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**ESTABELECIMENTO DAS CONDIÇÕES DE SELEÇÃO E CAPACITAÇÃO
ESPERMÁTICA, MEIOS DE FERTILIZAÇÃO E DE CULTIVO EMBRIONÁRIO
EM *Dasyprocta leporina* (LINNAEUS, 1758)**

MOSSORÓ

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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. Alessandra Fernandes Pereira

Co-orientador: Prof. Dr. Alexandre Rodrigues Silva

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LHARA RICARLIANY MEDEIROS DE OLIVEIRA

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*Dedico essa tese aos meus amados pais,
Leonara Cavalcante Medeiros de Oliveira
e Ricardo Jefferson Morais de Oliveira.*

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“Ao se olhar no espelho você
conclui: apesar de tudo, ainda é
você.”

Undertale

RESUMO

As tecnologias de reprodução assistida (TRAs) desempenham um papel fundamental na conservação de espécies silvestres, como a cutia-vermelha (*Dasyprocta leporina* Linnaeus, 1758), um roedor de grande importância ecológica em virtude do seu papel na dispersão de sementes no continente sul-americano. Essas tecnologias oferecem abordagens inovadoras para superar desafios reprodutivos, contribuindo para a preservação da diversidade genética dessas espécies. Nesse contexto, a compreensão dos processos de desenvolvimento embrionário em ambientes controlados *in vitro* torna-se essencial. Contudo, técnicas reprodutivas avançadas, como a fertilização *in vitro* (FIV), ainda carecem de estudos específicos voltados à elucidação das etapas iniciais envolvidas na manipulação dos gametas. Diante desse cenário, o presente estudo teve como objetivo investigar os processos reprodutivos da cutia-vermelha *in vitro*, especificamente a seleção e capacitação espermática, co-incubação de gametas e suplementação do meio de cultivo, a fim de aprofundar o conhecimento sobre sua biologia reprodutiva e, consequentemente, otimizar o desenvolvimento embrionário em sistemas de reprodução assistida. Para alcançar esses objetivos, a tese foi estruturada em quatro etapas. Na **primeira etapa**, foram comparados diferentes métodos de seleção espermática – gradiente de densidade, swim-up e centrifugação – visando identificar a técnica mais eficiente na melhoria da capacidade fecundante e viabilidade espermática para a FIV. A **segunda etapa** investigou os efeitos de agentes capacitantes, avaliando perfis de BSA de baixa e alta concentração (4 ou 15 mg/mL), na presença ou ausência de cloreto de cálcio (CaCl_2), ao longo de diferentes tempos de incubação (1, 3 ou 6 h). Na **terceira etapa**, foi analisada a influência do meio de cultivo na FIV, comparando os meios HTF, TYH e TALP quanto ao seu impacto sobre o desenvolvimento embrionário. Finalmente, a **quarta etapa** avaliou o meio mais adequado para o desenvolvimento embrionário, comparando SOF e KSOM, tanto para embriões produzidos por FIV quanto para aqueles obtidos por ativação oocitária artificial (AA). Os resultados da **primeira etapa** indicaram que todos os métodos de seleção foram eficazes na obtenção de espermatozoides viáveis para a FIV; porém, a centrifugação destacou-se por selecionar gametas com melhores parâmetros cinéticos, morfológicos e funcionais, além de manter um equilíbrio oxidativo favorável *in vitro*. Na **segunda etapa**, a presença de CaCl_2 mostrou-se essencial para a viabilidade e funcionalidade espermática, reduzindo a produção de espécies reativas de oxigênio (EROs) e promovendo altas taxas de capacitação (90,7%), reação acrossômica (93,8%) e hiperatividade (83,0%). Além disso, a menor concentração de BSA e as 6 h de incubação foram essenciais para maximizar os índices de capacitação nesta espécie. Os experimentos da **terceira etapa** indicaram que o meio TALP apresentou desempenho superior em relação aos meios HTF e TYH, proporcionando um ambiente com menor estresse oxidativo para embriões heterólogos. Esse efeito resultou no aumento das taxas de clivagem (71,1%), da proporção de embriões com ≥ 8 células (31,5%) e do desenvolvimento de mórulas (20,2%) após o desenvolvimento *in vitro*. Finalmente, a **quarta etapa** demonstrou que a combinação da FIV homóloga com o meio KSOM é a estratégia mais eficaz para a produção *in vitro* de embriões em cutias-vermelhas, resultando em maior qualidade dos zigotos (84,5%) e melhores taxas de desenvolvimento embrionário (clivagem total: 78,4%; mórula/clivados: 63,8%). Além disso, a AA apresentou desempenho superior na formação de mórulas no CIV com o KSOM (58,4%). Esse estudo também documentou, pela primeira vez, a produção *in vitro* de blastocisto em cutias (1,7%) no grupo FIV-KSOM. Em conclusão, os resultados deste estudo representam um passo importante no entendimento da biologia reprodutiva para a espécie e poderão servir como base para novos estudos voltados à reprodução assistida, com o propósito de preservar e auxiliar na continuidade das populações de cutias-vermelhas na América do Sul.

Palavras-chave: roedores cavídeos, fertilização *in vitro* heteróloga, produção de blastocistos e mórulas *in vitro*, espermatozoides epididimários, maturação e ativação oocitária artificial.

ABSTRACT

Assisted reproductive technologies (ARTs) play a fundamental role in the conservation of wildlife species, such as the red-rumped agouti (*Dasyprocta leporina* Linnaeus, 1758), a rodent of great ecological importance due to its role in seed dispersal across the South American continent. These technologies offer innovative approaches to overcoming reproductive challenges, contributing to the preservation of the genetic diversity of these species. In this context, understanding embryonic development processes in controlled *in vitro* environments becomes essential. However, advanced reproductive techniques, such as *in vitro* fertilization (IVF), still lack specific studies aimed at elucidating the initial stages involved in gamete manipulation. Given this scenario, the present study aimed to investigate the *in vitro* reproductive processes of the red-rumped agouti, specifically sperm selection and capacitation, gamete co-incubation, and culture medium supplementation, in order to deepen the understanding of its reproductive biology and, consequently, optimize its embryonic development in assisted reproduction systems. To achieve these objectives, the thesis was structured into four steps. In the **first stage**, different sperm selection methods—density gradient, swim-up, and centrifugation—were compared to identify the most efficient technique for improving sperm fertilization capacity and viability for IVF. The **second stage** investigated the effects of capacitating agents by evaluating low- and high-concentration BSA profiles (4 or 15 mg/mL), in the presence or absence of calcium chloride (CaCl₂), over different incubation times (1, 3, or 6 h). The **third stage** analyzed the influence of the culture medium on IVF, comparing HTF, TYH, and TALP media in terms of their impact on embryonic development. Finally, the **fourth stage** assessed the most suitable medium for embryonic development, comparing SOF and KSOM for embryos produced by IVF as well as those obtained through artificial oocyte activation (AA). The results of the **first stage** indicated that all selection methods were effective in obtaining viable sperm for IVF; However, centrifugation stood out by selecting gametes with superior kinetic, morphological, and functional parameters, while also maintaining a favorable oxidative balance *in vitro*. In the **second stage**, the presence of CaCl₂ was shown to be essential for sperm viability and functionality in the red-rumped agouti, reducing reactive oxygen species (ROS) production and promoting high rates of capacitation (90.7%), acrosome reaction (93.8%), and hyperactivity (83.0%). Additionally, the lower BSA concentration and the 6-h incubation period were crucial for maximizing capacitation rates in this species. The **third-stage** experiments indicated that the TALP medium outperformed HTF and TYH media by providing a lower oxidative stress environment for heterologous embryos. This effect resulted in increased cleavage rates (71.1%), a higher proportion of embryos with ≥8 cells (31.5%), and improved morula formation (20.2%) after *in vitro* development. Finally, the **fourth stage** demonstrated that the combination of homologous IVF with KSOM medium is the most effective strategy for *in vitro* embryo production in red-rumped agouti, leading to higher zygote quality (84.5%) and improved embryonic development rates (total cleavage: 78.4%; morula/cleaved: 63.8%). Furthermore, AA showed superior performance in morula formation on IVC with KSOM (58.4%). This study also documented, for the first time, *in vitro* blastocyst production in agouti (1.7%) in the IVF-KSOM group. In conclusion, the findings of this study represent an important step in understanding the reproductive biology of this species and may, in the future, serve as a foundation for new studies on assisted reproduction, with the aim of preserving and helping the continuity of red-rumped agouti populations in South America.

Keywords: Caviid rodents, heterologous *in vitro* fertilization, *in vitro* blastocyst and morula production, epididymal spermatozoa, oocyte maturation and artificial activation

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LISTA DE ABREVIATURAS E SIGLAS

°C	Graus Celsius
1PB	First polar body
2PB	Second polar body
AA	Artificial Activation (Ativação Artificial)
AA-KSOM	Artificial Activation with Potassium Simplex Optimized Medium
AA-SOF	Artificial Activation with Synthetic Oviductal Fluid medium
AFU	Arbitrary Fluorescence Units
ALH	Amplitude of Lateral Head
ANOVA	Análise de variância
AR	Acrosome Reaction
ARTs	Assisted Reproductive Technologies
ATP	Adenosina trifosfato
BCF	Beat Cross Frequency
Bl	Blastocisto
BSA	Albumina sérica bovina
CA	Califórnia
Ca ²⁺	Cálcio
CaCl ₂	Cloreto de cálcio
cAMP	Adenosina 3',5'-monofosfato cíclico
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CASA	Análise de Sêmen Assistida por Computador (<i>Computer Assisted Sperm Analysis</i>)
CEMAS	Centro de Multiplicação de Animais Silvestres
Cl ⁻	Cloro
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO ₂	Dióxido de carbono
COCs	Complexo <i>cumulus</i> oócito (<i>cumulus-oocyte complex</i>)
CT	Centrifugação (<i>Centrifugation</i>)
CTC	Clortetraciclina
CZB	Glucose-free and serum-free medium
D2	Dia dois

D5	Dia cinco
DG	Gradiente de densidade (<i>Density Gradient</i>)
DIV	Desenvolvimento <i>in vitro</i>
DNA	Ácido desoxirribonucleico
Dr.	Doutor
Dra.	Doutora
EDTA	Ácido etilenodiaminotetracético
EGF	Fator de Crescimento Epidermal (<i>Epidermal Growth Factor</i>)
EROs	Espécies Reativas de Oxigênio
et al	Colaboradores
FBS	Fetal Bovine Serum
FITC	Isotiocianato de fluoresceína
FIV	Fertilização <i>in vitro</i>
FSH	Hormônio folículo-estimulante
g	Grama
GO	Goiás
GSH	Glutathiona
h	Hora
H ₂ DCFDA	2',7'-Diclorofluoresceína
H33342	Hoechst 33342 (Bisbenzimidaz)
HBCa	15 mg/mL BSA com CaCl ₂ (<i>high BSA with calcium</i>)
HBSA	15 mg/mL BSA (<i>high BSA</i>)
HCl	Cloreto de hidrogênio
HCO ³⁻	Bicarbonato
he-IVF	Heterologous <i>In Vitro</i> Fertilization
he-FIV	Fertilização <i>in vitro</i> heteróloga
HEPES	Ácido 4-(2-hidroxiEtil)-1-piperazin Etanol Sulfônico
HOS	Hiposmótico
HOST	Teste hiposmótico (<i>Hypoosmotic Test</i>)
HTF	Human Tubal Fluid (fluido tubário humano)
HYP	Hyperactivation
Hz	Hertz
iB	Blastocisto inicial (<i>Initial Blastocyst</i>)

ICMBio	Instituto Chico Mendes de Conservação da Biodiversidade
ICs	Iniciação científica
IVC	<i>In Vitro</i> Culture
IVD	<i>In Vitro</i> Development
IVF	<i>In Vitro</i> Fertilization
IVF-KSOM	<i>In Vitro</i> Fertilization with Potassium Simplex Optimized Medium
IVF-SOF	<i>In Vitro</i> Fertilization with Synthetic Oviductal Fluid medium
IVM	<i>In Vitro</i> Maturation
KCl	Cloreto de potássio
Kg	Quilograma
KH ₂ PO ₄	Fosfato monopotássico
KSOM	Potassium Simplex Optimized Medium (<i>meio otimizado simples suplementado com potássio</i>)
KSOM-AA	Potassium Simplex Optimized Medium with Aminoacids
kx	Deflexão do cantilever
L	Litro
LBA	Laboratório de Biotecnologia Animal
LBCa	4 mg/mL BSA com CaCl ₂ (<i>low BSA with calcium</i>)
LBSA	4 mg/mL BSA (<i>low BSA</i>)
LCGA	Laboratório de Conservação de Germoplasma Animal
LH	Hormônio luteinizante
LIN	Linearity
M	Molar
m	Metro
M16	EmbryoMax modified M16 medium
M2	Modified Krebs-Ringer bicarbonate solution
Ma	Mestra
MCM	Meio mínimo capacitante (<i>Minimal Capacitating Medium</i>)
mg	Miligrama
MgSO ₄	Sulfato de magnésio
MII	Metáfase 2
mIU	Miliunidades internacionais
min	Minuto

MIV	Maturação <i>in vitro</i>
mL	Mililitro
mM	Milimolar
mmHg	Milímetro de mercúrio
MMM	Minimum Maintenance Medium
mmol	Milimole
mOsm	Miliosmole
MT6	Modified Tyrode's medium
mT-H	HEPES-buffered modified Tyrode's medium
Na	Sódio
Na ₂ HPO ₄	Fosfato dissódico
NaCl	Cloreto de sódio
NaHCO ₃	Bicarbonato de sódio
ng	Nanograma
nM	Nanomolar
no	Número
P	Probabilidade de significância
PBs	Polar bodies
PBS	Phosphate Buffered Saline (<i>tampão fosfato-salino</i>)
PCM	Phase-Contrast Microscopy
pH	Potencial Hidrogeniônico
PKA	Proteína quinase A
PM	Progressive motility
PNs	Pronúcleos
PPGCA	Programa de Pós-Graduação em Ciência Animal
Prof	Professor
Profa	Professora
ROS	Reactive Oxygen Species
s	Segundo
sAC	Adenil Ciclase
SE	Standard error
SEM	Scanning Electron Microscopy (<i>microscopia eletrônica de varredura</i>)
SOF	Synthetic Oviductal Fluid (<i>fluido do oviduto sintético</i>)

SP	Swim-up
sptz/mL	Espermatozoide por mililitro
STR	Straightness
TALP	Tyrode's Albumin Lactate Pyruvate (Tyrode-Albumina-Lactato-Piruvato)
TM	Total Motility
TM medium	Tyrode's medium
TRAs	Técnicas de Reprodução Assistida
TYH	Toyoda-Yokoyama-Hoshi medium
UFERSA	Universidade Federal Rural do Semi-Árido
USA	Estados Unidos da América
VAP	Velocity Average Pathway
VCL	Curvilinear Velocity
vs.	Versus
VSL	Velocity Straight Line
WCA	Without Capacitation Agent
WS	Without selection
ZP	Zona Pellucida
μL	Microlitro
μm	Micrometro
μM	Micromolar

LISTA DE SÍMBOLOS

®	Marca registrada
%	Porcentagem
+	Soma
=	Igual
±	Mais ou menos
<	Menor que
>	Maior que
×	Multiplicação
≥	Igual e maior que
~	Aproximadamente
/	Divido por
$\Delta\Psi_m$	Potencial de membrana mitocondrial

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CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1 INTRODUÇÃO

A *Dasyprocta leporina*, conhecida popularmente como cutia-vermelha, é um roedor histricomorfo de médio porte, amplamente distribuído pela América do Sul (DEUTSH; PUGLIA, 1988). Essa espécie desempenha um papel ecológico crucial, atuando como um eficiente dispersor de sementes e integrando a cadeia trófica dos ecossistemas onde está presente (HADLER et al., 2016). Diante da importância ecológica da espécie, diversas tecnologias de reprodução assistida (TRAs) vêm sendo desenvolvidas com o intuito de auxiliar na conservação e manejo das populações de *D. leporina*.

