ionophore	38 CL	Meintjes et al. (1997)

EN: endangered. LC: least concern. VU: vulnerable. CR: critically endangered (IUCN, 2021). ND: not determined. ESS: estrous sheep serum. ERS: estrous (white) rhinoceros serum. *Only centrifugation. CL: cleavage rate. BL: blastocyst rate.

When sperm selection methods in Iberian ibex (*Capra pyrenaica*) and European mouflon (*Ovis orientalis*) were compared, Capripure® (colloidal silica particles coated with silane) density-gradient centrifugation and dextran swim-up methods exhibited recovery rates of quality spermatozoa of 60.6% *vs.* 11.3% (*C. pyrenaica*) and 47.8% *vs.* 27.8% (*O. orientalis*), respectively. Therefore, density-gradient centrifugation is recommended for both species, highlighting the similarities among the different genera (Santiago-Moreno et al., 2014). Moreover, the Percoll gradient improved the linearity (64.5% *vs*. 28.5%) and viability (76.0% *vs*. 40.9%) of post-thaw sperm in Iberian red deer (García-Álvarez et al., 2016).

After sperm selection, proper oocyte-sperm interaction requires sperm capacitation, which is typically induced by different capacitating agents present in the IVF culture medium. Heparin has been widely used for sperm capacitation in domestic ruminants. However, although heparin for 1–4 h increases sperm capacitation in cattle, in the scimitar-horned oryx (*Oryx dammah*), which belongs to the same family, the rate of acrosome reaction did not increase after 6 h of incubation, suggesting that this species may require a longer incubation time (Roth et al., 1998). In contrast, when the effect of heparin (60 µg/mL) as a capacitating agent was evaluated in fallow deer (*Dama dama*), it led to an improved rate of acrosome reaction compared to the control medium (Fernández et al., 2013).

In general, equine sperm have difficulties in fertilizing oocytes *in vitro*, likely due to deficient capacitation (Leemans et al., 2019). Therefore, in domestic and wild horses, studies to improve the capacitation conditions for sperm are fundamental for the success of IVF. In Persian onager (*Equus hemionus onager*), the acrosome integrity was evaluated after sperm thawing, and good resistance of this species maintained 63% of intact acrosomes af- ter 24 h of incubation at 22ºC (Schook et al., 2013). Although capacitation allows fertilization to occur, studies on sperm capacitation in wild ungulates are still limited.

In vitro fertilization and embryo culture

During gamete co-incubation, sperm concentration and culture medium may influence the IVF results. In the gaur (*Bos gaurus*), a higher sperm concentration (5.0 × 10^6 sperm/mL) increased polyspermy by 40% when compared to a lower concentration (1.0 × 10^6 sperm/mL, Johnston et al., 1994). In red deer, a blastocyst formation rate of 9% after IVF with 2.0 × 10^6 sperm/mL was observed, while a sperm concentration of 1.0 × 10^6 sperm/mL could not generate blastocysts (Comizzoli et al., 2001 a). However, under optimized conditions in the same species, Berg et al. (2002) evaluated sperm concentrations ranging from 0.001 to 1.0 × 10^6 sperm/mL, and the best monospermic penetration rate was obtained using 0.4 × 10^6 sperm/mL (77.0%). These controversial data show the importance of continuous studies in different conditions to achieve more satisfactory results, allowing efficient fertilization without causing polyspermy.

The composition of the IVF medium should fa- vor the maintenance of gamete quality and stimulate fertilization. The most common IVF media in ungu-lates are synthetic oviduct fluid (SOF, Tervit et al., 1972), Tyrode's albumin lactate pyruvate (Parrish et al., 1988), FERT (Rath et al., 1999) and Brackett–Oli- phant (Brackett and Oliphant, 1975), which are sup-plemented with capacitation agents. However, these conditions have seldom been studied in wild ungulates. In red deer, when the addition of sheep serum (20%), heparin (20 µg/mL), and/or bovine serum albumin (BSA; 6 mg/mL) in the IVF medium was evaluated,only the medium containing sheep serum allowed an acceptable rate of monospermic fertilization (56.2%), similar to that observed in small domestic ruminants (Berg et al., 2002).

In horses, the success of IVF is very limited because of the difficulty in penetrating the zona pellucida *in vitro* (Hinrichs et al., 2002). Compared to other domestic mammals, conventional culture conditions have not been shown to be effective in activating sperm capacitance triggers that allow efficient fertilization (Leemans et al., 2019). Therefore, alternatives have been sought to overcome this barrier. Meintjes et al. (1997) used a drilling technique with an acid solution of the zona pellucida before co-incubation with Burchell's zebra (*Equus quagga burchelli*) sperm. In this case, only 41% (31/75) of the oocytes were cleaved, and only 2% (1/75) developed to the blastocyst stage. In this respect, advances achieved with the use of intracytoplasmic sperm injection (ICSI) in the IVF stage can strongly benefit horses. Recently, a 6% rate of blastocyst formation was observed using the ICSI of Burchell's zebra sperm in horse oocytes (Gambini et al., 2020).

Additionally, the ICSI methodology can increase the success of IVF in wild ungulates with low-quality gametes. In the white and black rhinoceros (*Ceratotherium simum* and *Diceros bicornis*, respectively), the use of IVF and ICSI for embryo production has been described; although, as it was only possible to obtain a 4-cell embryo after IVF in black rhinoceros, further reproductive studies are required (Hermes et al., 2009). Subsequently, ICSI was performed on southern white rhinoceros (*Ceratotherium simum simum*) and a rate of 14.5% for cleaved embryos and 8.4% for blastocysts was obtained (Hildebrandt et al., 2018).

The time of development to the blastocyst stage *in vitro* varies among species of domestic ungulates, being 7–8 days in cattle and sheep, and 6–7 days in pigs and horses (Piliszek and Madeja, 2018). Several *in vitro* culture protocols of domestic and wild ungulate embryos use SOF medium, although in domestic swine, the medium for porcine embryos (PZM) is widely used. Nevertheless, interestingly, collared peccary embryos showed good development *in vitro* using SOF medium (Borges et al., 2020).

Few species of wild ungulates have been studied until the stage of embryo culture in IVF, and consequently,

only some species have had embryos transferred and births (Figure 2). In many cases, embryonic development does not reach the blastocyst phase, or the rate of blastocyst formation is low (Table 2). This is likely due to the conditions used in the previous steps be-ing unsuitable for promoting efficient embryonic development. Moreover, the *in vitro* embryo culture (IVC) medium must also be carefully chosen to ensure successive cleavage of the embryos. Red sheep (*Ovis orientalis gmelini*) zygotes were cultured in Charles Rosenkrans 2 medium (CR2) or modified Brinster's ovum culture medium with bovine oviductal epithelial cells. These culture conditions did not lead to different cleavage rates, which ranged from 23.0% to 29.0%, and both media allowed the birth of offspring after embryo transfer (Flores-Foxworth et al., 1995). In red deer, the presence of ovine oviduct epithelial cells in SOF medium during IVC increased the rate of blasto- cyst formation compared to that in the medium alone (39.0% *vs*. 5.0%; Locatelli et al., 2005).

Techniques that assist in the establishment of IVF protocols

Parthenogenetic activation

A parthenogenetic or artificial oocyte activation strategy has been used as a model for evaluating experimental conditions that can influence embryonic development after IVF and ICSI in wild species (Yin et al., 2013). This technique can be used to assess oocyte competence and establish embryo culture media (Borges et al., 2020). Moreover, most mammals subjected to ICSI require an activation protocol to induce embryonic development. Thus, artificial oocyte activation can establish these protocols. Rao et al. (2010) evaluated the competence of mature oocytes from Indian blackbuck for embryonic development using parthenogenetic activation and obtained 58.0% cleavage and 13.0% blastocysts. In wild deer species, artificial activation was used to assess the competence of oocytes collected immediately or 12 h after animal death, with no significant effect observed on embryonic development (Brahmasani et al., 2013).