Em geral, as TRAs oferecem abordagens importantes para superar desafios reprodutivos, permitindo uma compreensão dos processos de fisiologia reprodutiva desses animais (BOLTON et al., 2022; GARDNER, 2021). Dentre as técnicas, as conduzidas em ambientes controlados *in vitro* fornecem informações fundamentais para a elucidação de aspectos reprodutivos (BESENFELDER; HAVLICEK, 2023), incluindo as interações entre gametas (CAÑÓN-BELTRÁN et al., 2023) e a cinética embrionária no estágio pré-implantacional (CAÑÓN-BELTRÁN et al., 2024).

Dentre os métodos mais empregados para a produção de embriões em tais estudos, destaca-se a fertilização *in vitro* (FIV). Essa biotecnologia proporciona uma compreensão mais aprofundada das interações entre gametas masculinos e femininos, uma vez que sua aplicação exige a otimização das etapas de maturação oocitária, seleção e capacitação espermática, além da determinação precisa do tempo e das condições ideais para a co-incubação e o desenvolvimento embrionário (HORIE et al., 2015).

Nesse contexto, a única tentativa documentada de aplicação da FIV em cutias foi realizada em uma espécie distinta, a cutia-de-dorso-escuro (*Dasyprocta prymnolopha*), por Ferraz et al. (2020). Nesse estudo, os autores utilizaram um protocolo convencional de FIV bovina, empregando um meio comercial, espermatozoides epididimários não capacitados e uma elevada concentração espermática de 25×10^6 espermatozoides/mL para a co-incubação com os oócitos. O protocolo adotado envolveu um tempo prolongado de FIV, com duração de 15 h, seguido pelo cultivo embrionário em meio fluido do oviduto sintético (SOF). Contudo, as taxas de sucesso foram relativamente baixas, registrando apenas 8% de clivagem no segundo dia de cultivo e 2,9% de mórulas no quinto dia, evidenciando a falta de eficiência da FIV para as cutias.

Assim, devido a eficiência da FIV ainda variar consideravelmente entre todas as espécies de roedores (IZQUIERDO-RICO et al., 2021), faz-se necessária uma investigação mais cuidadosa para viabilizar sua aplicação. Um dos principais entraves está na obtenção de um número suficiente de oócitos viáveis, uma limitação decorrente da escassez de estudos voltados para o aprimoramento das técnicas de recuperação oocitária *in vivo* em espécies silvestres (SWANSON, 2023). Diante desse desafio, a fertilização *in vitro* heteróloga (he-FIV) surge como uma estratégia alternativa promissora, permitindo a análise das interações reprodutivas *in vitro*, de forma similar à FIV convencional (homóloga), porém utilizando oócitos de espécies domésticas em associação com espermatozoides de animais silvestres (CAÑÓN-BELTRÁN et al., 2023).

Diante dessas informações, essa tese teve como objetivo investigar os aspectos reprodutivos de *D. leporina* em sistemas *in vitro*, por meio da otimização das estratégias de seleção e capacitação espermática, co-incubação de gametas e suplementação do meio de desenvolvimento embrionário, utilizando ambas FIV heteróloga e homóloga, visando aprimorar a produção de embriões viáveis na espécie. Os achados deste estudo poderão contribuir para a compreensão dos mecanismos fisiológicos subjacentes à reprodução da espécie, fornecendo subsídios para o desenvolvimento de biotecnologias aplicadas tanto para conservação, quanto para o manejo populacional de *D. leporina*.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 Panorama geral da espécie *Dasyprocta leporina*

A *Dasyprocta leporina*, conhecida popularmente como cutia-vermelha (**Figura 1**), é um roedor silvestre neotropical de médio porte (3–4 kg, ROWE; HONEYCUTT, 2002), amplamente distribuído na América do Sul, abrangendo desde a região amazônica até áreas do litoral central do Brasil, incluindo também a Venezuela e as Guianas (RUIZ-GARCÍA et al., 2022). Trata-se de uma espécie predominantemente terrestre e de hábitos diurnos (PATTON; EMMONS, 2015), cuja dieta é baseada principalmente em frutos, sementes, raízes e outros vegetais (OLIVEIRA; BONVICINO, 2011).



Figura 1. Imagem representativa de cutia-vermelha (*Dasyprocta leporina*) mantida no Centro de Multiplicação de Animais Silvestres da UFERSA. Fonte: própria.

Os indivíduos dessa espécie geralmente vivem em pares monogâmicos permanentes e apresentam comportamentos sociais frequentes em seu ambiente natural (KAISER et al., 2011). Eles são comumente encontrados em florestas com ambientes úmidos, situadas em altitudes mais baixas, bem como em florestas decíduas e secas (FERREGUETTI et al., 2018). A área de vida ocupada pela *D. leporina* costuma variar entre dois e três hectares, contendo locais fixos para repouso, alimentação e trilhas bem demarcadas utilizadas para deslocamento (SMYTHE, 1978).

A *D. leporina* pertence à subordem Hystricomorpha, à superfamília Caviioidea e à família Dasyproctidae, sendo integrante do gênero *Dasyprocta*, que compreende outras 12 espécies (**Figura 2**; TETA; REYES-AMAYA, 2021). Entre as espécies do gênero, observa-se uma variação quanto ao grau de ameaça à extinção, conforme as avaliações da União Internacional para a Conservação da Natureza (IUCN). Dentre elas, destacam-se como as mais ameaçadas atualmente *Dasyprocta mexicana* (REID, 2009) e *Dasyprocta ruatanica* (LEE et al., 2006). Embora o status de conservação de *D. leporina* tenha sido classificado como pouco preocupante em escala global há cerca de uma década (EMMONS; REID, 2016), estudos mais recentes indicam uma preocupante tendência de declínio populacional em determinadas regiões do Brasil. No estado de São Paulo, por exemplo, a espécie foi considerada como Quase Ameaçada (QA) em 2018, enquanto no Espírito Santo passou à categoria Vulnerável (VU) em 2022 (PERCEQUILLO et al., 2025). Cenários ainda mais críticos foram registrados no Parque Nacional da Tijuca, no Rio de Janeiro, onde a espécie foi enquadrada na categoria em extinção na natureza (EN) (ZUCARATTO; PIRES, 2015).

Esse declínio populacional é particularmente preocupante, uma vez que *Dasyprocta leporina* desempenha um papel ecológico crucial em seus habitats, sendo considerada a principal espécie dispersora de sementes por armazenamento, especialmente de sementes de grande porte (GALETTI et al., 2006). Esses animais acumulam frutos de árvores localmente abundantes para consumo futuro, e as sementes que não são recuperadas contribuem significativamente para a regeneração natural das florestas. De fato, supõe-se que a extinção de diversos mamíferos frugívoros de grande porte (megafauna) no final do Pleistoceno tenha deixado as cutias como herdeiras ecológicas na dispersão de várias espécies de plantas com frutos carnosos de grande tamanho (GUIMARÃES et al., 2008). A *D. leporina* tem sido, inclusive, registrada em processos de restauração de fragmentos florestais impactados por defaunação (GALETTI et al., 2010). Ainda, trata-se de uma espécie-chave na cadeia trófica, servindo de importante recurso alimentar para predadores, como a jaguatirica (*Leopardus pardalis*) (OLIVEIRA et al., 2010).

Além de sua relevância ecológica, a *D. leporina* possui considerável importância econômica e científica, sendo amplamente criada em cativeiro, especialmente nas regiões de Trinidad e Tobago, onde sua elevada demanda a torna uma das espécies de fauna silvestre mais caçadas (ESPINHEIRO et al., 2014). Do ponto de vista econômico, a espécie representa uma significativa fonte de proteína animal para comunidades rurais locais, sendo inclusive classificada como "*micro-livestock*" em virtude de seu potencial de produção em pequena escala (HARDOUIN et al., 2003). Sua carne, comercializada em mercados de vilarejos, é

Gênero <i>Dasyprocta</i>			
Espécies	Ilustração	Nome popular	Classificação IUCN
<i>Dasyprocta azarae</i>		Cutia-de-Azara	Sem dados (SD) 
<i>Dasyprocta coibae</i>		Cutia-de-Coiban	Quase ameaçada (QA) 
<i>Dasyprocta cristata</i>		Cutia-de-crista	Pouco preocupante (PP) 
<i>Dasyprocta fuliginosa</i>		Cutia-preta	Pouco preocupante (PP) 
<i>Dasyprocta guamara</i>		Cutia-do-Orinoco	Quase ameaçada (QA) 
<i>Dasyprocta kalinowskii</i>		Cutia-do-Peru	Sem dados (SD) 
<i>Dasyprocta leporina</i>		Cutia-vermelha	Pouco preocupante (PP) 
<i>Dasyprocta mexicana</i>		Cutia-mexicana	Criticamente ameaçada (CA) 
<i>Dasyprocta prymnolopha</i>		Cutia-de-dorso-preto	Pouco preocupante (PP) 
<i>Dasyprocta punctata</i>		Cutia centro-americana	Pouco preocupante (PP) 
<i>Dasyprocta ruatanica</i>		Cutia-da-Ilha-Ruatan	Ameaçada de extinção (AE) 
<i>Dasyprocta variegata</i>		Cutia-marrom	Sem dados (SD) 

Escala de classificação do nível de ameaça à extinção - IUCN

Sem dados

SD

PP

QA

VU

AE

CA

EN

EX

Extinto

Figura 2. Espécies que estão inseridas dentro do gênero *Dasyprocta* e seu índice de ameaça à extinção de acordo com a União Internacional de Conservação da Natureza (IUCN). SD: sem dados; PP: pouco preocupante; QA: quase ameaçada; VU: vulnerável; AE: ameaçada de extinção; CA: criticamente ameaçada; EN: extinto na natureza; EX: extinto. Fonte: própria.

reconhecida por seu elevado valor nutricional e proteico (JONES et al., 2021). Adicionalmente, a notável adaptabilidade da *D. leporina* às condições de cativeiro favorece sua utilização como ferramenta de conservação *ex situ*, viabilizando futuras reintroduções na natureza (JONES et al., 2023).

A partir desse conhecimento sobre a espécie, seu status de ameaça e sua relevância ecológica, econômica e científica, estudos visando conhecer sua fisiologia reprodutiva começaram a ser desenvolvidos como o primeiro passo para o desenvolvimento e aplicação de técnicas de reprodução assistida (TRAs), com o objetivo de subsidiar programas de manejo e reforço populacional voltados à preservação da cutia-vermelha.

2.2 Fundamentos da biologia reprodutiva de *D. leporina*

2.2.1 Aspectos básicos da biologia reprodutiva de cutias-vermelhas machos

Os primeiros estudos realizados em cutias-vermelhas machos tiveram como foco a caracterização da maturidade sexual da espécie. De acordo com Assis Neto et al. (2003), o período compreendido entre o nascimento e os cinco meses de idade corresponde à fase pré-púbere. Entre seis e oito meses de idade ocorre a fase de transição, entre a pré-puberdade e a puberdade, na qual se observam os primeiros espermátócitos primários e espermátides arredondadas. A puberdade nos machos de *D. leporina* é alcançada por volta dos nove meses de idade, sendo possível, nesse estágio, identificar todos os tipos celulares, bem como espermatozoides presentes no lúmen dos túbulos seminíferos, após os oito estágios do ciclo do epitélio seminífero.

Na sequência, Mollineau et al. (2006) iniciaram uma série de descrições anatômicas da espécie, caracterizando macroscopicamente o sistema reprodutor masculino. A média do comprimento, diâmetro e peso testiculares foi de $3,67 \pm 0,12$ cm, $1,67 \pm 0,04$ cm e $5,03 \pm 0,52$ g, respectivamente. Os testículos e epidídimos pareados estão posicionados em contato com os músculos abdominais, dentro de bolsas escrotaís. A cabeça do epidídimo encontra-se envolto por um corpo adiposo (**Figura 3A**). O canal deferente apresenta comprimento e diâmetro médios de $10,98 \pm 0,40$ cm e $0,14 \pm 0,01$ cm, respectivamente. Na porção terminal do canal deferente, seu diâmetro expande-se para formar a ampola. O pênis da espécie apresenta formato em “U”, com comprimento médio de $9,90 \pm 0,43$ cm. Além disso, a ausência de escroto em *D. leporina* foi previamente relatada por Menezes et al. (2003).

Posteriormente, Mollineau et al. (2008) descreveram, pela primeira vez, as características morfológicas dos espermatozoides da espécie, obtidos por meio de eletroejaculação. Os espermatozoides apresentaram cabeça ovalada, semelhante a uma pá, com ápice arredondado e base achatada (**Figura 3B**). As médias de comprimento da cabeça, peça intermediária, cauda e comprimento total dos espermatozoides foram de $5,23 \pm 0,04 \mu\text{m}$, $5,18 \pm 0,08 \mu\text{m}$, $37,52 \pm 0,24 \mu\text{m}$ e $47,96 \pm 0,25 \mu\text{m}$, respectivamente. As médias da largura da cabeça e da peça intermediária foram de $3,26 \pm 0,04 \mu\text{m}$ e $0,70 \pm 0,02 \mu\text{m}$, enquanto o volume médio de sêmen foi de $0,47 \pm 0,16 \text{ mL}$.

Em um trabalho subsequente, Mollineau et al. (2009) descreveram a anatomia microscópica das glândulas sexuais acessórias de *D. leporina*, destacando a presença de vesículas seminais lobuladas, glândulas coagulantes em formato de folha, glândulas prostáticas lobuladas e glândulas bulbouretrais em forma de ervilha. No que se refere ao desenvolvimento reprodutivo, Costa et al. (2010) verificaram que o ciclo espermatogênico na espécie tem duração média de $9,5 \pm 0,03$ dias, totalizando um período de aproximadamente $42,8 \pm 0,16$ dias para a espermatogênese completa. Arroyo et al. (2014) realizaram um estudo com o objetivo de descrever o epidídimo de *Dasyprocta leporina*, concluindo que esta estrutura é composta por células principais, basais, halo, apicais e claras, as quais apresentam alterações morfológicas e funcionais ao longo do desenvolvimento sexual.

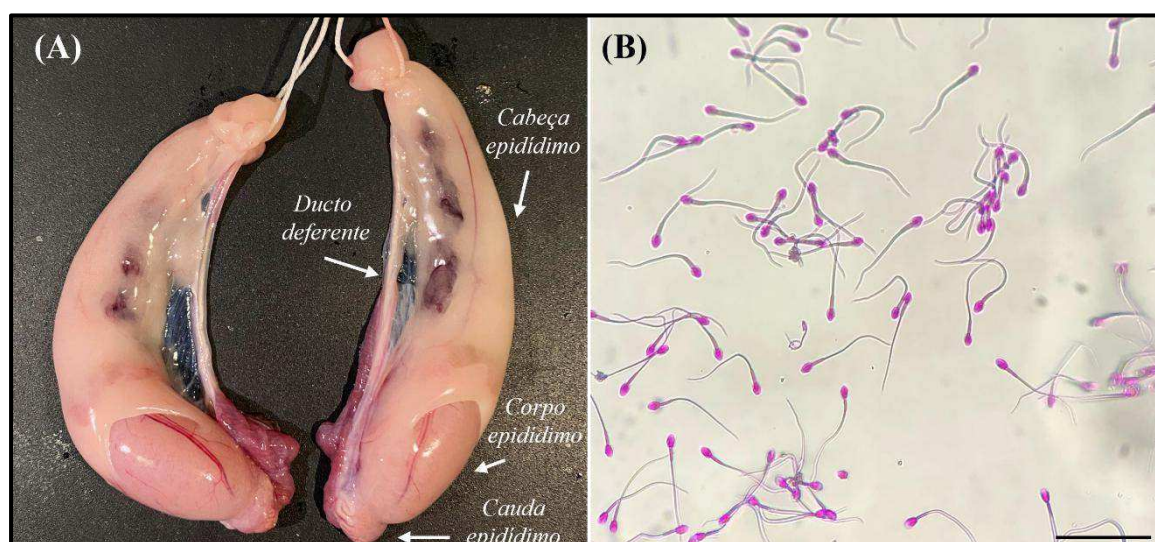


Figura 3. Características do sistema reprodutivo masculino de *D. leporina*. (A) Complexo testículo-epidídimo-ducto-deferente de cutias sexualmente maduras. (B) Morfologia espermática a partir da coloração com rosa de bengala. Barra de escala: $50 \mu\text{m}$. Fonte: própria.

Recentemente, foram descritas as alterações morfológicas e funcionais dos espermatozoides ao longo do trânsito epididimário em *D. leporina* (DANTAS et al., 2022). Os autores observaram que, apesar da morfologia espermática se manter relativamente constante entre as diferentes regiões do epidídimo, as médias do comprimento da cabeça, largura da cabeça e comprimento da cauda foram maiores na cabeça do epidídimo, diminuindo progressivamente ao longo do trânsito epididimário. Os espermatozoides provenientes da cauda do epidídimo apresentaram maior integridade de membrana, maior atividade mitocondrial e melhores parâmetros de motilidade total e progressiva, incluindo as velocidades média do percurso (VAP), retilínea (VSL), curvilínea (VCL), e proporção de espermatozoides rápidos (DANTAS et al., 2022).

2.2.2 Aspectos básicos da biologia reprodutiva de cutias-vermelhas fêmeas

O estudo pioneiro nas fêmeas espécie foi realizado por Almeida et al. (2003). Os autores descreveram os ovários da espécie como estruturas elipsoides, achatadas dorso ventralmente, com pequenas áreas translúcidas na superfície externa (**Figura 4A**). Neste estudo, foram observadas médias de peso, comprimento, largura e espessura de 0,082 g, 0,83 cm, 0,49 cm e 0,24 cm, respectivamente. Ainda, Fortes et al. (2005) contribuíram para a elucidação dos aspectos morfológicos da tuba uterina, caracterizando a presença de três regiões bem definidas: infundíbulo, ampola e istmo.

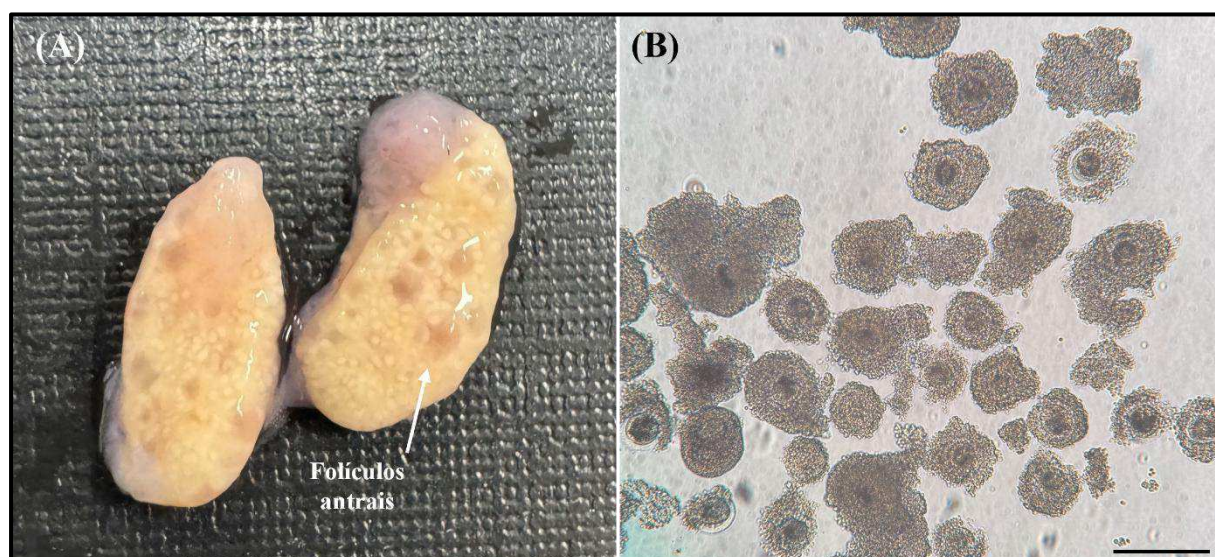


Figura 4. Características do sistema reprodutivo feminino de *D. leporina*. (A) Ovários de cutias-vermelhas sexualmente maduras. (B) Morfologia de oócitos imaturos de cutia obtidos pós fatiamento ovariano. Barra de escala: 100 μ m. Fonte: própria.

Singh et al. (2014), deu continuidade a esses trabalhos descrevendo toda a anatomia macroscópica e microscópica do trato reprodutivo da *D. leporina*. Dentre suas observações, evidenciaram que o córtex ovariano é constituído por tecido conjuntivo, no qual se encontram inseridos os folículos ovarianos. As tubas uterinas apresentam um aumento gradativo em largura da região próxima ao ovário em direção ao útero. Os cornos uterinos, dispostos em pares, convergem para formar um corpo uterino em formato de "Y", caracterizando um útero do tipo duplo, separado por um septo. A vagina apresentou comprimento médio de $7,0 \pm 11,4$ cm, sendo essa a porção mais longa do trato reprodutivo.

Posteriormente, Campos et al. (2015) descreveram o ciclo estral da espécie como sendo poliétrico contínuo, com duração média de $28,2 \pm 0,7$ dias, variando entre 24 e 31 dias. Adicionalmente, Oliveira et al. (2017) descreveram o período gestacional de *D. leporina*, concluindo que varia entre 103 e 110 dias, resultando no nascimento de 1,7 a 2 crias por ninhada.

Santos et al. (2018) realizaram a estimativa e morfometria da população de folículos pré-antrais ovarianos em *D. leporina*. A média da população folicular por par de ovários foi estimada em $4419,8 \pm 532,26$ e $5397,52 \pm 574,91$ para os ovários direito e esquerdo, respectivamente. Os diâmetros dos folículos, oócitos e núcleos foram: $18,62 \pm 3,40$ μm , $12,28 \pm 2,37$ μm e $6,10 \pm 0,93$ μm para folículos primordiais; $23,75 \pm 5,70$ μm , $14,22 \pm 3,00$ μm e $6,70 \pm 1,24$ μm para folículos primários; e $88,55 \pm 17,61$ μm , $52,85 \pm 17,56$ μm e $22,33 \pm 17,61$ μm para folículos secundários, respectivamente. A maioria dos folículos observados pertencia à categoria primordial (86,63%), seguida pelos primários (13,01%) e secundários (0,35%).

No mesmo trabalho, Santos et al. (2018) também descreveram, por meio de microscopia eletrônica de transmissão, as características ultraestruturas dos oócitos imaturos da cutia (**Figura 4B**), observando oócitos com nucléolo distinto e centralizado. O citoplasma dessas estruturas apresentou numerosas mitocôndrias arredondadas distribuídas de forma difusa, aparelho de Golgi bem desenvolvido, e gotas lipídicas.

Oliveira et al. (2019) investigaram a anatomia dos órgãos genitais de *D. leporina* em diferentes fases do ciclo estral. As principais diferenças encontradas foram: durante a fase de proestro, foram observados folículos em desenvolvimento e corpo lúteo em regressão; na fase de estro, evidenciaram-se folículos de Graaf; durante o metaestro, foi identificado o corpo hemorrágico; e na fase de diestro, observou-se a presença de corpo lúteo maduro.

Finalmente, Praxedes et al. (2023) descreveram as características morfométricas de oócitos imaturos recuperados por fatiamento ovariano, apresentando as seguintes médias: diâmetro externo do oócito (90,9 μm), espessura da zona pelúcida (14,8 μm), diâmetro do

ooplasma (65,0 μm), comprimento do espaço perivitelínico (7,7 μm) e área interna do oócito (2.599 μm^2).

As investigações acerca do sistema reprodutor das fêmeas de *Dasyprocta leporina* tiveram um desenvolvimento mais tardio em comparação aos estudos voltados para o sistema reprodutor masculino da espécie. Contudo, a partir dessas valiosas informações reprodutivas de ambos os sexos, foi possível avançar na implementação de etapas preliminares que contribuem para o estabelecimento das TRAs e de suas aplicações propriamente ditas em *D. leporina*.

2.3 Avanços experimentais voltados à aplicação de TRAs em *D. leporina*

Para a aplicação das TRAs, além do aprimoramento específico para cada espécie de interesse, torna-se imprescindível o desenvolvimento e a padronização de técnicas complementares que viabilizem a implementação integral dessas biotecnologias reprodutivas. Dentre as técnicas auxiliares, já foram desenvolvidas para *D. leporina* a recuperação espermática e oocitária, criopreservação de tecidos e células germinativas, protocolos de indução do estro e estratégias de preservação de folículos pré-antrais.

2.3.1 Avanços de TRAs em cutias-vermelhas machos

O primeiro avanço na recuperação espermática de *D. leporina* foi realizado por Mollineau et al. (2008), que tentou obter espermatozoides por meio da eletroejaculação. Os autores obtiveram uma eficiência de 30% dos ejaculados contendo espermatozoides. Dois anos depois, uma segunda tentativa foi realizada, com a alteração do protocolo de voltagem, e observou-se uma eficiência de 40% dos ejaculados contendo espermatozoides (MOLLINEAU et al., 2010).

Na sequência, Castelo et al. (2015b) investigaram as interações entre diferentes protocolos de eletroejaculação, associados a estímulos seriados ou contínuos, e após um total de 40 tentativas, no melhor grupo, 57% dos ejaculados apresentaram espermatozoides. Mais recentemente, Lima et al. (2023) compararam diferentes protocolos anestésicos para a eletroejaculação e, embora todos tenham sido eficazes na indução da ejaculação (100%), a maioria dos ejaculados foi azoospermica (85%). A presença de espermatozoides foi observada em apenas 10 a 30% dos grupos experimentais, sendo a combinação de cetamina/xilazina a mais eficiente.

Diante desses desafios, buscaram-se alternativas para a obtenção de espermatozoides em *D. leporina*. Silva et al. (2011) foram os primeiros a descrever a recuperação espermática epididimária na espécie, utilizando a técnica de lavagem retrógrada na cauda do epidídimo, com sucesso, obtendo 91,5% de motilidade total e uma concentração espermática de $1,1 \times 10^9$ spz/mL. Posteriormente, Castelo et al. (2015a) também obtiveram resultados satisfatórios com essa técnica, alcançando motilidade total de 96,2% e concentração de $1,4 \times 10^9$ spz/mL. Esse método continua demonstrando eficiência, como evidenciado por Castelo et al. (2023), que reportou motilidade de 96,2% e concentração de $0,8 \times 10^9$ spz/mL em seus experimentos.

Tentativas de conservar esse material genético masculino também foram realizadas. Silva et al. (2011) conduziram a primeira tentativa de criopreservação de espermatozoides epididimários de *D. leporina*, e após o descongelamento, observou-se motilidade de 26,5% e vigor de 2,6 no melhor grupo, que utilizou o diluidor ACP-109c (ACP Biotecnologia). Em seguida, Castelo et al. (2015a) aprimoraram esses índices testando diferentes crioprotetores, constatando que o uso de 3% de glicerol ou 3% de dimetilsulfóxido (DMSO) mantiveram maiores valores de motilidade espermática pós-descongelamento (47,5%) e vigor (3,3).

Na tentativa de otimizar ainda mais os protocolos de criopreservação, Castelo et al. (2023) compararam amostras de espermatozoides epididimários e ejaculados. As amostras epididimárias apresentaram maiores valores de volume, concentração e número total de espermatozoides em comparação àquelas obtidas por eletroejaculação. Contudo, após a criopreservação, não houve diferença entre as fontes, demonstrando que ambas podem ser eficazmente preservadas com o protocolo estabelecido em 2015.

Finalmente, estudos também foram conduzidos visando à preservação de tecidos testiculares para futuras aplicações em biotecnologias reprodutivas. Silva et al. (2022) verificaram que a vitrificação em superfície sólida, utilizando DMSO e etilenoglicol (EG), permitiu a adequada preservação do potencial proliferativo de espermatogônias, células de Leydig e células de Sertoli em *D. leporina*.

2.3.2 Avanços de TRAs em cutias-vermelhas fêmeas

O primeiro passo em fêmeas foi dado por Wanderley et al. (2012), que investigaram a criopreservação de tecido ovariano de *D. leporina* e concluíram que folículos pré-antrais inclusos no tecido ovariano poderiam ser criopreservados com sucesso utilizando 1,5 M de propanodiol, como crioprotetor. Em seguida, a indução de estro em fêmeas de cutia-vermelha foi estudada por Peixoto et al. (2018). As fêmeas que receberam prostaglandina, estando na fase

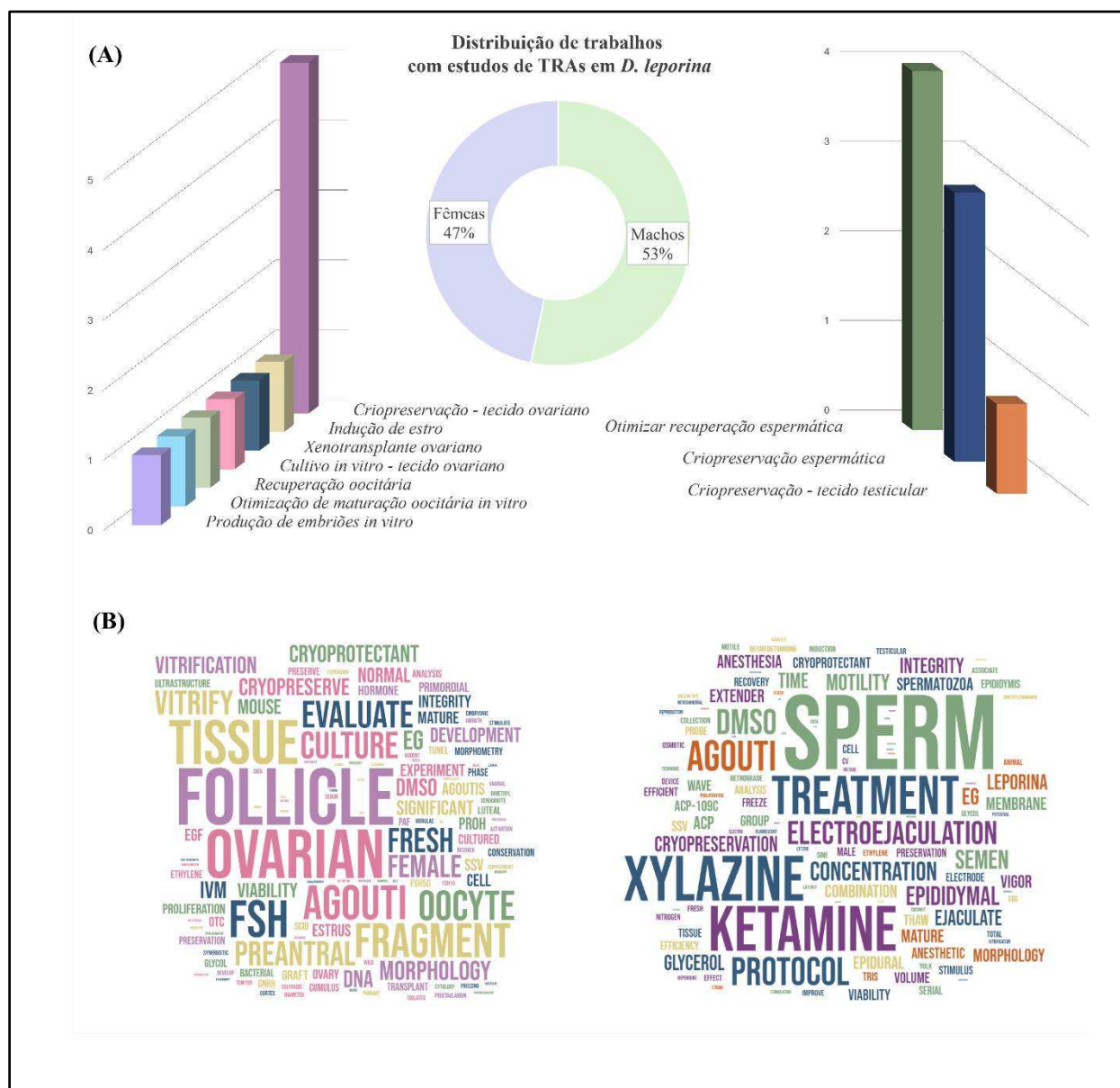
lútea no início do tratamento, manifestaram estro entre três e seis dias após a administração. Em contrapartida, fêmeas tratadas com a combinação de prostaglandina + análogo do hormônio liberador de gonadotrofina (GnRH) apresentaram estro quatro dias após o tratamento. Os autores consideraram os protocolos semelhantes e com eficiência limitada.