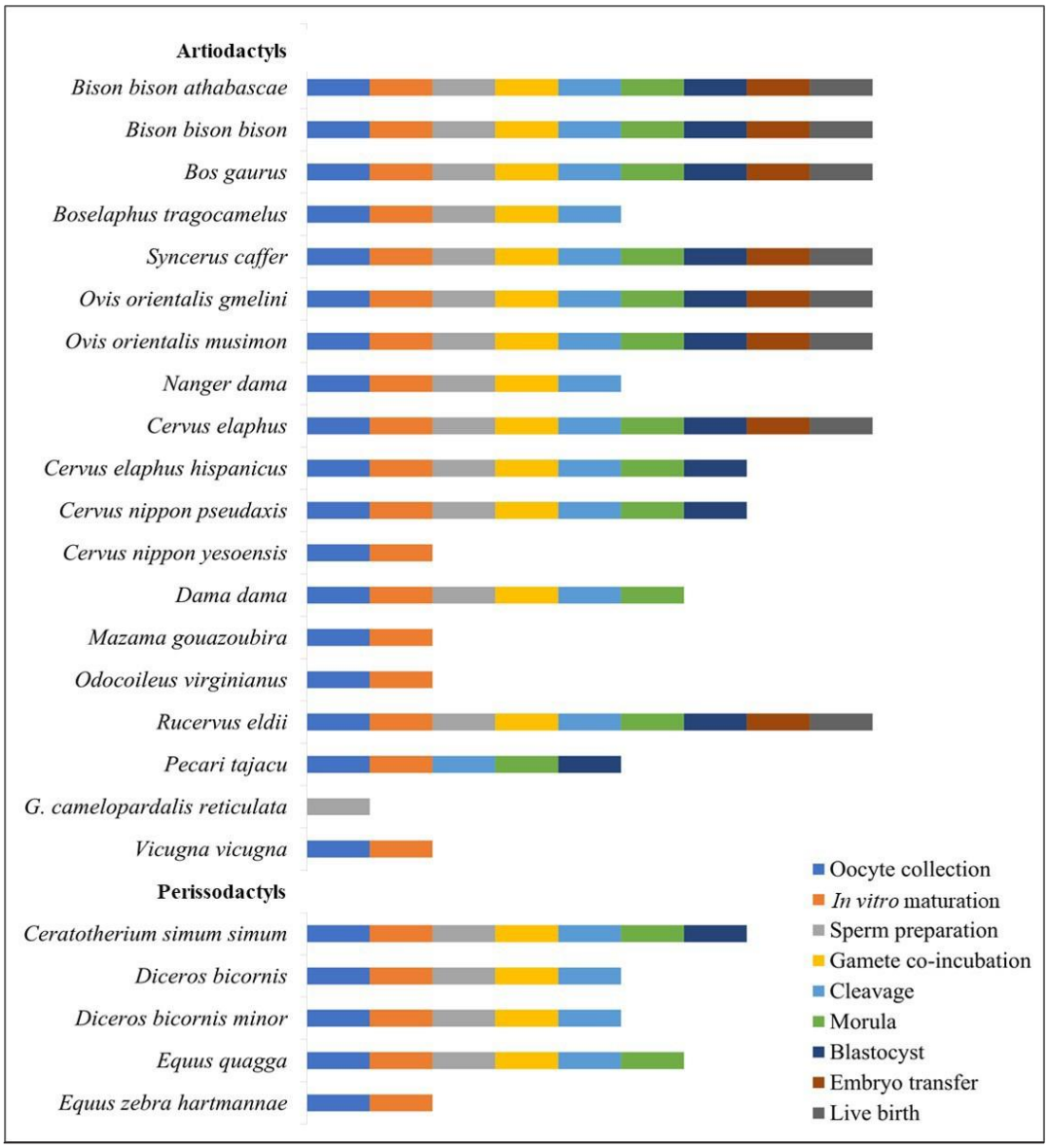

Figure 2. Development of *in vitro* fertilization (IVF) steps in several wild ungulate's species

For this type of application, it is important to establish an ideal activation protocol for different species. Yin et al. (2013) evaluated two chemical activation protocols for sika deer oocytes and obtained a higher rate of blastocysts (32.4%) using a combination of ionomycin and 6-dimethylaminopurine (6-DMAP) when compared to ethanol and 6-DMAP. They compared the quality of activated embryos with embryos produced *in vivo* and observed no difference, demonstrating the efficiency of artificial oocyte activation. Similarly, in collared peccary, we tested ionomycin and four secondary activator combinations (6-DMAP, 6-DMAP plus cytochalasin B, cycloheximide, and cycloheximide plus cytochalasin B), and ionomycin and 6-DMAP were superior in producing blastocysts (23.5%) and could be used to establish culture conditions for ICSI and cloning by somatic cell nuclear transfer (Borges et al., 2020).

Heterologous in vitro fertilization

Studies on IVF conditions in wild ungulates are scarce likely because of the challenges in obtaining sufficient mature oocytes for evaluating different experimental groups. To this end, heterologous IVF could be used, as it has already been applied as an alternative tool to evaluate sperm fertility and the response of spermatozoa to different cryopreservation and processing protocols. Hence, this technique was useful for assessing the longevity of frozen–thawed oryx sperm pre-incubated for 12 and 24 h before co-incubation with oocytes, obtaining 56.2% and 19.4% of embryos with ≥8-cell stage (O'Brien and Roth, 2000). Pradieé et al. (2018) evaluated the effect of different methods of cryopreserving Iberian ibex epididymal sperm on fertilizing capacity using bovine oocytes and observed similar cleavage rates for freezing (31.3%) and vitrification (45.1%), demonstrating that both methods can be used.

Heterologous IVF was used for sika deer sperm and zona-free bovine oocytes to assess IVF media and characterize sperm-oocyte interactions; this technique was highlighted as a tool for sperm selection that confers high development potential before homologous IVF or to understand unsuccessful homologous IVF (Comizzoliet al., 2001 b). In this context, the male reproductive seasonality of the North American bison (*Bison bison*) was also evaluated using heterologous IVF with bovine oocytes. Sperm recovered in the reproductive season led to a higher rate of blastocyst formation (30.5% *vs.* 13.7%, Krishnakumar et al., 2015).

Another interesting study with heterologous IVF aimed to evaluate the effectiveness of the cattle IVF protocol for African antelope species: springbok (*Antidorcas marsupialis*), impala (*Aepyceros melampus*), and blesbok (*Damaliscus dorcus phillipsi*). The authors observed that the protocol enabled rates above 50% penetration and cleavage, but improvements are needed to obtain embryos at the blastocyst stage in these species (Chatiza et al., 2013). This reinforces the fact that protocols of domestic species can be used as a starting point for studies on wild species.

Furthermore, many closely related species can produce viable hybrid individuals in nature, such as hybrids of sika deer (*Cervus nippon*; 2n = 68) and red deer (*Cervus elaphus*; 2n = 68), and in genetic improvement trials, such as American bison (*Bison bison*; n = 60) with domestic cattle (2n = 60) (Gabryś et al., 2021). This knowledge is also useful for studies with IVF, as observed by Meintjes et al. (1997), who produced zebroid embryos using Burchell's zebra (*Equus quagga burchellii*) sperm and Hartmann's zebra (*Equus zebra hartmannae*) oocytes for evaluating culture conditions. However, Owiny et al. (2009) evaluated the hybridization potential of African buffalo (*Syncerus caffer caffer*) sperm and cattle oocytes for genetic improvement purposes but was not successful in producing blastocysts, likely because of chromosomal disparity.