A partir de 2018, Praxedes et al. iniciaram uma série de estudos voltados à conservação do tecido ovariano de *D. leporina*. Inicialmente, os autores avaliaram o desenvolvimento de tecido ovariano vitrificado da cutia-vermelha após xenotransplante em camundongos fêmeas *C57Bl/6* com imunodeficiência combinada grave (SCID). A atividade ovariana retornou aproximadamente 20 dias após o transplante em 4 de 5 camundongos que receberam tecido ovariano fresco e em 1 de 6 que receberam tecido vitrificado (PRAXEDES et al., 2018). Embora o protocolo tenha se mostrado funcional, os autores sugeriram a otimização da criopreservação para a espécie. Portanto, em 2020, os autores avaliaram diferentes crioprotetores e suas combinações na criopreservação de tecido ovariano da cutia-vermelha, relatando sucesso com a concentração de 3 M de EG para a preservação da morfologia dos folículos pré-antrais (PRAXEDES et al., 2020).

Em continuidade, visando aprofundar o entendimento sobre a criopreservação ovariana, Praxedes et al. (2021a) avaliaram o efeito de sistemas abertos e fechados na vitrificação do tecido ovariano quanto à carga microbiológica e preservação dos folículos pré-antrais. Os autores concluíram que ambos os sistemas foram igualmente eficientes na preservação do tecido, especialmente na morfologia folicular e integridade do DNA; contudo, o sistema fechado demonstrou ser menos propenso à proliferação bacteriana.

Mais recentemente, Praxedes et al. (2023a) investigaram o cultivo *in vitro* desses folículos pré-antrais após vitrificação em sistemas abertos e fechados. O estudo demonstrou que o meio TCM199 suplementado com 50 ng/mL de hormônio folículo-estimulante (FSH) foi eficiente na manutenção da viabilidade *in vitro* dos folículos tanto frescos quanto vitrificados.

Adicionalmente, Praxedes et al. (2023b) buscaram otimizar o meio de maturação *in vitro* (MIV) de oócitos da espécie e desenvolver um protocolo para a produção de embriões oriundos de oócitos ativados artificialmente. Inicialmente, os autores conseguiram com sucesso coletar oócitos imaturos *post-mortem* pelo método de fatiamento ovariano. Ao finalizar os experimentos, recomendaram a combinação de 10 mIU/mL de FSH e 10 ng/mL de EGF no meio MIV, alcançando uma taxa de maturação nuclear oocitária de 52,0% e 100,0% de expansão das células do *cumulus*. Além disso, após desenvolvimento embrionário, a taxa de clivagem foi de 43,2% no segundo dia e uma produção de mórulas de 8,3% no quinto dia no grupo com melhor desempenho.



2.4 Aspectos reprodutivos da *D. leporina* em comparação com roedores murídeos e cavídeos

A ordem Rodentia é a mais numerosa entre os mamíferos placentários, composta por mais de 2.000 espécies e representando cerca de 40% de todas as espécies de mamíferos (CARLETON; MUSSER, 2005). Apesar da proximidade filogenética entre as espécies (ROWE; HONEYCUTT, 2002), diferenças morfofisiológicas marcantes são evidentes ao compararmos suas características específicas. Essas diferenças reprodutivas observadas entre a

D. leporina e outros roedores, podem ser a chave para compreender quais aspectos precisam ser otimizados para aumentar a eficiência das TRAs aplicadas à nossa espécie de interesse.

Inicialmente, ao compararmos os machos dessas espécies (**Quadro 1**), podemos observar diferenças significativas na morfologia espermática. Em cavídeos de médio a grande porte, como a cutia-vermelha, a paca e a capivara, é comum a presença de uma cabeça ovalada sem gancho. Por outro lado, em pequenos murídeos, como o camundongo, o rato e o hamster-sírio, a cabeça espermática apresenta formato falciforme e possui ganchos no ápice. Além disso, nota-se uma diferença marcante no tamanho das caudas espermáticas quando comparamos a *D. leporina* com murídeos de pequeno porte, sendo a cauda do rato e do hamster-sírio quase o dobro em comprimento.

Essas diferenças podem estar diretamente relacionadas com o sucesso na seleção espermática, uma vez que velocidades mais elevadas podem ser alcançadas por espermatozoides com cabeças hidrodinamicamente mais eficientes e caudas de comprimento adequado para a deslocamento. Portanto, modificações nas proporções entre comprimento da cabeça, largura da cabeça e tamanho da cauda resultam em uma estrutura otimizada para desempenhar seu papel de forma eficiente em cada espécie, dentro de seu ambiente *in vivo* (VAREA et al., 2013).

Já nas fêmeas (**Quadro 2**), as variações são ainda mais expressivas, com espécies atingindo a maturidade sexual em apenas 1 mês de idade, como o hamster-sírio, em contraste com os 7 meses necessários para *D. leporina*. Nós observamos a mesma disparidade ao compararmos a duração do ciclo estral: na cutia-vermelha, o ciclo dura em média 28 dias, enquanto na toupeira-pelada, outro roedor de médio porte, dura apenas 1 dia. Essas características influenciam diretamente a frequência com que essas fêmeas podem ficar prenhes ao longo do ano, bem como a duração do seu período gestacional. A partir desses dados, é possível observar que roedores de médio a grande porte necessitam de mais dias para completar o desenvolvimento embrionário (cutia-vermelha: 110 dias; paca: 120 dias; capivara: 150 dias), enquanto pequenos murídeos requerem um tempo significativamente menor (camundongo: 18 dias; rato: 21 dias; hamster-sírio: 16 dias).

396 **Quadro 1.** Características reprodutivas de roedores macho de diferentes espécies

Espécies de roedores	Maturidade sexual	Ciclo espermatogênico	Espermatogênese completa	Morfologia espermiática	Tamanho do espermatozoide		
					Cabeça	Peça	Cauda
Cutia-vermelha (<i>Dasyprocta leporina</i>) ¹	9-10 meses	9 dias	43 dias	Cabeça ovalada - sem gancho	5,2 µm	5,1 µm	37,5 µm
Porquinho-da-índia (<i>Cavia porcellus</i>) ²	1-2 meses	8 dias	47 dias	Cabeça arredondada - sem gancho	12,2 µm	11,4 µm	89,6 µm
Preá (<i>Galea spixii</i>) ³	1-3 meses	SD	SD	Falciforme – sem gancho	4,6 µm	5,1 µm	44,2 µm
Paca (<i>Cuniculus paca</i>) ⁴	8-12 meses	11 dias	52 dias	Cabeça ovalada - sem gancho	7,5 µm	5,3 µm	30,7 µm
Capivara (<i>Hydrochoerus hydrochaeris</i>) ⁵	15-24 meses	12 dias	54 dias	Cabeça ovalada - sem gancho	6,8 µm	6,9 µm	44,2 µm
Camundongo (<i>Mus musculus</i>) ⁶	1-2 meses	8 dias	34 dias	Falciforme – com gancho	7,4 µm	22,3 µm	118 µm
Rato (<i>Rattus norvegicus</i>) ⁷	2-3 meses	12 dias	52 dias	Falciforme – com gancho	7,1 µm	67,4 µm	175 µm
Hamster-sírio (<i>Mesocricetus auratus</i>) ⁸	1-2 meses	12 dias	30 dias	Falciforme – com gancho	6,3 µm	49,5 µm	174,7 µm
Toupeira-pelada (<i>Heterocephalus glaber</i>) ⁹	7-9 meses	SD	SD	Multilobado sem gancho	3,9 µm	1,0 µm	28 µm

397 Referências: ¹Mollineau et al. (2008); Costa et al. (2010); ²Simões et al. (2016); Quispe-Ccasa et al. (2023); ³Santos et al. (2012); Santos et al. (2014); Silva
398 et al. (2017); ⁴Costa et al. (2010); Santos et al. (2020); ⁵Paula et al. (1999); Batalha; Oba, 2006; ⁶Costa et al. (2018). ⁷ Clouthier et al. (1996); Weber et al.
399 (2014); ⁸Miething et al. (1998); Gu et al. (2019); ⁹Van der Horst et al. (2011). SD: sem dados.

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403 **Quadro 2.** Características reprodutivas de roedores fêmeas de diferentes espécies

Espécies de roedores	Maturidade sexual	Duração do ciclo estral	População folicular	Tamanho do oócito imaturo	Período gestacional	Média de ninhada/prenhez
Cutia-vermelha (<i>Dasyprocta leporina</i>) ¹	6-7 meses	28,2 dias	4.908,2	90 µm	103-110 dias	1 a 2 filhotes
Porquinho-da-índia (<i>Cavia porcellus</i>) ²	2 meses	16 dias	101,9	78 µm	68 dias	2 a 3 filhotes
Preá (<i>Galea spixii</i>) ³	2-3 meses	15,8 dias	416,0	75 µm	59 dias	2 a 4 filhotes
Paca (<i>Cuniculus paca</i>) ⁴	8-12 meses	32,5 dias	SD	SD	120 dias	1 a 2 filhotes
Capivara (<i>Hydrochoerus hydrochaeris</i>) ⁵	7-12 meses	7,5 dias	SD	SD	150 dias	1 a 4 filhotes
Camundongo (<i>Mus musculus</i>) ⁶	1-2 meses	4 dias	2.330,2	80 µm	18-22 dias	6 a 10 filhotes
Rato (<i>Rattus norvegicus</i>) ⁷	3-4 meses	5 dias	305,5	79 µm	21 dias	6 a 12 filhotes
Hamster-sírio (<i>Mesocricetus auratus</i>) ⁸	0,5-1 mês	4 dias	315,6	117 µm	16 dias	10 filhotes
Toupeira-pelada (<i>Heterocephalus glaber</i>) ⁹	7-9 meses	1 dia	SD	101 µm	70 dias	11 a 28 filhotes

404 Referências: ¹Campos et al. (2015); Oliveira et al. (2017); Santos et al. (2018); ²Grégoire et al. (2012); Fernández et al. (2022); ³Santos et al. (2015); Praxedes
 405 et al. (2017); Oliveira et al. (2008); ⁴Reis et al. (2011); ⁵López et al. (2008); Miglino et al. (2013); ⁶Ajayi; Akhigbe, 2020; ⁷Marcondes et al. (2002); ⁸AL-
 406 Murshidi et al. (2015); ⁹Jarvis; Sherman, 2002. SD: sem dados.

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Além disso, essa mesma relação de "alto versus baixo" também se reflete no tamanho médio das ninhadas desses roedores, que varia de 1 a 4 crias nas espécies de médio porte, chegando a 6–12 crias em pequenos murídeos. Destacando-se dessa tendência, a toupeira-pelada apresenta a maior ninhada entre os roedores, com até 28 crias por gestação.

A partir dessas informações apresentadas na fundamentação, é possível identificar diferenças significativas nas características reprodutivas entre a *D. leporina* e outros roedores, além da evidente carência de estudos que elucidem aspectos-chave da sua reprodução. Considerando que tais diferenças podem impactar na escolha das etapas iniciais das TRAs, como a FIV, e, conseqüentemente, influenciar na eficiência dessa técnica entre as diferentes espécies, torna-se imprescindível investigar o estado da arte da FIV em roedores. Esta investigação foi amplamente discutida no artigo de revisão que compõe esta tese e, a partir das lacunas identificadas, foi possível delinear a estratégia de desenvolvimento das etapas que integram essa biotécnica reprodutiva aplicada a *D. leporina*.

3 JUSTIFICATIVA

A fertilização *in vitro* tem se consolidado como uma ferramenta importante para o avanço do conhecimento sobre a fisiologia reprodutiva de mamíferos, especialmente aqueles de interesse para conservação e manejo sustentável. Para espécies silvestres, como a *Dasyprocta leporina*, a FIV representa uma oportunidade promissora não apenas para a preservação genética e a ampliação das populações em cativeiro, mas também para aprofundar a compreensão sobre as particularidades reprodutivas da espécie. Contudo, apesar dos avanços aplicadas a roedores, a FIV na *D. leporina* ainda enfrenta inúmeros desafios, principalmente em virtude da ausência de protocolos otimizados para esta espécie.

Entre os principais entraves está a escassez de estudos voltados para a padronização das etapas críticas da FIV, como a seleção e capacitação espermática, o tempo de co-incubação dos gametas e a suplementação do meio de desenvolvimento *in vitro*, que são fundamentais para maximizar as taxas de fertilização e o desenvolvimento embrionário. A experiência prévia com a cutia-de-dorso-escuro (*D. prymnolopha*) demonstrou a limitação de resultados ao se empregar protocolos convencionais de FIV bovina, reforçando a necessidade de abordagens específicas para a espécie em estudo.

Diante desse cenário, a utilização de estratégias complementares, como a FIV heteróloga, pode fornecer subsídios valiosos para o refinamento dos protocolos, permitindo a análise das interações entre gametas e das condições ideais para o desenvolvimento embrionário em cutia-vermelha. Portanto, ao otimizar as etapas da FIV para *D. leporina*, espera-se viabilizar sua aplicação prática com aumento da produção *in vitro* de embriões de qualidade, e contribuir significativamente para os esforços de conservação e manejo populacional da espécie, fortalecendo o papel das biotecnologias reprodutivas na manutenção da diversidade genética e sustentabilidade dos ecossistemas onde essa espécie exerce papel ecológico relevante.

4 HIPÓTESES CIENTÍFICAS

I – A centrifugação simples preserva melhor a integridade estrutural dos espermatozoides de *D. leporina* e resulta em uma maior taxa de desenvolvimento embrionário pós he-FIV em comparação aos métodos de gradiente de densidade e *swim-up*.

II – A capacitação *in vitro* de espermatozoides de *D. leporina* ocorre com maior eficiência quando incubados por apenas 1 h em meio MCM suplementado com alta concentração de BSA (15 mg/mL) e cloreto de cálcio (CaCl₂) .

III – O meio HTF promove maior eficiência na he-FIV com espermatozoides de *D. leporina*, resultando em um desenvolvimento embrionário superior em comparação aos meios TYF e TALP.

IV – A FIV homóloga e o meio KSOM promove um desenvolvimento *in vitro* mais eficiente de embriões de *D. leporina* em comparação à ativação oocitária artificial e o meio SOF.

V - A utilização de oócitos maturados *in vitro*, aliada às otimizações espermáticas previamente estabelecidas, favorece a formação de blastocistos durante o desenvolvimento *in vitro* na FIV homóloga de *D. leporina*.

5 OBJETIVOS

5.1 OBJETIVO GERAL

Estabelecer protocolos para a produção *in vitro* de embriões em *D. leporina*, por meio da comparação de diferentes estratégias de seleção e capacitação espermática, bem como da avaliação dos efeitos de distintos meios de fertilização e desenvolvimento embrionário sobre o desenvolvimento pré-implantacional.

5.2 OBJETIVOS ESPECÍFICOS

I – Avaliar o impacto dos métodos de seleção espermática (gradiente de densidade *vs.* *swim-up* *vs.* centrifugação simples) sobre a qualidade dos espermatozoides, considerando parâmetros funcionais, estruturais, taxa de fecundação e desenvolvimento embrionário utilizando um modelo de FIV heteróloga em *D. leporina*.