Heterologous ICSI can also provide important information for improving embryo production *in vitro*.This technique has been applied to assess the fertility of freeze-dried sperm of giraffe (*Giraffa camelopardalis reticulata*), using mouse oocytes, with 100% pronucleus formation observed (Kaneko et al., 2014). Gambini et al. (2020) provided valuable information regarding the manipulation of zebra sperm and used porcine oocytes to estimate its ability to induce activation after ICSI, which showed 30% pronucleus formation. After this evaluation, the sperm were injected into the oocytes of horses and were able to develop as far as the blastocyst stage. This result highlights that horses can be models for wild equines, as oocytes support hybrid development without compromising the expression and localization of cell differentiation markers and blastocyst cell number (Gambini et al., 2020).

Heterologous ICSI also played a key role in the development of a protocol for rhinoceros-homologous gametes. Hildebrandt et al. (2018) injected rhinoceros sperm into pig oocytes to assess fertility and the need for artificial activation. There was an increase of 30% to 90% in the fertilization rate, and the need to activate oocytes after ICSI of rhinoceros with low performance was noted.

Final considerations

IVF can be a valuable tool for the conservation of wild ungulates. In these animals, the greatest advances in IVF in terms of obtaining embryos and births occurred in species of artiodactyl from the Bovidae and Cervidae families. Among the reasons for this, we highlight the diversity of well-studied domestic models and the availability of individuals of the wild species for studies. In the other families of wild ungulates, the results are preliminary, typically with low efficiency and reproducibility.

Many factors must be considered for successful IVF to be achieved in these animals. Initially, it is necessary to promote research to understand the fundamental characteristics of reproduction and physiology of gametes and embryos from different families of wild ungulates.It is important to use advances made in domestic species

to acquire more information about the wild species. For this, techniques such as heterologous IVF, ICSI, and artificial activation can be explored in greater depth. Copying protocols from domestic models to wild species is not an effective strategy, although reproductive informa-tion for the species of interest should be combined with information from available domestic model(s). Thus, it is possible to create appropriate experimental designs to make the best use of the limited availability of gametes to establish the ideal culture and manipulation conditions for different species.

It is unlikely that IVF/embryo transfer will become a routine technique for wild ungulate management; however, it is undoubtedly an alternative that presents numerous advantages over other reproductive techniques. Recent efforts to establish IVF techniques are encouraging and will be central to the conservation of endangered ungulates and management of abundant species with economic value.

References

Andrabi S.M.H., Maxwell W.M.C. (2007). A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim. Reprod. Sci., 99: 223–243.

Benham H.M., McCollum M.P., Nol P., Frey R.K., Clarke P.R., Rhyan J.C., Barfield J.P. (2021). Production of embryos and a live offspring using *post-mortem* reproductive material from bison (*Bison bison bison*) originating in Yellowstone National Park, USA. Theriogenology, 160: 33–39.

Berg D.K., Asher G.W. (2003). New developments reproductive technologies in deer. Theriogenology, 59: 189–205.

Berg D.K., Thompson J.G., Asher G.W. (2002). Development of *in vitro* embryo production systems for red deer (*Cervus elaphus*): part 2. The timing of *in vitro* nuclear oocyte maturation. Anim. Reprod. Sci., 70: 77–84.

Berlinguer F., González R., Succu S., Del Olmo A., Garde J.J., Es- peso G., Gomendio M., Ledda S., Roldan E.R. (2008). *In vitro* oocyte maturation, fertilization and culture after ovum pick-up in an endangered gazelle (*Gazella dama mhorr*). Theriogenology,69: 349–359.

Borges A.A., Santos M.V.O., Queiroz Neta L.B., Oliveira M.F., Silva A.R., Pereira A.F. (2018). *In vitro* maturation of collared peccary (*Pecari tajacu*) oocytes after different incubation times. Pesq. Vet. Bras., 38: 1863–1868.

Borges A.A., Santos M.V.O., Nascimento L.E., Lira G.P.O., Praxedes É.A., Oliveira M.F., Silva A.R., Pereira A.F. (2020). Production of collared peccary (*Pecari tajacu* Linnaeus, 1758) parthenogenic embryos following different oocyte chemical activation and *in vitro* maturation conditions. Theriogenology, 142: 320–327.

Brackett B.G., Oliphant G. (1975). Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod., 12: 260–274.

Brahmasani S.R., Yelisetti U.M., Katari V., Komjeti S., Lakshmikantan U., Pawar R.M., Sisinthy S. (2013). Developmental abil- ity after parthenogenetic activation of *in vitro* matured oocytes collected postmortem from deers. Small Rumin. Res., 113: 128–135.

Cervantes M.P., Palomino J.M., Anzar M., Mapletoft R.J., Adams G.P. (2016). *In vivo* and *in vitro* maturation of oocytes collected from superstimulated wood bison (*Bison bison athabascae*) during the anovulatory and ovulatory seasons. Anim. Reprod. Sci., 173: 87–96.

Cervantes M.P., Palomino J.M., Anzar M., Mapletoft R.J., Mastromonaco G.F., Adams G.P. (2017). *In vitro*–production of embryos using immature oocytes collected transvaginally from superstimulated wood bison (*Bison bison athabascae*). Theriogenology, 92: 103–110.

Chatiza F.P., Bartels P., Nedambale T.L., Wagenaar G.M. (2013). Sperm–egg interaction and functional assessment of springbok, impala and blesbok cauda epididymal spermatozoa using a domestic cattle *in vitro* fertilization system. Anim. Reprod. Sci., 143: 8–18.

Chaves M.G., Miragaya M.H., Capdevielle E.F., Rutter B., Giuliano S.M., Agüero A. (2004). *In vitro* maturation of vicuna oocytes recovered by surgical aspiration of follicles from superstimulated ovaries. Biocell, 28: 545.

Comizzoli P., Mermillod P., Mauget R. (2000). Reproductive biotechnologies for endangered mammalian species. Reprod. Nutr. Dev., 40: 493–504.

Comizzoli P., Mermillod P., Cognie Y., Chai N., Legendre X., Mauget R. (2001 a). Successful *in vitro* production of embryos in the red deer (*Cervus elaphus*) and the sika deer (*Cervus nippon*). Theriogenology, 55: 649–659.

Comizzoli P., Mauget R., Mermillod P. (2001 b). Assessment of *in vitro* fertility of deer spermatozoa by heterologous IVF with zona-free bovine oocytes. Theriogenology, 56: 261–274.

Fernández S., Sestelo A., Rivolta M., Córdoba M. (2013). Capacitation and acrosome reaction induction on thawed *Dama dama* deer spermatozoa: glycine effect as cryopreservation diluent supplement. Zool. Sci., 30: 1110–1116.

Flores-Foxworth G., Coonrod S.A., Moreno J.F., Byrd S.R., Kraemer D.C., Westhusin M. (1995). Interspecific transfer of IVM IVF-derived red sheep (*Ovis orientalis gmelini*) embryos to domestic sheep (*Ovis aries*). Theriogenology, 44: 681–690.

Gabryś J., Kij B., Kochan J., Bugno-Poniewierska M. (2021). Interspecific hybrids of animals – in nature, breeding and science – a review. Ann. Anim. Sci., 21: 403–415.

Gambini A., Duque Rodríguez M., Rodríguez M.B., Briski O., Flores Bragulat A.P., Demergassi N., Losinno L., Salamone D.F. (2020). Horse ooplasm supports *in vitro* preimplantation development of zebra ICSI and SCNT embryos without compromising YAP1 and SOX2 expression pattern. Plos One, 15: e0238948.