II – Analisar o efeito de diferentes concentrações de BSA (alta: 15 mg/mL *vs.* baixa: 4 mg/mL), presença ou ausência de cloreto de cálcio (CaCl₂) e a influência do tempo de incubação (1, 3 e 6 h) sobre parâmetros associados à funcionalidade, viabilidade e aquisição da capacitação espermática em *D. leporina*.

III – Comparar a eficiência do desenvolvimento embrionário durante a FIV heteróloga com oócitos de porquinho-da-índia e espermatozoides de *D. leporina* utilizando diferentes meios de co-incubação (TYH *vs.* HTF *vs.* TALP).

IV – Analisar a eficiência da FIV homóloga e da ativação oocitária artificial (AA), além do melhor meio de desenvolvimento embrionário (SOF *vs.* KSOM) a fim de otimizar a produção *in vitro* de embriões de *D. leporina*, por meio da avaliação da morfologia zigótica, cinética embrionária e níveis de estresse oxidativo.

V – Promover a produção *in vitro* de blastocistos em *D. leporina* por meio do aperfeiçoamento das etapas espermáticas estabelecidas nos objetivos específicos anteriores, utilizando a fertilização *in vitro* homóloga.

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**CAPÍTULO 2 - INSIGHTS AND IMPLICATIONS FROM A COMPARATIVE
ANALYSIS OF *IN VITRO* FERTILIZATION SUCCESS IN CAVIOIDEA AND
MUROIDEA RODENTS**

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Insights and implications from a comparative analysis of *in vitro* fertilization success in Caviioidea and Muroidea rodents

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Abstract

In vitro fertilization (IVF) is an assisted reproduction technique widely used due to its capacity to generate more offspring within a brief timeframe while simultaneously providing fundamental physiological data through gamete interactions. However, its suboptimal development in most species is challenging when IVF is applied to rodents. Despite numerous studies focusing on the Caviioidea and Muroidea superfamilies, some species struggle to achieve satisfactory cleavage rates, whereas others obtain over 90% efficiency with live offspring. Efforts to enhance these rates include adopting methodologies commonly used in other mammals or developing entirely new protocols. Nonetheless, the distinct morphophysiological differences among these animals necessitate careful consideration to avoid overgeneralization during optimization. Therefore, we aimed to review what has been established for the two most researched rodent superfamilies, Caviioidea and Muroidea, regarding the steps of IVF, such as gamete manipulation and embryonic development, to understand better the protocol

diversification in the rodent order and how it affects IVF efficiency. After an extensive analysis of data accumulated over the years, it becomes evident that the Muroidea superfamily is used more extensively in reproductive studies than the Cavoidea. Furthermore, IVF procedures achieve total efficiency in only one rodent species, the laboratory mouse (*Mus musculus*). Consequently, developing a successful IVF technique across rodent species requires substantial modifications to optimize species-specific early steps—a challenge complicated by difficulties in obtaining viable gametes during the initial stages and the limited research interest in species without significant commercial value, such as wild rodents.

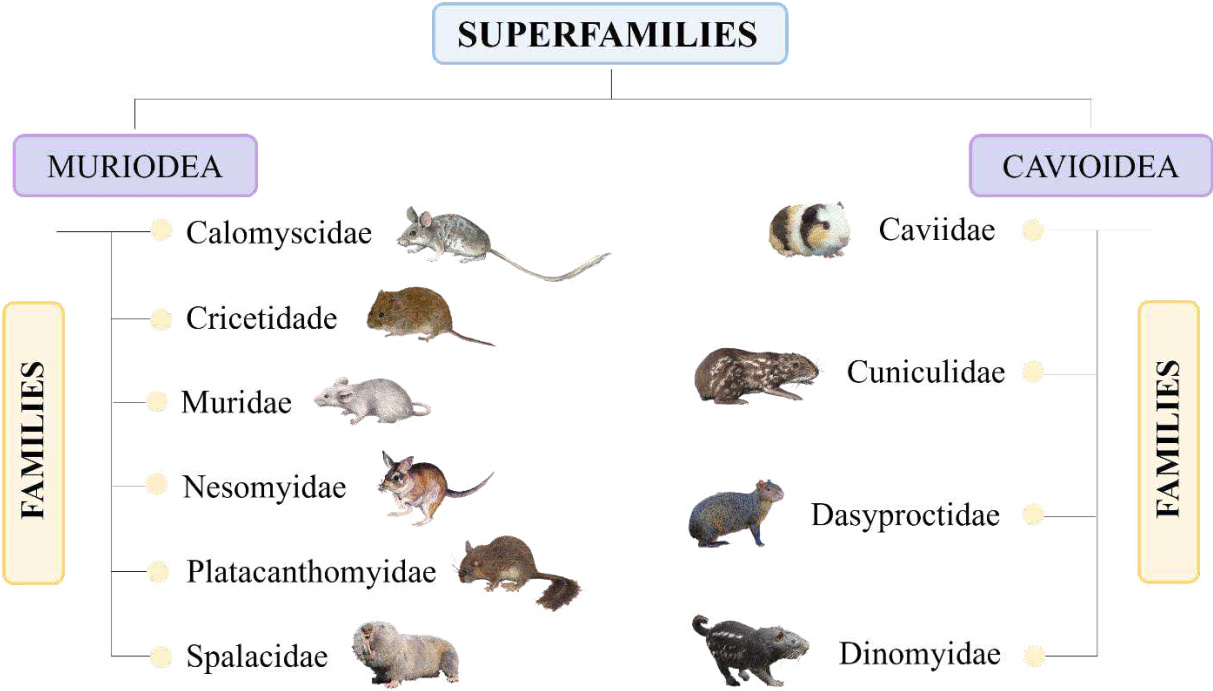
Keywords: Genetic diversity preservation, Assisted Reproductive Technologies, wild rodent conservation, *in vitro* embryo production, oocyte maturation and epididymal sperm capacitation

Introduction

In vitro fertilization (IVF) of rodents serves two primary goals: (i) elucidating the reproductive physiology of different species and (ii) increasing the number of offspring.¹ Initially, the study of physiology is possible by observing the behavior of these gametes in a controlled environment that emulates *in vivo* conditions, providing information from the interactions of these cells during co-incubation to the embryonic development phases up to the blastocyst stage.² Moreover, as a tool for reproductive enhancement in rodents, IVF is used to sustain populations in the laboratory, such as *Mus musculus*, and thus more easily meet the population numbers required for research using these animals.¹ Furthermore, it can be used to conserve endangered wild species, being an option to quickly increase the number of descendants.³

Most of the studies aimed at optimizing this biotechnique are concentrated in just two superfamilies: Cavoidea and Muroidea (**Figure 1**). This is because they represent the most

876 used species in laboratory studies, such as the laboratory mouse (Muroidea; *Mus musculus*), rat
877 (Muroidea; *Rattus norvegicus*), hamster (Muroidea; *Mesocricetus auratus*), and guinea pig
878 (Cavioidea; *Cavia porcellus*).⁴ The importance of these animals lies in their essential role in the
879 ecosystem, acting as seed dispersers and forming a crucial foundation for various food chains.⁵
880 They have also been extensively involved in scientific research for logistical reasons, such as
881 low feeding and maintenance costs, easy breeding, large litter sizes, and a short generation
882 turnover.⁶ However, even within these superfamilies, the small murids are the only species to
883 regard IVF as a routine and efficient technique.⁷



884
885 **Figure 1.** Representative image of the families that comprise the two large rodent superfamilies
886 (Cavioidea and Muroidea) included in the optimization of IVF protocols

887
888 These success gaps between the species may be linked to a long history of clashing approaches,
889 with authors either elucidating all the initial steps or using previously established protocols as
890 a basis. Horie et al.⁸ promoted an efficient protocol for prairie voles (*Microtus ochrogaster*),
891 where initially, superovulation and collection of matured oocytes in females was conducted,

followed by collection and capacitation of epididymal sperm, gamete incubation, and *in vitro* culture in different embryonic development medium. Approximately 32.6% blastocysts were obtained in this study, and two offspring were born after embryo transfer. In contrast, Ferraz et al.⁹ performed an IVF in black-rumped agouti (*Dasyprocta prymnolopha*) using a protocol for cattle, where immature oocytes were obtained, followed by *in vitro* maturation for 24 h, collection of epididymal sperm, gamete co-incubation for 15 h, and embryo development in a commercial embryo medium. The authors reported a 2.9% morula rate; no embryos or offspring were generated. Presumably, this phenomenon occurs due to the significant morphophysiological differences presented by these species that prevent the optimal application of fertilization among rodents of these superfamilies when combined with a low number of research for reproductive characteristics, such as follicular fluid composition, estrus duration and synchronization, gamete characteristics and the mechanisms necessary for their interaction, and dynamics for embryonic development.

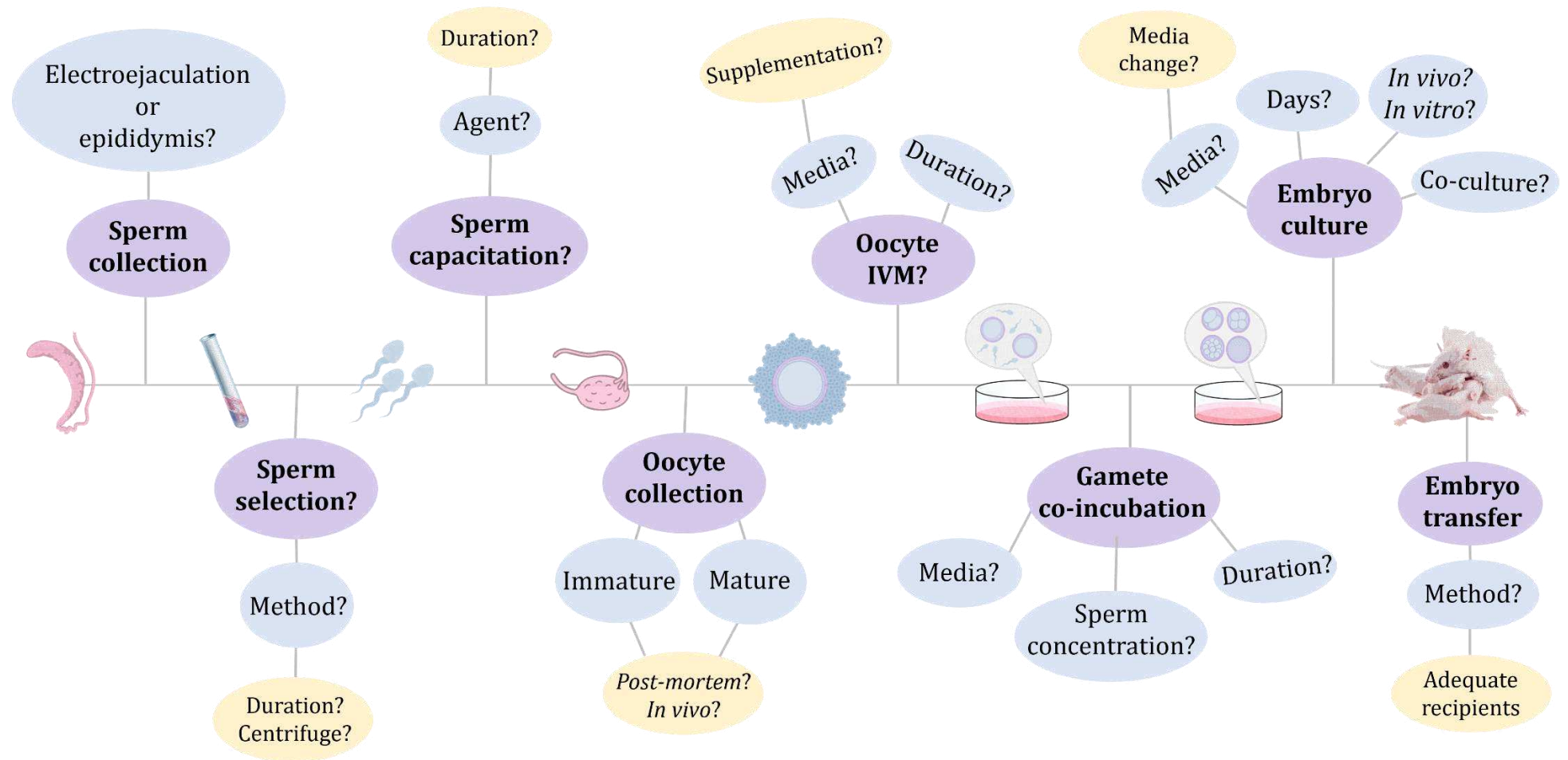
Therefore, this review aimed to discern the current phase of IVF optimization in rodents from the Caviioidea and Muroidea superfamilies. By scrutinizing the various stages of IVF, this review seeks to understand why this technique has not yet attained consistency among these animals, drawing on observations from studies conducted over the past several years.

Navigating the complexities of sperm preparation for IVF

Fundamentals of sperm collection and selection

The intricacies of instituting an effective IVF procedure in Caviioidea and Muroidea species (**Figure 2**) become apparent before sperm manipulation since there are disparities even in the methodologies employed for acquiring sperm cells. Different authors obtained varied results in both rodents' superfamilies when applying electroejaculation or epididymal sperm recovery.¹⁰

GAMETES TO OFFSPRING: VARIABLES SURROUNDING RODENTS IVF



917

918 **Figure 2.** Representative scheme highlighting the steps necessary to optimize an IVF protocol in rodents.

Although there are reports of success with electroejaculation in mice (*Mus musculus*), the technique is considered more difficult and expensive than obtaining cauda epididymal sperm.^{11,12} As a result, smaller species, such as Muroidea rodents, have their spermatozoa preferably collected after anesthesia/euthanasia, followed by recovery of the vasal-epididymal complex with associated testicles.¹³ There is a high success rate linked to this type of collection, with sperm motility above 80% in different species, such as the Cairo spiny mouse (*Acomys cahirinus*³), large Japanese field mouse (*Apodemus speciosus*¹⁴), Japanese grass vole (*Microtus montebelli*¹⁵), East European vole (*Microtus rossiaemeridionalis*¹⁵), and common vole (*Microtus arvalis*¹⁵).

In contrast, despite electroejaculation being the most advocated technique for spermatozoa recovery in Cavioidea rodents due to the larger size of the animals, efficiency has varied within the superfamily and even within the same species. Benavides et al.¹⁶ successfully performed electroejaculation in guinea pigs (*Cavia porcellus*) and obtained an ejaculate with 95% spermatozoa with a motility of 90.8%. On the other hand, with the first collections in red-rumped agouti (*Dasyprocta leporina*), the authors observed an efficiency of only 30–41% of ejaculates containing spermatozoa with 50% motility.^{17,18} Castelo et al.¹⁹ raised these numbers in *D. leporina* to 57% ejaculate-containing cells, with motility above 60%. Currently, there is still no proven efficiency using this method in *D. leporina*, as the recovered samples only presented half the volume and concentration compared to samples obtained from the epididymis by retrograde flushing, making the latter the method of choice for these animals.²⁰ The necessity of euthanizing these animals for sperm collection could be considered a significant limitation in procuring samples, contributing to the sluggish advancement of the IVF steps for these rodents.

In addition to optimal collection, it is necessary to examine fundamental characteristics of the cells, such as sperm morphology. This is particularly challenging because species within the Caviioidea and Muroidea superfamilies exhibit substantial differences in their sperm cells. For Caviioidea, among ten widely studied species, such as guinea pigs (*Cavia porcellus*), yellow-toothed cavies (*Galea musteloides*), chacoan mara (*Dolichotis salinicola*)²¹, and red-rumped agoutis (*Dasyprocta leporina*)¹⁷, we observed a predominance of oval and paddle-like heads, with no hooks, ranging from 5 to 14 μm . Their midpiece and tail length are noticeably smaller than Muroidea, ranging from 3 to 12 μm and 33 to 92 μm , respectively. In contrast, a study conducted with about 170 species from three families and ten subfamilies of Muroidea rodents showed predominantly flattened sperm heads with a long apical hook and a size that was generally smaller (3–6 μm) than Caviioidea. On the other hand, their midpiece (17–63 μm) and tail length (75–175 μm) were longer than the other rodent superfamily.^{22,23}

These small morphology discrepancies can be key aspects for IVF improvement, influencing the choice of sperm selection methods since morphological patterns are closely linked to the success of spermatozoa cells in the female tract *in vivo*.²⁴ For Muroidea rodents, the swim-up (SW) method seems to be the most efficient (**Table 1**), as seen by Banafshi et al.⁷ comparing two selection methods in mouse (*Mus musculus*) epididymal sperm, where half of the samples were selected by a density gradient (DG, 45/90%), followed by incubation for 15 min at 37 °C and centrifugation at 300×g for 10 min, twice. The other half were selected by the SW protocol, where the cells were kept tilted for 15 min at 37 °C and centrifuged at 300×g for 5 min. After selection, a motility rate of 76.5% for DG and 82.3% for SW was reported. Regarding IVF, the DG group obtained a rate of 59.8% of 2-cell cleavage and SW 70.0%, proving that the swim-up is the most suitable method for mice. However, there are variations for the SW method even within the murine, where some studies report longer times, as done in the Algerian mouse (*Mus*

969 **Table 1.** Sperm collection and selection methods applied in Muroidea and Caviioidea rodents for IVF

Superfamilies	Family	Species	Sperm collection	Selection methods	Selection duration	Centrifugation parameters	Motility after selection (%)	Authors
Muroidea	Muridae	<i>Mus musculus</i>	Cauda epididymis	Density gradient (45/90%)	*	300×g – 10 min (2x)	76.5	Banafshi ⁷
				Swim up	15 min	300×g – 5 min	82.3	
	Muridae	<i>Mus spretus</i>	Cauda epididymis	Swim-up	60 min	Without centrifugation	70.0	Hasegawa ²⁵
	Muridae	<i>Rattus norvegicus</i>	Cauda epididymis	Density gradient (40/80%)	15 min	1500×g – 15 min	ND	Safitri Purnobasuki ²⁶
						200×g – 20 min		
	Muridae	<i>Acomys cahirinus</i>	Cauda epididymis	Swim-up	30 min	Without centrifugation	90.0	McKenna ³
	Muridae	<i>Rattus norvegicus</i>	Cauda epididymis	Swim-up	15 min	500×g – 10 min	80.0	Agnihotri ²⁷

	Cricetidae	<i>Mesocricetus auratus</i>	Cauda epididymis	Swim-up	3 min	Without centrifugation	80.0	Hirose ²⁸
	Cricetidae	<i>Microtus ochrogaster</i>	Epididymis	Swim-up	30 min	Without centrifugation	50.0	Horie ⁸
	Cricetidae	<i>Peromyscus maniculatus</i>	Epididymis	Swim-up	10 min	Without centrifugation	ND	Veres ²⁹
Cavioidea	Dasyproctidae	<i>Dasyprocta prymnolopha</i>	Cauda epididymis	Centrifugation	*	200×g – 5 min	ND	Ferraz ⁹
	Caviidae	<i>Cavia porcellus</i>	Cauda epididymis	Density gradient (40/80%)	*	250×g – 10 min	77.8	Cañón-Beltrán ³⁰
	Caviidae	<i>Cavia porcellus</i>	Vas deferens	Centrifugation	*	600×g – 3 min (2×)	ND	Cañón-Beltrán ³¹
	Caviidae	<i>Cavia porcellus</i>	Cauda epididymis	Density gradient (35/85%)	*	600×g – 18 min 300×g – 10 min	50.0	Ni ³²

spretus), where the cells were in a controlled environment for 60 min and showed 70% motility²⁵, and species that required an extremely short time, such as the golden hamster (*Mesocricetus auratus*) that successfully recovered spermatozoa with 80% motility after 3 min of SW.²⁸

Although fewer studies conduct sperm selection in specimens from the Caviioidea superfamily, the methodologies that seem to stand out are simple centrifugation and DG (**Table 1**). These studies may suggest that spermatozoa with smaller midpiece and tail length require a higher separation force, as seen by Ni et al.³², who combined a DG (35/85%) protocol with two centrifugation regimes of 600×g and 300×g for extended periods (18 min) and recovered viable sperm with 50% motility in guinea pigs. Thus, while sperm retrieval and selection constitute one of the preliminary steps in optimizing IVF due to the presumed ease of material collection, it becomes evident that this assertion holds true only for specific species. Consequently, contemporary research requires more *in vivo* studies to elucidate one efficient way to handle these vital samples, ensuring technical progress.

The critical role of sperm capacitation

In different species, the next elucidated step for sperm is capacitation. This step is often skipped in Caviioidea and Muroidea rodents, as some authors claim that the selection process alone is enough to prepare these cells for fertilization.^{7,33} Meanwhile, some authors emphasize the importance of mimicking the female's environment to ensure greater similarity with events that occur *in vivo*, requiring the addition of bicarbonate (HCO_3^-) and its modulators, such as bovine serum albumin (BSA) and calcium chloride (CaCl_2).³⁴ These capacitating agents have already been used in both superfamilies, in different concentrations, and with variations in the time of exposure to the male gamete (**Table 2**).

Table 2. Sperm capacitation conditions applied in Muroidea and Caviioidea rodents for IVF

Superfamilies	Family	Species	Capacitation agent	Capacitation medium	Capacitation time	Capacitation evaluation	Capacitation rate (%)	Authors
Muroidea	Muridae	<i>Mus spretus</i>	0.11 mg/mL hypotaurine + 0.3% BSA	HTF medium	1 h	ND	ND	Hasegawa ²⁵
		<i>Mus musculus</i>						
		<i>Mus mattheyi</i>						
	Muridae	<i>Mus pahari</i>	ND	HTF medium	30 min.	ND	ND	Izquierdo-Rico ³⁵
		<i>Mastomys coucha</i>						
	Muridae	<i>Mus musculus</i>	25 mM HCO ₃ [−] + 3 mg/mL BSA	TYH medium	1:30 h	Acrosome reaction: FITC	35.0	Balbach ³⁶
	Muridae	<i>Mus musculus</i>	25 mM HCO ₃ [−] + 5 mg/mL BSA	TYH medium	3 h	ND	40.0	Hidalgo ³⁷
Muridae	<i>Mus musculus</i>							DelBarco-Trillo ³⁸
	<i>Mus spretus</i>							
	<i>Mus spicilegus</i>	15 mM HCO ₃ [−]	mT-H medium	1:30 h	ND	ND		

Muridae	<i>Mus musculus</i>		25 mM HCO ₃ [−]	mT-H medium	2:30 h	ND	ND	Martín-Coello ³⁹
	<i>Mus spretus</i>							
	<i>Mus spicilegus</i>							
Cricetidae	<i>Cricetulus griseus</i>		20 μM D-penicillamine + 100 μM hypotaurine + 1 μM epinephrine	mT-H medium	2 to 4 h	ND	ND	Tourmente ⁴⁰
	<i>Mesocricetus auratus</i>							
	<i>Phodopus campbelli</i>							
	<i>Phodopus sungorus</i>							
	<i>Phodopus roborovskii</i>							
Cricetidae	<i>Mesocricetus auratus</i>	3.4 mM Ca ²⁺ + 15 mg/mL BSA	TALP medium	6 h	Acrosome reaction: PCM	77.0	Hirose ²⁸	
Cavioidea	Caviidae	<i>Cavia porcellus</i>	10 mg/mL heparin + 6 mg/mL BSA	FERT-TALP	2 h	Acrosome reaction: FITC	28.4	Cañón-Beltrán ^{30,41}
	Caviidae	<i>Cavia porcellus</i>	1% BSA	HTF medium	2 h	ND	ND	Cañón-Beltrán ³¹

Caviidae	<i>Cavia porcellus</i>	1.71 mM CaCl_2	MCM medium	1 h	Acrosome reaction: CTC	55.0	Ramírez-Ramírez ⁴²
Caviidae	<i>Cavia porcellus</i>	1.71 mM CaCl_2	TALP medium	2 h	Acrosome reaction: CTC	80.0	Cordero- Martínez ⁴³
Caviidae	<i>Cavia porcellus</i>	1.71 mM CaCl_2	MCM medium	1:30 h	ND	34.2	Delgado- Buenrostro ⁴⁴
Caviidae	<i>Cavia porcellus</i>	2.0 mM Ca^{2+} + 25 mM HCO_3^-	MCM medium	6 h	Acrosome reaction: PCM	45.0	Chen ³⁴
Caviidae	<i>Cavia porcellus</i>	2.0 mM Ca^{2+} + 4 mg/mL BSA	MCM medium	6 h	Acrosome reaction: PCM	60.0	Ni ³²

997 ND: no data available. BSA: bovine serum albumin. HCO_3^- : sodium bicarbonate. HTF: human tubal fluid medium. TYH: Toyoda, Yokoyama,
998 Hoshi medium. mT-H: HEPES-buffered modified Tyrode's medium. TALP: Tyrode, albumin, lactate, and pyruvate medium. MCM: minimum
999 capacitation medium. FITC: Fluorescein isothiocyanate. PCM: phase-contrast microscopy. CTC: chlortetracycline staining.

Muroidea rodent *in vitro* capacitation seems to occur better in 25 mM bicarbonate. However, the addition of BSA appears to vary between species. In Algerian mice (*Mus spretus*), the authors report the need for only 0.3% BSA per 1 h to have a satisfactory capacitation rate for IVF.²⁵ Contrary to what was established by Hidalgo et al.³⁷, when promoting capacitation in mice (*Mus musculus*), a concentration of 5 mg/mL BSA was used in association with bicarbonate for a more extended period (3 h), achieving a capacitation rate of approximately 40%. Likewise, Hirose et al.²⁸ used a high profile of BSA (15 mg/mL) in association with 3.4 mM Ca^{2+} for an even longer capacitation window (6 h), obtaining excellent results in Syrian hamsters (*Mesocricetus auratus*), reaching rates above 70%.

The data for capacitation in Caviioidea species is slightly different. Studies in guinea pigs focus on adding CaCl_2 to the capacitation medium, combined with 25 mM sodium bicarbonate (HCO_3^-). Chen et al.³⁴ and Ni et al.³² emphasized in *Cavia porcellus* epididymal sperm that adding $\text{Cl}^-/\text{Ca}^{2+}$ was essential for capacitation and acrosome reaction. In their first experiment, they maintained a constant HCO_3^- concentration but removed the chloride (Cl^-), resulting in a 30% lower capacitation rate than the control group. In their second experiment, removing calcium (Ca^{2+}) from the medium promoted a 15% reduction in the rate of acrosomal reactions, demonstrating that it is essential to assess the impact of these factors present in the *in vivo* environment and add them during *in vitro* techniques. In addition, despite the similarity between protocols in this superfamily, there is still a lot of variation between the results adjusting only one parameter, where when using 1.71 mM CaCl_2 , different authors achieved rates of 34.2%⁴⁴, 55%⁴², and 80%⁴³ just increasing the sperm exposure periods in the capacitation medium of choice.

Notwithstanding recent advancements, considerable gaps persist in the comprehensive understanding of handling rodent spermatozoa. Research involving Caviioidea species is significantly underrepresented compared to the extensive work on sperm selection. Given that sperm preparation is a foundational stage of the process, the combination of deficient knowledge of sperm capacitation and inadequate selection methods can be one of the causes that prevent the increased effectiveness of IVF in these rodents.

Addressing obstacles in female gamete retrieval for IVF

The rodents of these two superfamilies show slight differences in their *cumulus*-oocyte complexes (COCs) morphology. Regarding the size, smaller species representing the superfamily Muroidea, such as mice (*Mus musculus*), have a COC size of around 65 μm ⁴⁵, while larger species of the superfamily Caviioidea, such as the black-rumped agouti (*Dasyprocta prymnolopha*) and red-rumped agouti (*Dasyprocta leporina*), have COCs of approximately 100 and 90 μm , respectively.^{9,46} Additionally, the definition of a viable COC may vary according to its characteristics. In contrast, in black-rumped agouti (*D. prymnolopha*), a compact *cumulus* structure containing more than three layers of cells and ooplasm with fine and homogeneous granulations filling the interior of the zona pellucida is preferable.⁴⁷ Still, for guinea pigs (*C. porcellus*), an ideal oocyte has granular cytoplasm and four or more layers of *cumulus* cells.⁴⁸ These specificities surrounding the female gamete can dictate how these structures will interact with the environment in which they are inserted, requiring different supplementations to reach maturation and how they will be recovered from the animals (**Table 3**).

1045 **Table 3.** Oocyte collection and *in vitro* maturation in Muroidea and Caviioidea rodents

Superfamilies	Family	Species	Collection method	Females (<i>n</i>)	Number of oocytes/females recovered	IVM duration	MII (%)	Authors
Muroidea	Muridae	<i>Rattus norvegicus</i>	Slicing	57	8 oocytes	30 h	ND	Oliveira ⁴⁹
	Muridae	<i>Rattus norvegicus</i>	Slicing	74	7 oocytes	30 h	55.7	Paim ⁵⁰
	Muridae	<i>Rattus norvegicus</i>	Slicing	72	5 oocytes	24 h	36.6	Alcoba ⁵¹
	Muridae	<i>Mus musculus</i>	Follicle aspiration	3	25 oocytes	17 h	93.0	Martín-Coello ³⁹
		<i>Mus spretus</i>		3	20 oocytes		86.0	
		<i>Mus spicilegus</i>		6	11 oocytes		91.0	
Caviioidea	Caviidae	<i>Cavia porcellus</i>	Follicle aspiration	110	15 oocytes	17 h	78.3	Cañón-Beltrán ⁴¹
	Caviidae	<i>Cavia porcellus</i>	Slicing	48	14 oocytes	24 h	52.5	Yao ⁵²

Caviidae	<i>Cavia porcellus</i>	Follicle aspiration	ND	564 oocytes*	17 h	75.9	Cañón-Beltrán ⁵³
Cuniculidae	<i>Cuniculus paca</i>	LOPU	8	45 oocytes	24 h	54.1	Barros ⁵⁴
Dasyproctidae	<i>Dasyprocta prymnolopha</i>	Slicing	17	17 oocytes	ND	ND	Ferraz ⁴⁷
Dasyproctidae	<i>Dasyprocta prymnolopha</i>	Slicing	17	17 oocytes	24 h	27.4	Ferraz ⁹
Dasyproctidae	<i>Dasyprocta leporina</i>	Slicing	21	18 oocytes	24 h	52.1	Praxedes ⁴⁶

1046 ND: no data available. LOPU: Laparoscopic ovum-pick up. *Total oocyte recovery without the number of females available for proportion.

Most of the research done for the COC recovery from the Muroidea superfamily is with the laboratory mouse (*Mus musculus*). In this species, the oocytes are recovered already matured, with well-established protocols that promote superovulation with intraperitoneal injection of gonadotrophin to secure more samples from these small rodents.⁵⁵ However, this is not a reality for wild rodents or even less studied laboratory species, such as hamsters (*Mesocricetus*), as few studies have shed light on understanding the hormonal cycles of these females and how to increase this sample gain efficiently. Significant progress in this field has only begun recently, as demonstrated by the research of Yao et al.⁵², who, using a protocol based on mice with human menopausal gonadotrophin subcutaneously injected into the back of the guinea pigs' necks, obtained an average of six oocytes per female, where the non-super stimulated group produced an average of only two oocytes per guinea pig. Therefore, in light of these challenges, concerted efforts appear to have been directed within both superfamilies to secure COCs, even in their immature states, to enhance the efficacy of their *in vitro* maturation (IVM).

Why does IVM optimization fall short of enhancing IVF efficiency?