García-Álvarez O., Maroto-Morales A., Berlinguer F., Fernández-Santos M.D.R., Esteso M.C., Mermillod P., Ortiz J.A., Ramon M., Pérez-Guzmán M.D., Garde J.J., Soler A.J. (2011). Effect of storage temperature during transport of ovaries on *in vitro* embryo production in Iberian red deer (*Cervus elaphus hispanicus*). Theriogenology, 75: 65–72.

García-Álvarez O., Soler A., Maulen Z., Maroto-Morales A., Iniesta-Cuerda M., Martín-Maestro A., Fernández-Santos M.R., Garde J. (2016). Selection of red deer spermatozoa with different cryoresistance using density gradients. Reprod. Domest. Anim., 51: 895–900.

Gordon I.J., Hester A.J., Festa-Bianchet M. (2004). The manage- ment of wild large herbivores to meet economic, conserva- tion and environmental objectives. Reprod. Domest. Anim., 41: 1021–1031.

Hermes R., Göritz F., Portas T.J., Bryant B.R., Kelly J.M., Maclellan L.J., Keeley T., Schwarzenberger F., Walzer C., Schnorrenberg A., Spindler R.E., Saragusty J., Kaandorp S., Hildebrandt T.B. (2009). Ovarian superstimulation, transrectal ultrasound–guided oocyte recovery, and IVF in rhinoceros. Theriogenology, 72: 959–968.

Herrick J.R. (2019). Assisted reproductive technologies for endangered species conservation: developing sophisticated protocols with limited access to animals with unique reproductive mechanisms. Biol. Reprod., 100: 1158–1170.

Hildebrandt T.B., Hermes R., Colleoni S., Diecke S., Holtze S., Renfree M.B., Stejskal J., Hayashi K., Drukker M., Loi P., Göritz F., Lazzari G., Galli C. (2018). Embryos and embryonic stem cells from the white rhinoceros. Nat. Commun., 9: 1–9.

Hinrichs K., Love C.C., Brinsko S.P., Choi Y.H., Varner D.D. (2002). *In vitro* fertilization of *in vitro*-matured equine oocytes: effect of maturation medium, duration of maturation, and sperm calcium ionophore treatment, and comparison with rates of fertilization *in vivo* after oviductal transfer. Biol. Reprod., 67: 256–262.

IUCN – International Union for Conservation of Nature and Natural Resources (homepage on the Internet). Red list of threatened

species (cited 2021 March 01). Available from: http://www.iucnredlist.org/details/41777/0.

Johnston L.A., Parrish J.J., Monson R., Leibfried-Rutledge L., Susko-Parrish J.L., Northey D.L., Rutledge J.J., Simmons L.G. (1994). Oocyte maturation, fertilization and embryo development *in vitro* and *in vivo* in the gaur (*Bos gaurus*). Reproduction, 100: 131–136.

Kaneko T., Ito H., Sakamoto H., Onuma M., Inoue-Murayama M. (2014). Sperm preservation by freeze-drying for the conservation of wild animals. PloS One, 9: e113381.

Krishnakumar S., Whiteside D.P., Elkin B., Thundathil J.C. (2015). Effect of reproductive seasonality on gamete quality in the North American bison (*Bison bison bison*). Reprod. Domest. Anim., 50: 206–213.

Leemans B., Stout T.A., Schauwer C., Heras S., Nelis H., Hoogewijs M., Soom A.V., Gadella B.M. (2019). Update on mammaliansperm capacitation: how much does the horse differ from other species? Reproduction, 157: 181–197.

Li Z., Song X., Yin S., Yan J., Lv P., Shan H., Cui K., Liu H., Liu Q. (2021). Single-Cell RNA-seq revealed the gene expression pattern during the *in vitro* maturation of donkey oocytes. Genes, 12: 1–15.

Liu B., Cui Y., Yu S. (2013). Effect of swim-up and percoll treatment on sperm quality and *in vitro* embryo development in yak. J. Integr. Agric., 12: 2235–2242.

Locatelli Y., Cognié Y., Vallet J.C., Baril G., Verdier M., Poulin N., Legendre X., Mermillod P. (2005). Successful use of oviduct epithelial cell coculture for *in vitro* production of viable red deer (*Cervus elaphus*) embryos. Theriogenology, 64: 1729–1739.

Locatelli Y., Hendriks A., Vallet J.C., Baril G., Duffard N., Bon N., Ortiz K., Scala C., Maurel M-C., Mermillod P., Legendre X. (2012). Assessment LOPU–IVF in Japanese sika deer (*Cervus nippon nippon*) and application to Vietnamese sika deer (*Cervus nippon pseudaxis*) a related subspecies threatened with extinction. Theriogenology, 78: 2039–2049.

Macías-García B., González-Fernández L., Matilla E., Hernández N., Mijares J., Sánchez-Margallo F.M. (2018). Oocyte holding in the Iberian red deer (*Cervus elaphus hispanicus*): Effect of initial oocyte quality and epidermal growth factor addition on *in vitro* maturation. Reprod. Domest. Anim., 53: 243–248.

Mahesh Y.U., Rao B.S., Suman K., Lakshmikantan U., Charan K.V., Gibence H.R.W., Shivaji S. (2011). *In vitro* maturation and fertilization in the nilgai (*Boselaphus tragocamelus*) using oocytes and spermatozoa recovered post-mortem from animals that had died because of foot and mouth disease outbreak. Reprod. Domest. Anim., 46: 832–839.

Malakoutikhah S., Fakheran S., Hemami M.R., Tarkesh M., Senn J. (2020). Assessing future distribution, suitability of corridors and efficiency of protected areas to conserve vulnerable ungulates under climate change. Divers. Distrib., 26: 1383–1396.

Mastromonaco G.F., Songsasen N. (2020). Reproductive technolo- gies for the conservation of wildlife and endangered species. In: Reproductive technologies in animals, Presicce G.A. (ed.). Academic Press, Rome, Italy, pp. 99–117.

Meintjes M., Bezuidenhout C., Bartels P., Visser D.S., Meintjes J., Loskutoff N.M., Fourie F. L., Barry D.M., Godke R.A. (1997). *In vitro* maturation and fertilization of oocytes recovered from free-ranging Burchell's zebra (*Equus burchelli*) and Hartmann's zebra (*Equus zebra hartmannae*). J. Zoo Wildl. Med., 28: 251–259.

Mohr D., Cohnstaedt L.W., Topp W. (2005). Wild boar and red deer affect soil nutrients and soil biota in steep oak stands of the Eifel. Soil Biol. Biochem., 37: 693–700.

O'Brien J.K., Roth T.L. (2000). Functional capacity and fertilizing longevity of frozen–thawed scimitar–horned oryx (*Oryx dammah*) spermatozoa in a heterologous *in vitro* fertilization system. Reprod. Fertil. Dev., 12: 413–421.

Owiny O.D., Barry D.M., Agaba M., Godke R.A. (2009). *In vitro* production of cattle×buffalo hybrid embryos using cattle oocytes and African buffalo (*Syncerus caffer caffer*) epididymal sperm. Theriogenology, 71: 884–894.

Palomino J.M., Mastromonaco G.F., Cervantes M.P., Mapletoft R.J., Anzar M., Adams G.P. (2020). Effect of season and superstimulatory treatment on *in vivo* and *in vitro* embryo production in wood bison (*Bison bison athabascae*). Reprod. Domest. Anim., 55: 54–63.

Parrish J.J., Susko-Parrish J., Winer M.A., First N.L. (1988). Capacitation of bovine sperm by heparin. Biol. Reprod., 38: 1171–1180.

Piliszek A., Madeja Z.E. (2018). Pre-implantation development of domestic animals. Curr. Top. Dev. Biol., 128: 267–294.

Pradieé J., Sánchez-Calabuig M.J., Castaño C., O'Brien E., Esteso M.C., Beltrán-Breña P., Maillo V., Santiago-Moreno J., Rizos D. (2018). Fertilizing capacity of vitrified epididymal sperm from Iberian ibex (*Capra pyrenaica*). Theriogenology, 108, 314–320.

Ptak G., Clinton M., Barboni B., Muzzeddu M., Cappai P., Tischner M., Loi P. (2002). Preservation of the wild European mouflon: thefirst example of genetic management using a complete program of reproductive biotechnologies. Biol. Reprod., 66: 796–801.

Pukazhenthi B.S. (2016). Saving wild ungulate diversity through enhanced management and sperm cryopreservation. Reprod. Fertil. Dev., 28: 1133–1144.

Rao B.S., Mahesh,Y.U., Lakshmikantan U.R., Suman K., Charan K.V., Shivaji S. (2010). Developmental competence of oocytes recovered from *postmortem* ovaries of the endangered Indian blackbuck (*Antilope cervicapra*). J. Reprod. Dev., 56: 623–629.

Rath D., Long C.R., Dobrinsky J.R., Welch G.R., Schreier L.L., Johnson L.A. (1999). *In vitro* production of sexed embryos for gender preselection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. J. Anim. Sci., 77: 3346–3352.

Ratto M., Gomez C., Berland M., Adams G.P. (2007). Effect of ovarian superstimulation on COC collection and maturation in alpacas. Anim. Reprod. Sci., 97: 246–256.

Rizos D., Ward F., Duffy P.A.T., Boland M.P., Lonergan P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. Mol. Reprod. Dev., 61: 234–248.

Rola L.D., Zanetti E.S., Del Collado M., Peroni E.D.F.C., Duarte J.M.B. (2021). Collection and *in vitro* maturation of *Mazama gouazoubira* (brown brocket deer) oocytes obtained after ovarian stimulation. Zygote, 29: 216–222.

Roth T.L., Weiss R.B., Buff J.L., Bush L.M., Wildt D.E., Bush M. (1998). Heterologous *in vitro* fertilization and sperm capacitation in an endangered African antelope, the scimitar–horned oryx (*Oryx dammah*). Biol. Reprod., 58: 475–482.

Santiago-Moreno J., Esteso M.C., Castaño C., Toledano-Díaz A., Rodríguez E., López-Sebastián A. (2014). Sperm selection by Capripure® density-gradient centrifugation versus the dextran swim-up procedure in wild mountain ruminants. Anim. Reprod. Sci., 149: 178–186.

Schook M.W., Wildt D.E., Weiss R.B., Wolfe B.A., Archibald K.E., Pukazhenthi B.S. (2013). Fundamental studies of the reproduc- tive biology of the endangered Persian onager (*Equus hemionus onager*) result in first wild equid offspring from artificial insemination. Biol. Reprod., 89: 41–51.

Siriaroonrat B., Comizzoli P., Songsasen N., Monfort S.L., Wildt D.E., Pukazhenthi B.S. (2010). Oocyte quality and estradiol supplementation affect *in vitro* maturation success in the white-tailed deer (*Odocoileus virginianus*). Theriogenology, 73: 112–119.

Sontakke S.D. (2018). Monitoring and controlling ovarian activities in wild ungulates. Theriogenology, 109: 31–41.

Stoops M.A., O'Brien J.K., Roth T.L. (2011). Gamete rescue in the African black rhinoceros (*Diceros bicornis*). Theriogenology, 76: 1258–1265.

Tervit H.R., Whittingham D.G., Rowson L.E.A. (1972). Successful culture *in vitro* of sheep and cattle ova. Reproduction, 30: 493–497.

Thongphakdee A., Berg D.K., Tharasanit T., Thongtip N., Tipkan- tha W., Punkong C., Tongthainan D., Noimoon S., Maikeaw U., Kajornklin N., Siriaroonrat B., Comizzoli P., Kamolnorranath S. (2017). The impact of ovarian stimulation protocol on oocyte quality, subsequent *in vitro* embryo development, and pregnancy after transfer to recipients in Eld's deer (*Rucervus eldii thamin*). Theriogenology, 91: 134–144.

Trasorras V.L., Chaves M.G., Miragaya M.H., Pinto M., Rutter B., Flores M., Agüero A. (2009). Effect of eCG superstimulation and buserelin on cumulus–oocyte complexes recovery and mat- uration in llamas (*Lama glama*). Reprod. Domest. Anim., 44: 359–364.

Tulake K., Yanagawa Y., Takahashi Y., Katagiri S., Higaki S., Koyama K., Wang X., Li H. (2014). Effects of ovarian storage condition on *in vitro* maturation of Hokkaido sika deer (*Cervus nippon yesoensis*) oocytes. Jpn. J. Vet., 62: 187–192.

Velamazan M., Perea R., Bugalho M.N. (2020). Ungulates and ecosystem services in Mediterranean woody systems: A semi-quantitative review. J. Nat. Conserv., 55: 125837.

Yin Y., Tang L., Zhang P., Kong D., Wang Z., Guan J., Song G., Tan B., Li Z. (2013). Optimizing the conditions for *in vitro* maturation and artificial activation of sika deer (*Cervus nippon hortulorum*) oocytes. Reprod. Domest. Anim., 48: 27–32.

Received: 24 VIII 2021
Accepted: 4 I 2022

ANEXO B – COMPROVANTE DE SUBMISSÃO PARA REPRODUCTIVE BIOLOGY

Confirm co-authorship of submission to Reproductive Biology

Caixa de entrada ×

Reproductive Biology <em@editorialmanager.com> qua., 8 de fev. 16:08 (há 17 horas)

para mim

*This is an automated message. *

Journal: Reproductive Biology
Title: Effects of different selection techniques on physiological and functional quality of sperm collected from collared peccaries
Corresponding Author: Dr. Prof. Alexsandra Fernandes Pereira
Co-Authors: Maria Valéria de Oliveira Santos; Andréia Maria da Silva; Leonardo Vitorino Costa de Aquino; Lhara Ricarliany Medeiros de Oliveira; Samara Sandy Jeronimo Moreira; Moacir Franco de Oliveira, Dr.; Alexandre Rodrigues Silva, Dr.
Manuscript Number:

Dear Maria Valéria de Oliveira Santos,

Dr. Prof. Alexsandra Fernandes Pereira submitted this manuscript via Elsevier's online submission system, Editorial Manager, and you have been listed as a Co-Author of this submission.
Elsevier asks Co-Authors to confirm their consent to be listed as Co-Author and track the papers status. In order to confirm your connection to this submission, please click here to confirm your co-authorship:

https://www.editorialmanager.com/repbio/l.asp?i=125903&l=SDLYWFHR

ANEXO C – COMPROVANTES DE SUBMISSÃO PARA ANIMALS

[Animals] Manuscript ID: animals-2237476 - Submission Received

Caixa de entrada ×

Editorial Office <animals@mdpi.com> 7 de fev. de 2023 15:37 (há 2 dias)
para Alexsandra, mim, Andreia, Leonardo, Lhara, João, Moacir, Alexandre

Dear Professor Pereira,

Thank you very much for uploading the following manuscript to the MDPI submission system. One of our editors will be in touch with you soon.