The primary stage in developing an optimal IVM methodology for diverse rodent species involves acquiring viable immature oocytes, which can quickly become the first major difficulty. Collection can be performed *postmortem* when working with a large number of laboratory animals, such as mice (*Mus musculus*), with a standard process after cervical dislocation, where the ovaries can be excised, and immature oocytes for the IVM can be obtained by gently teasing the cortical region of the ovaries.⁵⁶ On the other hand, the optimal approach would entail *in vivo* recovery in cases where the animal population is limited, whether wild-strained murines or wild caviids. This *in vivo* methodology ensures the survival of the egg donors, allowing multiple collections over the lifespan of a single animal.⁵⁷

Only one has successfully implemented laparoscopic ovum pick-up among those who have optimized IVM steps to establish IVF in their rodent species. Barros et al.⁵⁷ described a successful oocyte recovery in the lowland paca (*Cuniculus paca*), where eight adult females were anesthetized (25 mg/kg ketamine and 0.5 mg/kg midazolam) and had their follicles (1 mm) aspirated with an 18-gauge needle attached to a vacuum system with pressure not exceeding 65 mmHg. They were successful in 27 procedures, where 445 follicles were observed (14 ± 2.23 per animal) and 366 were aspirated (11.44 ± 1.82 per animal). One hundred and forty oocytes were recovered, with a mean of 4.38 ± 0.86 oocytes per paca, with an overall oocyte recovery rate being 38.25%. Meanwhile, the recovery of postmortem immature oocytes is still the method of choice (**Table 3**) for other wild rodents such as the black-rumped agouti (*Dasyprocta prymnolopha*), red-rumped agouti (*Dasyprocta leporina*), Algerian mice (*Mus spretus*), and spiny mouse (*Acomys cahirinus*), significantly limiting the experimental samples of these authors, which may delay the progress in optimizing their IVF protocols.^{39,9,3,46}

Between Caviioidea and Muroidea rodents, the former, despite being advanced in all other stages of IVF, do not present more significant updates for IVM optimization. This can be explained by the practicality of obtaining matured oocytes through broad functional protocols, as mentioned previously.⁵⁸ Nevertheless, we can observe varied results, whether very successful, as seen by Martín-Coello et al.³⁹, when promoting IVM for 24 h in three different species of murines (*Mus musculus*, *Mus spretus*, and *Mus spicilegus*), obtained metaphase II (MII) rates ranging from 86–93% (**Table 3**). As well as gradual increases in the percentage of success, as was seen with rats (*Rattus norvegicus*), which initially used a protocol with only 24 h of IVM, the authors achieved MII rates of only 36.6%.⁵¹ But in 2015, Paim et al.⁵⁰ increased the IVM time of rats to 30 h while maintaining the same supplements in the medium, observing a mature oocyte rate of 55.7%, an increase of almost 20% in a few years (**Figure 3**).

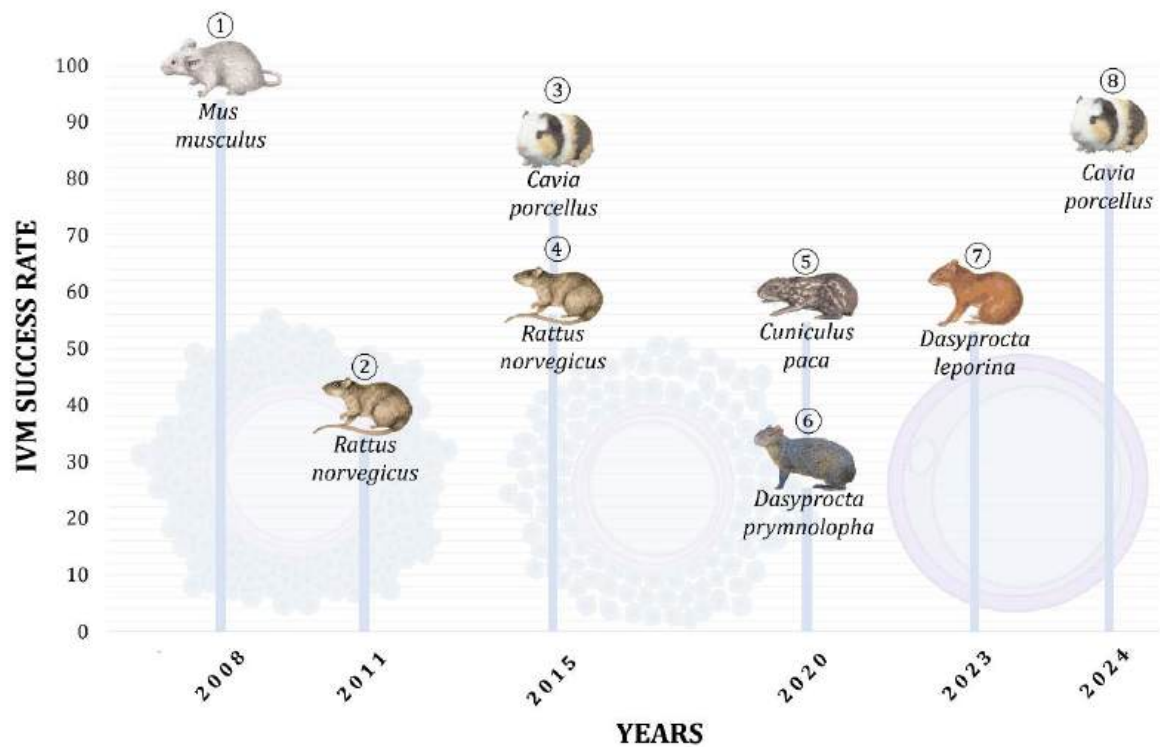


Figure 3. Representative graph demonstrates the efficiency of the oocyte *in vitro* maturation in different species of Muroidea and Cavoidea rodents over the years. **1)** Martín-Coello et al.³⁹; **2)** Alcoba et al.⁵¹; **3)** Cañón-Beltrán et al.⁵³; **4)** Paim et al.⁵⁰; **5)** Barros et al.⁵⁴; **6)** Ferraz et al.⁹; **7)** Praxedes et al.⁴⁶; **8)** Cañón-Beltrán et al.⁴¹.

This gradual efficiency increase phenomenon can be observed in Cavoidea species (**Figure 3**). In guinea pigs (*Cavia porcellus*), the authors consolidated the methodology by applying a 17-h IVM, where 75.9%⁵³ turned into 78.3% of matured COCs⁴¹, emphasizing the success of the protocol. In the genus *Dasyprocta*, the efficiency of *in vitro* maturation increased by almost 30%, as the first results in *D. prymnolopha* were only 27.4% MII⁹, and in *D. leporina* was improved to 52.1% of oocytes on the MII nuclear stage.⁴⁶ However, these outcomes can be explained by the variances in protocol applications, such as incorporating different supplements in the media for each species and the different IVM durations.

The first example can be seen in the IVM outcomes of guinea pig, where despite Cañon-Beltran et al.⁴¹ having obtained a rate above 78% MII, Yao et al.⁵², with the same species managed to get only 52.5% of the oocytes to mature *in vitro*, but with a more extended IVM protocol, reaching 24 h. Thus, knowing the ideal time the gamete would take *in vivo* to complete its maturation can enhance the reproductive biotechnologies of interest. Accounting for temporal aspects, some authors design their experiments with this aim. Three different times of oocyte maturation were tested (16 vs. 20 vs. 24 h), as in the IVM of black-rumped agouti (*Dasyprocta prymnolopha*), defining the best time for the species as 24 h.⁹ Due to this previous work in the genus *Dasyprocta*, Praxedes et al.⁴⁶ optimized different parameters, such as the influence of various concentrations of follicle-stimulating hormone (10, 50, or 75 mIU/mL, FSH) and the synergistic effects of adding an epidermal growth factor (10, 50, or 100 ng/mL, EGF) in the maturation medium, concluding that the combination of 10 mIU/mL FSH and 10 ng/mL EGF for 24 h is ideal for red-rumped agouti oocytes.

Therefore, although *in vitro* oocyte maturation represents a crucial step in understanding the behavior of female gametes *in vitro* and serves as an alternative to using fully mature and fertilization-ready cells, this technique still exhibits incredibly variable efficiency among these rodents, with rates far from the ideal, unlike the consistent success seen in *Mus musculus*. These factors and the challenges in obtaining sufficient samples to increase the number of studies hinder the technique from reaching its full potential in optimizing IVF protocols for rodents within the Caviioidea and Muroidea superfamilies.

The true state of rodent IVF: diverse efficiency and limited success

Initially, the sperm concentration to be added to the IVF droplet is one of the most critical factors, as excess cells can induce oxidative stress and polyspermy.^{59,60} There is no established

standard for Muroidea species (**Table 4**), leading to a wide variation in the concentrations used. For instance, for Algerian mice (*Mus spretus*), authors used a low concentration of 0.032×10^6 (or 32,000 spz/mL) during co-incubation with mature oocytes, achieving approximately a 15% cleavage rate and a 10% efficiency in birth rate post-embryo transfer.²⁵ Conversely, a higher concentration of 1.0×10^6 (or 1,000,000 spz/mL) was used in the IVF of the Japanese grass vole (*Microtus montebelli*), resulting in a 2.7% blastocyst formation rate and a birth efficiency rate of 36.7% following embryo transfer.⁶⁵

A significant disparity in sperm concentrations is evident among the three studies that successfully established IVF protocols in Caviodea rodents. Ferraz et al.⁹ adapted a bovine protocol for *D. prymnolopha*, employing a 25×10^6 spz/mL concentration with IVM oocytes, yielding a low cleavage rate of 2.9%. In contrast, Cañón-Beltrán et al.^{31,41} adapted mouse protocols for application in guinea pigs (*C. porcellus*), opting for a much lower concentration of 1.0×10^6 spz/mL in the IVF droplet with mature oocytes. This approach resulted in cleavage rates exceeding 80% for two-cell embryos and, subsequently, a 29.5% rate of morula formation.

Another critical factor to consider is co-incubation duration during fertilization, as extended exposure can induce oxidative stress in the *in vitro* environment.⁶⁶ The Muroidea superfamily exhibits one wild range of appropriate co-incubation times, ranging from 3 to 8 h. The optimal duration for these rodents appears to be around 6 h, as evidenced by Meguro et al.¹⁴ in their study on the large Japanese field mouse (*Apodemus speciosus*). They used a standard concentration of 1.0×10^6 spz/mL with previously matured oocytes for a 6-h co-incubation period, yielding a 29.4% rate of 4-cell stage embryos after *in vitro* development. Longer durations are observed in earlier studies, such as in the southern African mastomys (*Mastomys coucha*) in 1998, where a lower sperm concentration of 0.1×10^6 spz/mL was used over an 8

1162 **Table 4.** Different conditions of *in vitro* fertilization and embryo production in Muroidea and Cavoidea rodents

Superfamilies	Family	Species	Sperm concentration (sptz/mL)	Co-incubation medium	IVF duration	IVC medium	Efficiency rate (%)	Live birth (% efficiency)	Authors
Muroidea	Muridae	<i>Mus musculus</i>	0.67×10^6	TALP	5 h	TALP	67.3 (2 cells)	No	Suzuki; Fujinoki ⁶¹
	Muridae	<i>Mus musculus</i>	*	HTF	5 h	KSOM-AA	70.0 (2 cells)	Yes – (20)	Banafshi ⁷
	Muridae	<i>Mus spretus</i>	0.032×10^6	HTF	4 h	CZB	15.2 (2 cells)	Yes – (10)	Hasegawa ²⁵
		<i>Mus musculus</i>					81.2 (2 cells)		
	Muridae	<i>Mus mattheyi</i>	1.0×10^6	HTF	5 h	KSOM-AA	0.0 (2 cells)	No	Izquierdo-Rico ³⁵
		<i>Mus pahari</i>					3.5 (2 cells)		
		<i>Mastomys coucha</i>					0.8 (2 cells)		
	Muridae	<i>Rattus norvegicus</i>	*	M16	5 h	M16	86.0 (Bl)	No	Safitri; Purnobasuki ²⁶
	Muridae	<i>Acomys cahirinus</i>	0.25×10^6	MT6	3 h	MT6	*	Yes – (*)	McKenna ³

								Meguro ¹⁴
Muridae	<i>Apodemus speciosus</i>	1.0×10^6	HTF	6 h	KSOM	29.4 (4 cells)	No	
	<i>Mus musculus molossinus</i>					97.4 (2 cells)	Yes – (100)	
	<i>Mus musculus musculus</i>					97.4 (2 cells)	Yes – (83)	
Muridae	<i>Mus musculus domesticus</i>	0.5×10^6	HTF	5 h	CZB	96.3 (2 cells)	Yes – (83)	Mochida ¹
	<i>Mus musculus castaneus</i>					84.6 (2 cells)	Yes – (75)	
Muridae	<i>Mus spicilegus</i>	1.0×10^6	HTF	4 h	HTF	87.0 (2 cells)	No	Martín-Coello ³⁹
Muridae	<i>Mastomys coucha</i>	0.1×10^6	HTF	8 h	*	43.7 (2 cells)	No	Nohara ⁶²
Cricetidae	<i>Mesocricetus auratus</i>	0.022×10^6	TALP	6 h	*	*	No	Hirose ²⁸
Cricetidae	<i>Microtus ochrogaster</i>	*	HTF	5 h	G-1 PLUS	32.6 (Bl)	Yes – (50)	Horie ⁸

	Cricetidae	<i>Microtus fortis</i>	0.8×10^6	HTF	6 h	HTF	93.0 (iB)	No	Liu ⁶³
	Cricetidae	<i>Calomys musculus</i>	1.0×10^6	M2	4 h	*	65.3 (2 cells)	No	Lasserre ⁶⁴
		<i>Calomys laucha</i>					74.6 (2 cells)		
	Cricetidae	<i>Microtus montebell</i>	1.0×10^6	M2	6 h	*	2.7 (Bl [#])	Yes – (36.7)	Wakayama ⁶⁵
Cavioidea	Caviidae	<i>Cavia porcellus</i>	1.0×10^6	TM	18-22 h	SOF	29.5 (morula)	No	Cañón-Beltrán ⁴¹
	Caviidae	<i>Cavia porcellus</i>	1.0×10^6	HTF	5 h	KSOM	81.4 (2 cells)	No	Cañón-Beltrán ³¹
	Dasyproctidae	<i>Dasyprocta prymnolopha</i>	25×10^6	*	15 h	SOF	2.9 (morula)	No	Ferraz ⁹

1163 * no data available. #: *in vivo* development. IVC: *in vitro* culture of embryos. HTF: human tubal fluid medium. TM: Tyrode's medium. MT6:
 1164 Modified Tyrode's medium. M16: EmbryoMax modified M16 medium. TALP: Tyrode, albumin, lactate, and pyruvate medium. M2: modified
 1165 Krebs-Ringer bicarbonate solution. KSOM: optimized simplex medium with potassium. KSOM-AA: optimized simplex medium with potassium
 1166 and amino acids. CZB: glucose-free and serum-free medium. G-1 PLUS: commercial medium for the culture of human embryos. SOF: synthetic
 1167 oviductal fluid. Bl: blastocyst. iB: initial blastocyst

hour window, resulting in a cleavage rate exceeding 43%.⁶² A similar disparity in co-incubation times is evident for Caviioidea species, as seen in sperm concentration choices. Using a cattle-adapted protocol, Ferraz et al.⁹ opted for a longer IVF duration of 15 h, whereas Cañón-Beltrán et al.^{31,41} chose a shorter co-incubation period of 5 h.

A third parameter that influences the success of the gamete's co-incubation *in vitro* is the choice of the appropriate medium. Due to their varied compositions, they can perform different functions in different species, as they differ in their energy, protein, and ionic sources.⁶⁷ For Caviioidea rodents, the medium used was the Human Tubal Fluid (HTF) in guinea pigs by Cañón-Beltrán et al.³¹, where the authors reported an 81.4% cleavage success rate after IVF. Likewise, for Muroidea rodents, most studies use HTF in different rodents, such as *Mus musculus*⁷, *Apodemus speciosus*¹⁴, and *Microtus ochrogaster*⁸, obtaining efficiency rates ranging from 29.4 to 70.0%.