Journal name: Animals
Manuscript ID: animals-2237476
Type of manuscript: Article
Title: Effects of Capacitating Media and Incubation Time on Collared Peccary Sperm Quality, Acrosome Reaction, and Fertilizing Ability
Authors: Maria Valéria Oliveira Santos, Andreia Maria da Silva, Leonardo V. C. Aquino, Lhara R. M. Oliveira, João B. F. Souza-Junior, Moacir F. Oliveira, Alexandre R. Silva, Alexsandra F. Pereira *
Received: 7 February 2023
E-mails: valeriasnts07@gmail.com, andreia.m.silva@hotmail.com, leonardovt@live.com, lharagirs@hotmail.com, souza.jr@ufersa.edu.br, moacir@ufersa.edu.br, alexrs@ufersa.edu.br, alexsandra.pereira@ufersa.edu.br
Submitted to section: Animal Reproduction, https://www.mdpi.com/journal/animals/sections/Animal_Reproduction
Effective Option regarding Wildlife Resources Protection: Assisted Reproductive Technology

ANEXO D – COMPROVANTES DE REVISÃO DO INGLÊS DOS ARTIGOS

editage

Editing Certificate

This document certifies that the manuscript listed below has been edited to ensure language and grammar accuracy and is error free in these aspects. The edit was performed by professional editors at Editage, a division of Cactus Communications. The author's core research ideas were not altered in any way during the editing process. The quality of the edit has been guaranteed, with the assumption that our suggested changes have been accepted and the text has not been further altered without the knowledge of our editors.

MANUSCRIPT TITLE

Embryo production via in vitro fertilization in wild ungulates: Progress and perspectives

AUTHORS

Maria Valéria de Oliveira Santos, Alexandre Rodrigues Silva, Alexsandra Fernandes Pereira

ISSUED ON

December 13, 2021

JOB CODE

LEESP_18_2

THE EDITAGE Promise QUALITY GUARANTEE

Vikas Narang

Vikas Narang
Chief Operating Officer - Editage

editage

Editage, a brand of Cactus Communications, offers professional English language editing and publication support services to authors engaged in over 1300 areas of research. Through its community of experienced editors, which includes doctors, engineers, published scientists, and researchers with peer review experience, Editage has successfully helped authors get published in internationally reputed journals. Authors who work with Editage are guaranteed excellent language quality and timely delivery.

GLOBAL :
+1(833) 979-0061 | request@editage.com

BRAZIL :
08000474773 | contato@editage.com

CACTUS

impact.science

researcher.life

lifesciences.cactusglobal.com

editage

Editing Certificate

This document certifies that the manuscript listed below has been edited to ensure language and grammar accuracy and is error free in these aspects. The edit was performed by professional editors at Editage, a division of Cactus Communications. The sections titled references, figures, tables, figure-legends and tables-legends were not edited by Editage upon the author's request.

The author's core research ideas were not altered during the editing process. Editage guarantees the quality of editing with the assumption that our suggested changes have been accepted and the edited text has not been altered without the knowledge of our editors.

MANUSCRIPT TITLE

Effects of capacitating media and incubation time on collared peccary sperm quality, acrosomal exocytosis, and fertilizing ability

AUTHORS

Santos, MVO; Silva, AM; Aquino, LVC; Oliveira, LRM; Souza-Junior, JB; Oliveira, MF; Silva, AR; Pereira, AF

ISSUED ON

December 12, 2022

JOB CODE

LEESP_33

THE EDITAGE Promise QUALITY GUARANTEE

Vikas Narang

Vikas Narang
Chief Operating Officer - Editage

editage

Editage, a brand of Cactus Communications, offers professional English language editing and publication support services to authors engaged in over 1300 areas of research. Through its community of experienced editors, which includes doctors, engineers, published scientists, and researchers with peer review experience, Editage has successfully helped authors get published in internationally reputed journals. Authors who work with Editage are guaranteed excellent language quality and timely delivery.

GLOBAL :
+1(833) 979-0061 | request@editage.com

BRAZIL :
08000474773 | contato@editage.com

CACTUS

impact.science

researcher.life

lifesciences.cactusglobal.com

editage

Editing Certificate

This document certifies that the manuscript listed below has been edited to ensure language and grammar accuracy and is error free in these aspects. The edit was performed by professional editors at Editage, a division of Cactus Communications. The sections titled references, figures and figure-legends were not edited by Editage upon the author's request.

The author's core research ideas were not altered during the editing process. Editage guarantees the quality of editing with the assumption that our suggested changes have been accepted and the edited text has not been altered without the knowledge of our editors.

MANUSCRIPT TITLE

In vitro production of heterologous embryos in collared peccaries using sperm selected by different techniques

AUTHORS

MVO Santos, AM Silva, LVC Aquino, LRM Oliveira, SSJ Moreira, MF Oliveira, AR Silva, AF Pereira

ISSUED ON

December 24, 2022

JOB CODE

LEESP_34

THE EDITAGE Promise QUALITY GUARANTEE

Vikas Narang

Vikas Narang
Chief Operating Officer - Editage

editage

Editage, a brand of Cactus Communications, offers professional English language editing and publication support services to authors engaged in over 1300 areas of research. Through its community of experienced editors, which includes doctors, engineers, published scientists, and researchers with peer review experience, Editage has successfully helped authors get published in internationally reputed journals. Authors who work with Editage are guaranteed excellent language quality and timely delivery.

GLOBAL :
+1(833) 979-0061 | request@editage.com

BRAZIL :
08000474773 | contato@editage.com

CACTUS

impact.science

researcher.life

lifesciences.cactusglobal.com

ANEXO E – CERTIFICADO COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Mossoró, 15 de outubro de 2019.

PARECER 30/2019

Certificamos que o projeto intitulado ***"DESENVOLVIMENTO DE ESTRATÉGIAS PARA A OTIMIZAÇÃO DA FECUNDAÇÃO IN VITRO EM CATETOS, PECARI TAJACU (LINNAEUS, 1758)"*** *protocolo n. 23091.010737/2019-33 sob a responsabilidade de Alexsandra Fernandes Pereira, o qual envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da lei 11.794 de 8 de outubro de 2009 e com as normas editadas pelo Conselho Nacional de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal Rural do Semi-Árido –UFERSA em reunião de 08/10/2019.*

Vigência do projeto	*Novembro de 2019 a agosto de 2021*
Espécie/linhagem	Pecari tajacu
N. de Animais	*12*
Peso/idade	*Adultos*
Sexo	*8 machos e 5 fêmeas*
Origem	*CEMAS/UFERSA*

Emanuelle Fontenele Rabelo

Coordenadora CEUA-UFERSA

BR 110 – Km 47 – Bairro Pres. Costa e Silva
CEP 59625-900 – Mossoró – RN – (84) 3317-8360
http://www2.ufersa.edu.br/portal/comissoes/ceua

ANEXO F – SISTEMA DE AUTORIZAÇÃO E INFORMAÇÃO EM BIODIVERSIDADE

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 71834-1	Data da Emissão: 25/10/2019 17:22:56	Data da Revalidação*: 25/10/2020
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Alexsandra Fernandes Pereira	CPF: 913.071.983-68
Título do Projeto: DESENVOLVIMENTO DE ESTRATÉGIAS PARA A OTIMIZAÇÃO DA FECUNDAÇÃO IN VITRO EM CATETOS, Pecari tajacu (LINNAEUS, 1758)	
Nome da Instituição: UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	CNPJ: 24.529.265/0001-40

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Etapa 1: Avaliação dos métodos de seleção espermática	11/2019	01/2020
2	Etapa 2: Avaliação dos métodos de capacitação espermática	05/2020	05/2021
3	Etapa 3: FIv homologa	05/2022	12/2022
4	Colheita e criopreservação de sêmen	11/2019	11/2021
5	Etapa 3: Avaliação das condições de FIV heterologa	05/2021	05/2022

Equipe

#	Nome	Função	CPF	Nacionalidade
1	Alexandre Rodrigues Silva	Colaborador	702.982.543-87	Brasileira
2	MOACIR FRANCO DE OLIVEIRA	Colaborador	325.949.504-59	Brasileira

Observações e ressalvas

1	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
2	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infraestrutura da unidade.
3	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
4	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio n° 03/2014 ou na Instrução Normativa ICMBio n° 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
5	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
6	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.

Este documento foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

Código de autenticação: 0718340120191025 **Página 1/3**

Ministério do Meio Ambiente - MMA

Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio

Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 71834-1	Data da Emissão: 25/10/2019 17:22:56	Data da Revalidação*: 25/10/2020
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Alexsandra Fernandes Pereira	CPF: 913.071.983-68
Título do Projeto: DESENVOLVIMENTO DE ESTRATÉGIAS PARA A OTIMIZAÇÃO DA FECUNDAÇÃO IN VITRO EM CATETOS, Pecari tajacu (LINNAEUS, 1758)	
Nome da Instituição: UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	CNPJ: 24.529.265/0001-40

Observações e ressalvas

7	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.

Locais onde as atividades de campo serão executadas

#	Descrição do local	Município-UF	Bioma	Caverna?	Tipo
1	Centro de Multiplicação de Animais Silvestres	Mossoró-RN	Caatinga	Não	Fora de UC Federal

Atividades

#	Atividade	Grupo de Atividade
1	Coleta/transporte de amostras biológicas ex situ	Atividades ex-situ (fora da natureza)

Atividades X Táxons

#	Atividade	Táxon	Qtde.
1	Coleta/transporte de amostras biológicas ex situ	Pecari tajacu	-

Materiais e Métodos

#	Tipo de Método (Grupo taxonômico)	Materiais
1	Amostras biológicas (Outros mamíferos)	Fragmento de tecido/órgão

Destino do material biológico coletado

#	Nome local destino	Tipo destino
1	UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	Criadouro científico

Este documento foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

Código de autenticação: 0718340120191025

Ministério do Meio Ambiente - MMA

Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio

Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 71834-1	Data da Emissão: 25/10/2019 17:22:56	Data da Revalidação*: 25/10/2020
De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.		

Dados do titular

Nome: Alexsandra Fernandes Pereira	CPF: 913.071.983-68
Título do Projeto: DESENVOLVIMENTO DE ESTRATÉGIAS PARA A OTIMIZAÇÃO DA FECUNDAÇÃO IN VITRO EM CATETOS, Pecari tajacu (LINNAEUS, 1758)	
Nome da Instituição: UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	CNPJ: 24.529.265/0001-40

Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de Amostra	Qtde.	Data

* Identificar o espécime do nível taxonômico possível.

Este documento foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

Código de autenticação: 0718340120191025

ANEXO G – RESUMO APRESENTADO 19TH INTERNATIONAL CONGRESS ON ANIMAL REPRODUCTION

Reproduction in exotic animals and wild species

ESTABLISHING THE PERCOLL GRADIENT TECHNIQUE FOR SPERM SELECTION FROM COLLARED PECCARIES (PECARI TAJACU LINNAEUS, 1758)

M.V. De Oliveira Santos [1], A.M. Da Silva [2], L.R. Medeiros De Oliveira [1], L. Vitorino Costa De Aquino [1], S.S. Jerônimo Moreira [2], É. Almeida Praxedes [1], M. Franco De Oliveira [2], A. Rodrigues Silva [2], A. Fernandes Pereira [1]

[1]*Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoro, RN, Brazil*

[2]*Laboratory of Animal Germplasm Conservation, Federal Rural University of Semi-Arid, Mossoro, RN, Brazil*

BACKGROUND-AIM

The collared peccary is a wild ungulate whose population is disappearing in some South American biomes. The development of assisted reproductive techniques as the in vitro fertilization (IVF) could contribute to peccary conservation; however, techniques should be stablished for the species peculiarities. The Percoll gradient has been used to select sperm from different mammals and shows variations in its concentrations that can influence the sperm quality. Thus, we evaluated the effect of different Percoll gradient concentrations on the quality and reactive oxygen species (ROS) levels of peccary sperm.

METHODS

The semen was collected from six males by electroejaculation and divided into three groups: fresh control, 45/90% and 35/70% Percoll gradients (centrifuged at 900g/15 min). The pellet obtained after selection was centrifuged to remove the Percoll. Sperm were evaluated for kinetic motility patterns, membrane integrity associated with mitochondrial activity and ROS levels. Data were expressed as mean ± SE and analyzed by ANOVA followed by Tukey test ($P<0.05$).

RESULTS

The total and progressive motilities were similar between the control (95.0 ± 1.8 and 70.8 ± 6.0%) and 45/90% (87.5 ± 3.6 and 75.3 ± 5.2%); however, the motilities were significantly lower in the 35/70% group (8.2 ± 2.1 and 3.3 ± 1.4%). The 35/70% gradient also negatively influenced other kinetic parameters. Moreover, the 45/90% gradient presented the highest values for velocity average pathway (96.8 ± 9.4 vs. 40.7 ± 6.1 vs. 69.7 ± 7.5 µm/sec), velocity straight line (88.4 ± 8.9 vs. 27.3 ± 7.0 vs. 56.0 ± 6.6 µm/sec) and linearity (66.0 ± 4.7 vs. 35.7 ± 6.7 vs. 46.0 ± 3.1%) compared to 35/70% and control, respectively. The membrane integrity associated with mitochondrial activity was similar between 45/90% gradient and control (71.5 ± 5.6 vs. 80.0 ± 3.1%), however, it was lower in the 35/70% (13.2 ± 2.7%). The ROS levels did not differ among treatments (control: 1.00 ± 0.04; 45/90%: 1.12 ± 0.05; 35/70%: 1.05 ± 0.06 fluorescence units).

CONCLUSIONS

In conclusion, the 45/90% Percoll gradient is efficient to select collared peccary sperm with better kinetic motility patterns, without causing membrane damage and oxidative stress. These results are fundamental for the development of embryo production by IVF in peccaries.

ANEXO H – RESUMO APRESENTADO EM VIII INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

Animal Reproduction

In vitro production of hybrid embryos derived from collared peccary semen selected by different methods and swine oocytes

M.V.O. Santos[1*], A.M. Silva[2], L.V.C. Aquino[1], L.R.M. Oliveira[1], S.S.J. Moreira[2], M.F. Oliveira[3], A.R. Silva[2], A.F. Pereira[1]

[1]Laboratory of Animal Biotechnology, UFERSA, Mossoró, RN, Brazil; [2]Laboratory of Animal Germoplasm Conservation, UFERSA, Mossoró, RN, Brazil; [3]Laboratory of Applied Animal Morphophysiology, UFERSA, Mossoró, RN, Brazil

The collared peccary (*Pecari tajacu* Linnaeus, 1758) is a wild ungulate that adapt well to captivity and its population can benefit from the use of assisted reproduction techniques, such as *in vitro* fertilization (IVF). Sperm selection is an important step in the success of IVF and different selection methods can influence sperm quality. Percoll gradient (PG) and washing by centrifugation (WC) techniques have distinct selection mechanisms and are routinely used in the IVF of domestic mammals, but not yet stablished for the peccaries. Therefore, we aimed to compare the efficiency of PG and WC in the sperm selection on the production of hybrid embryos using collared peccary semen and swine oocytes. All procedures were approved by the ethics committee of UFERSA (no. 30/2019) and Institute Chico Mendes for Conservation of Biodiversity (no. 71834-1). Fresh semen was collected from six collared peccaries by electroejaculation and diluted for selection (100 x 10^6 sperm/mL) in SPTL medium (100 mM NaCl, 3.1 mM KCl, 0.4 mM NaH_2PO_4, 21.6 mM sodium lactate, 25 mM $NaHCO_3$, 0.5 mM caffeine, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 0.6% albumin bovine serum, 1 mM sodium pyruvate, and 1% antibiotic– antimycotic solution). Immediately after collection (control group) and selection (PG or WC), sperm were evaluated for kinetic motility patterns. For selection by PG, 250 µL of semen was added over a 45/90% Percoll gradient and centrifuged at 900*g* for 15 min. The pellet obtained was centrifuged (300*g*/ 3 min) to remove the Percoll. For selection by WC, 1.0 mL of semen was subjected to three centrifugations of 100*g* for 3 min. After selection for both methods, sperm concentration was adjusted, and sperm (3 × 10^5 sperm/mL) were co-incubated for 6 h with swine oocytes previously submitted to *in vitro* maturation for 42–44 h. The IVF medium consisted of 114 mM NaCl, 3.2 mM KCl, 0.35 mM NaH_2PO_4, 10 mM sodium lactate, 5 mM glucose, 25 mM $NaHCO_3$, 2 mM caffeine, 4.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Hepes, 0.3% albumin bovine serum, 0.11 mM sodium pyruvate, and 1% antibiotic–antimycotic solution. After IVF, the presumed zygotes were cultured *in vitro* for six days in synthetic oviduct fluid. On Days 2 and 6, the cleaved embryos and morula were quantified, respectively. Data were expressed as mean ± standard error and kinetic motility patterns were analyzed by ANOVA followed by Tukey test ($P < 0.05$). All other data were assessed with a chi-squared test ($P < 0.05$). All kinetic patterns of motility were similar between the control and WC groups. Already PG improved parameters of straight-line velocity - VAP (98.7 µm/sec *vs*. 61.7 µm/sec *vs*. 72.6 µm/sec), amplitude of lateral head displacement - ALH (4.2 µm *vs*. 7.3 µm *vs*. 7.2 µm), straightness - STR (92.0% *vs*. 71.3% *vs*. 72.5%) and linearity (72.3% *vs*. 40.3% *vs*. 46.0%) when compared to control group and WC, respectively. However, PG significantly increased the number of static sperm compared to control group and WC ($P < 0.05$). The cleavage rate did not differ between selection methods, with a total of 26.3% ± 3.4 (35/133) for WC and 25.2% ± 4.5 (34/135) for PG. More than 88% ± 4.1 (30/34) of cleaved hybrid embryos were classified as having more than 3 cells. The total percentage of embryos that reached the morula stage was 10.5% ± 2.6 (14/133) and 8.1% ± 2.5 (11/135) for the WC and PG groups, respectively. The rate of morula per cleaved embryo was 40% ± 14.2 (14/35) with WC selection and 32.3% ± 13.5 (11/34) using PG. There was no difference in the development of morula among the experimental groups. In summary, although PG improves some motility kinetic parameters, both selection methods were efficient to isolate motile sperm capable of fertilizing porcine oocytes *in vitro*. PG and WC allowed the development of hybrid embryos and can be used for selection of collared peccary sperm. These results are essential and initial for the development of embryo production by IVF in collared peccaries. **Acknowledgements:** CAPES, CNPq

*E-mail: valeriasnts07@gmail.com


XXIV Congresso Brasileiro de Reprodução Animal
VIII International Symposium on Animal Biology of Reproduction - Joint Meeting
CBRA
O Colégio Brasileiro de Reprodução Animal certifica que o trabalho intitulado "In vitro production of hybrid embryos derived from collared peccary semen selected by different methods and swine oocytes", dos autores "M.V.O. Santos, A.M. Silva, L.V.C. Aquino, L.R.M. Oliveira, S.S.J. Moreira, M.F. Oliveira, A.R. Silva, A.F. Pereira ", apresentado oralmente, foi premiado em 1o lugar na categoria "Reprodução de suínos".
Dr. Marcelo Rezende Luz
Presidente do CBRA e do XXIV CBRA e VIII ISABR – Joint Meeting
Parceiros empresariais:
Apoio institucional:
Realização:
CBRA
Colégio Brasileiro de Reprodução Animal
WTA
PremieRpet
minitube
Biogénesis Bagó
A evolução da saúde animal
ourofino saúde animal
imv TECHNOLOGIES
Bolupharma
CNPq
CRMV MG
Escola de Veterinária UFMG

ANEXO I – PÔSTER APRESENTADO EM RÉSEAU QUÉBÉCOIS EN REPRODUCTION COM PARTE DOS RESULTADOS OBTIDOS DURANTE O ESTÁGIO DOUTORADO SANDUÍCHE - CNPq

The use of eugenol as an antioxidant for the *in vitro* maturation of porcine oocytes

Maria Valéria De Oliveira Santos[1], Karina Gutierrez[2], Alexsandra Fernandes Pereira[1], Vilceu Bordignon[2]

[1]Laboratory of Animal Biotechnology, Federal Rural University of Semi-Arid, Mossoró, Brazil
[2]Department of Animal Science, McGill University, Sainte-Anne-de-Bellevue, Quebec, Canada

RQR Réseau Québécois en reproduction

BACKGROUND-AIM

Eugenol (EG), an antioxidant from *Syzygium aromaticum*, has shown promising results for *in vitro* development of bovine embryos when it was supplemented during *in vitro* maturation (IVM) (Oliveira et al. 2021, doi:10.1071/AN21197). However, it has not been tested for IVM of other species, such pigs, which are more susceptible to oxidative stress due to their higher content of lipids. Therefore, the objective is to investigate if EG can improve the efficiency of swine embryo production in vitro.

METHODS

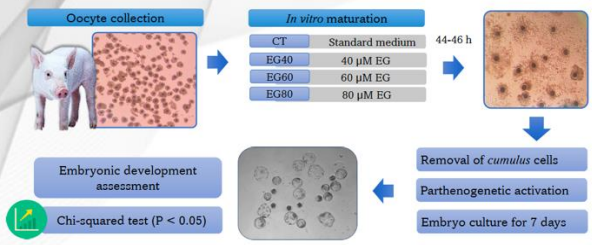

Fig. 1. *In vitro* production of embryos by parthenogenetic activation to evaluate different eugenol concentrations during *in vitro* maturation of oocytes from slaughterhouse ovaries.

ACKNOWLEDGMENT

CNPq CAPES

RESULTS

The cleavage rate was not significantly affected by EG supplementation, ranging from 53.6 to 63.0%. The blastocyst rates were not significantly different between CT, EG 40µM, and EG 60µM groups but were significantly lower in the EG 80µM group. Supplementation with 60µM EG significantly increased the rate of expanded blastocysts and decreased the rate of initial blastocysts compared to the other groups.

Table 1. Effect of different EG concentrations during *in vitro* maturation of porcine oocytes on embryonic development.

Groups	Cleavage/ Oocytes	Blastocyst/ Oocytes	Blastocyst/ Cleavage
CT	53.6 ± 5.1 (147/274)a	26.3 ± 2.1 (72/274)a	49.0 ± 6.5 (72/147)a
EG40	58.4 ± 6.1 (156/267)a	28.5 ± 3.4 (76/267)a	48.7 ± 4.5 (76/156)a
EG60	55.9 ± 4.9 (152/272)a	32.4 ± 3.5 (88/272)a	57.9 ± 4.5 (88/152)a
EG80	57.2 ± 3.2 (163/285)a	18.9 ± 3.3 (54/285)b	33.1 ± 7.2 (54/163)b

a,b: Different superscript letters are significantly different (P < 0.05). Mean % ± SE (n).

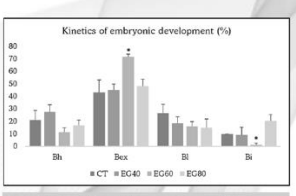

Fig. 2. Effect of different EG concentrations on IVM of porcine oocytes on the kinetics of embryonic development. * P < 0.05.

CONCLUSION

The results suggest that porcine embryo quality was improved by supplementing IVM medium with 60µM EG. However, additional studies are required to better evaluate the impact on embryo cell viability, as well as to assess EG during *in vitro* fertilization and embryo culture.