From these observations, we can conclude that the IVF in these two rodents' superfamilies is far from being considered efficient for everyone. In murines, of the fifteen works presented during this review, six species (*Calomys laucha*, *Microtus fortis*, *Microtus ochrogaster*, *Mus musculus*, *Mus spicilegus*, and *Rattus norvegicus*) showed cleavage rates above 70% after embryonic development. Only four species (*Microtus fortis*, *Microtus montebell*, *Microtus ochrogaster*, and *Rattus norvegicus*) showed blastocyst formation. Even within the genus *Mus*, under identical IVF conditions, Izquierdo-Rico et al.³⁵ noted disparities in embryonic development rates, spanning from 81.2% for 2-cell cleavage in *Mus musculus* to 3.5% and 0% in *Mus pahari* and *Mus matthevi*, respectively. Additionally, among the protocols used, only 5/15 species (*Acomys cahirinus*, *Mus musculus*, *Mus spretus*, *Microtus ochrogaster*, and *Microtus montebell*) had offspring after embryo transfer. However, within Caviioidea rodents,

the generation of blastocysts has not been documented to date, exhibiting a halt in development after morula formation, and no offspring have been produced thus far.

In summary, establishing a successful IVF technique in rodents requires significant modifications to optimize several species-specific initial steps. It is imperative not to rely on exact replicas of protocols designed for other mammals, even those within the same families or genus as the study species.

Conclusions

Despite recent advances in increasing the efficiency of the initial stages of IVF, as the best method for sperm collection, selection, and capacitation, followed by oocyte collection and *in vitro* maturation, and the best conditions for gamete co-incubation and embryo culture, there is still a long way to go to guarantee high efficiency for Muroidea and Cavoidea rodents. One of the primary reasons for the limited advancement in these methodologies is the lack of studies dedicated to elucidating the protocols involved. This is primarily due to the challenges encountered during the initial stages of collecting viable male and female gametes. Additionally, the low interest in investigating species that do not offer significant commercial returns, such as wild rodents, further impedes the progress of these reproductive biotechniques.

Moreover, this review highlights the discrepancy in the number of species studied between the two superfamilies. In Muroidea, more species (20) are explored for reproductive studies compared to Cavoidea (4). However, even within the Muroidea superfamily, research is concentrated on only two families (Muridae and Cricetidae). Thus, the notion that fertilization is a common and optimized technique for rodents holds true only for the laboratory mouse (*Mus musculus*).

1218

1219 In conclusion, for IVF to be regarded as a viable and promising option for these rodents, it is
 1220 imperative to prioritize morphophysiological studies and advance research aimed at
 1221 understanding their *in vivo* environment. This understanding is essential for effectively
 1222 translating *in vivo* conditions to the *in vitro* environment, thereby ensuring species-specific
 1223 success for each animal of interest, whether the goal is conservation or commercial
 1224 reproduction.

1225

1226 **Conflicts of interest**

1227 The authors declared no potential conflicts of interest with respect to the research, authorship,
 1228 and/or publication of this article.

1229

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**CAPÍTULO 3 - COMPARATIVE ANALYSIS OF SPERM SELECTION METHODS
AND THEIR IMPACT ON *IN VITRO* FERTILITY IN THE RED-RUMPED AGOUTI
(*Dasyprocta leporina*)**

ARTIGO EXPERIMENTAL 1

SUBMETIDO NA RESEARCH IN VETERINARY SCIENCE

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Comparative analysis of sperm selection methods and their impact on *in vitro* fertility in the red-rumped agouti (*Dasyprocta leporina*)

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Highlights

- The centrifugation method achieved the highest progressive motility.
- Centrifugation maintained plasma membrane integrity and mitochondrial activity.
- ROS levels were lower and GSH levels higher in centrifugation, showing a strong correlation.
- He-IVF produced cleaved zygotes and morulae.
- Fertilization rates were similar across all sperm selection methods.

Abstract

Despite advances in sperm manipulation techniques for the red-rumped agouti, methodologies for selecting these cells before *in vitro* fertilization (IVF) remain insufficiently explored. This study evaluated the effects of various sperm selection methods on morphological and functional parameters, oxidative stress status, and fertilization potential using heterologous oocytes (he-IVF). Epididymal spermatozoa were recovered by retrograde flushing and processed using four distinct protocols: without selection (WS group), centrifugation (CT group), density gradient (DG group), and swim-up (SP group). Sperm recovery was significantly lower following selection compared to the WS group ($P < 0.05$). The CT method yielded the highest progressive motility (64.6%), surpassing WS (32.2%), DG (47.0%), and SP (48.4%) ($P < 0.05$). Regarding membrane functionality maintenance, CT (85.9%) was comparable to WS (90.7%) and superior to the other groups ($P < 0.05$). Furthermore, the CT protocol maintained a greater proportion of cells with intact plasma membranes and normal mitochondrial activity ($P < 0.05$). A strong ROS/GSH correlation was observed in the CT group, with significantly reduced ROS levels and increased GSH, outperforming the moderate correlations seen in DG and SP ($P < 0.05$). Lastly, he-IVF resulted in cleaved embryos and morulae, though fertilization rates did not differ between groups ($P > 0.05$). In conclusion, centrifugation was the most effective method for maintaining sperm functionality and oxidative balance, while all selection protocols-maintained sperm fertilization capacity. This study enhances our understanding of oocyte-sperm interactions in the red-rumped agouti, contributing to advancing assisted reproduction techniques.

Keywords: wildlife fertility; assisted reproduction; male germplasm; sperm preparation; heterologous oocytes.

1. Introduction

The red-rumped agouti (*Dasyprocta leporina* Linnaeus, 1758) is a wild rodent from the superfamily Cavoidea (Deutsch and Puglia, 1988). Although this species is widely distributed across the American continent and plays a crucial ecological role as an effective seed disperser, its populations are significantly declining due to habitat fragmentation and hunting pressures (Mittelman et al., 2021). Faced with this problem, germplasm investigation has become an interesting option for developing conservation strategies and their broader implications in reproductive science (Oliveira and Pereira, 2025). Given their phylogenetic proximity to other endangered *Dasyprocta* rodents, these studies in red-rumped agouti can provide valuable insights for preserving biodiversity. Additionally, a better understanding of their reproductive physiology may contribute to optimizing Assisted Reproductive Technologies (ARTs) with potential applications beyond wildlife conservation, including human medicine (Sharpe, 2018).

Currently, research on this species has predominantly focused on spermatozoa, encompassing various aspects, such as sperm recovery and evaluation (Mollineau et al., 2008; Castelo et al., 2015), cryopreservation of epididymal spermatozoa (Castelo et al., 2015), characterization of morphological and functional changes in sperm during epididymal transit (Dantas et al., 2022^{ab}), and the effects of weather changes on sperm characteristics (Dantas et al., 2024). Nevertheless, the handling of these cells remains suboptimal, with inconsistent efficiency observed even during electroejaculation (Castelo et al., 2015). As a result, the use of epididymal sperm is still required in contemporary studies and practices (Dantas et al., 2024).

In this context, some preparation is necessary before using recovered epididymal sperm samples for *in vitro* fertilization (IVF) in ART optimization studies. Spermatozoa are often subjected to various physical manipulations, such as pipetting, mixing, and centrifugation (Varisli et al.,

2009), during the selection protocols for IVF. In the case of the red-rumped agouti, previous studies have shown that rodent spermatozoa are particularly susceptible to mechanical stress; therefore, these protocols can inflict significant damage on sperm cells (Varisli et al., 2009; Kim et al., 2013). Nevertheless, implementing these sperm selection protocols is essential, as epididymal samples are inevitably contaminated with epithelial cells, red blood cells, leukocytes, bacteria, and cellular debris, all of which can adversely affect sperm viability, motility, osmotic balance, and enzymatic activity, while also promoting the production of reactive oxygen species (ROS) (Chatdarong et al., 2010).

Therefore, applying simpler and faster selection methods could be the key to maintaining the viability of these cells, thereby safeguarding their fertility potential following processing. Among the protocols most used in laboratory rodents, density gradient selection with Percoll[®] (DG) efficiently removes the non-viable sperm fraction (Ni et al., 2009). However, the Percoll[®] can adhere to the sperm membranes, requiring subsequent intensive washing, which can harm the spermatozoa (Am-In et al., 2013). Another preparation technique is swim-up (SP), which separates motile spermatozoa capable of moving away from the bottom of the sample (Banafshi et al., 2021). It is simple and economical, but the sperm recovery rate is significantly lower than other methods (Navarro-Serna et al., 2021). Centrifugation (CT) is one of the most used methods for rats, mice, and guinea pigs (Kim et al., 2013; Cañón-Beltrán et al., 2023). Its combination of low centrifugal forces with short processing time makes it one of the most efficient for separating viable populations of epididymal sperm and concentrating samples (Cañón-Beltrán et al., 2021). However, the impact of the centrifugation method can influence IVF outcomes across different rodent species. Cañón-Beltrán et al., 2021 reported high cleavage rates exceeding 81% following centrifugation in guinea pigs. In contrast, in black-rumped agoutis (*Dasyprocta prymnolopha*), Ferraz et al. (2020) achieved a post-IVF development rate

of only 8.6%, highlighting potential species-specific differences in response to this sperm selection method.

Furthermore, there is a significant gap in understanding how these interventions affect epididymal spermatozoa from red-rumped agouti. Subtle variations in sperm morphology have been observed among the extensively studied species within the Caviioidea superfamily, which may influence the selection of appropriate sperm preparation methods. These morphological characteristics are intricately associated with the fertilization potential of spermatozoa *in vivo*, highlighting their importance in developing effective ARTs (Sanchez et al., 2013). It is crucial to evaluate post-selection sperm regarding various morphological and functional parameters, including motility, changes in mitochondrial activity and both membrane and DNA damages, oxidative status, and fertilizing capacity of these gametes, since a higher oocyte interaction and ability to generate embryos would be essential for ARTs. Therefore, this study aimed to evaluate the influence of different sperm selection protocols for red-rumped agouti spermatozoa by analyzing sperm morpho-functional parameters, oxidative stress, and sperm ability to fertilize guinea pig oocytes using heterologous *in vitro* fertilization (he-IVF).

2. Material and methods

The chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except those mentioned otherwise. .

2.1. Animals and sperm recovery

The red-rumped agoutis and guinea pigs were kept at the Center of Multiplication of Wild Animals (CEMAS/UFERSA). They were fed commercial rabbit feed, fresh drinking water was available, and a 12-h natural photoperiod was used.

Six sexually mature male red-rumped agouti specimens (one per replicate) were captured and administered premedication consisting of ketamine (15 mg/kg; Ketalar, Pfizer, São Paulo, Brazil) combined with xylazine (1 mg/kg; Rompun, Bayer, São Paulo, Brazil). After 15 min, anesthesia was induced via intramuscular administration of sodium thiopental (50 mg/kg; Thiopentax, Cristalia, São Paulo, Brazil). Euthanasia was subsequently conducted through intravenous administration of potassium chloride (1 mL/kg; Equiplex, Goiânia, Goiás, Brazil) following established protocols (Castelo et al., 2015).

The testes-epididymis paired complex was recovered (Figure 1-A) and transported to the laboratory in heated saline solution (37 °C, NaCl 0.15 M). The caudal region was excised, and the epididymal spermatozoa were obtained by retrograde flushing with 1.0 mL of saline solution (Castelo et al., 2015). The recovered fluid was kept in a water bath (37 °C) to be used immediately afterward.

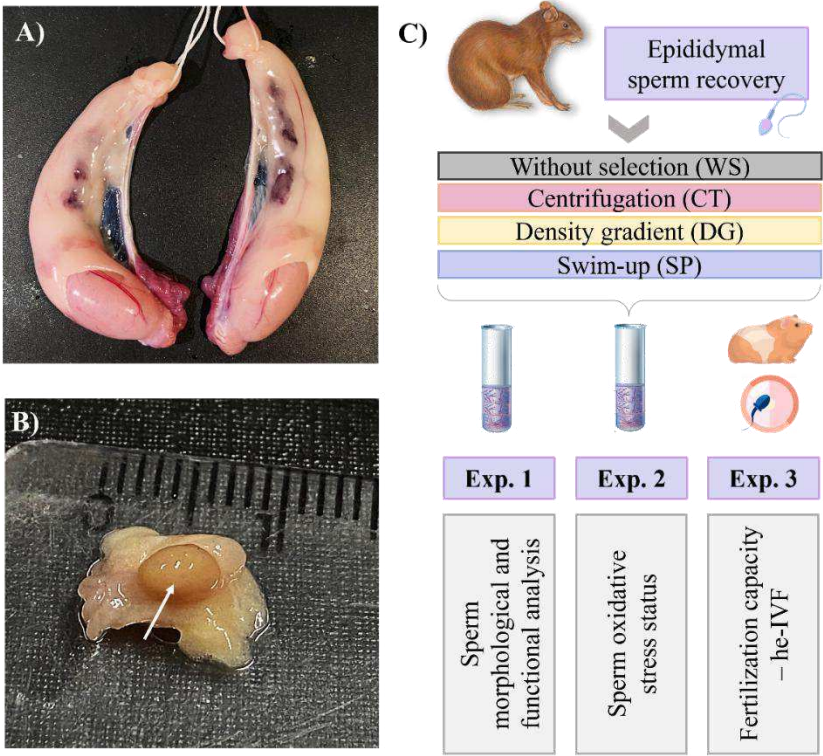


Figure 1. Sperm selection and *in vitro* fertilization assessment in red-rumped agouti. **(A)** Testes-epididymis-vas deferens complex recovered from sexually adult red-rumped agouti for epididymal sperm recovery. **(B)** Representative micrography of a guinea pig ovary with antral follicles (white arrow). **(C)** Experimental design: for effects of the different protocols for red-rumped agouti sperm selection through sperm analysis and he-IVF with guinea pig oocytes.

2.2. Experimental design

To evaluate the most efficient spermatozoa separation procedure for red-rumped agoutis, epididymal spermatozoa were processed by different methods: centrifugation (CT), density gradient (DG), and swim-up (SP). After selection, the samples were subjected to three experiments to assess sperm morphology and functionality, oxidative stress status, and fertilization capacity with heterologous IVF (he-IVF) using guinea pig oocytes (Figure 1-B). The control group without selection (WS) was immediately evaluated after collection (Figure 1-C).

2.3. Spermatozoa selection procedures

2.3.1. Centrifugation

Spermatozoa samples were diluted to a concentration of 100×10^6 sperm/mL in minimum maintenance medium (MMM, 105.8 mM NaCl, 25 mM NaHCO₃, 5.56 mM glucose, 21.6 mM Na lactate, 25 mM HEPES, 0.25 mM sodium pyruvate, 10 µg/mL phenol red, and 1% antibiotic antimycotic solution). For the separation, a 1:1 solution with spermatozoa and MMM was placed in a 15 mL plastic tube and centrifuged twice (300× g for 3 min, room temperature). The supernatant was discarded, and the cells were suspended in 200 µL MMM for analysis or he-IVF (Cañón-Beltrán et al., 2021).

2.3.2. *Density gradient 35-85%*

A Percoll[®] gradient density protocol of 35-85% was used as suggested for rodents by Ni et al. (2009). In a 1.5 mL conical microtube, 500 μ L of 85% Percoll[®] and 35% Percoll[®] was carefully dispensed on top of each other to avoid mixing. The microtube was equilibrated at 38.5 °C under 6.5% CO₂ for 2 h. Diluted spermatozoa were layered over the gradient and centrifuged at 300× g for 15 min (37 °C). The supernatant was discarded, and the pellet was resuspended in 500 μ L MMM medium, which was subsequently centrifuged (300× g for 10 min) to remove Percoll[®]. Finally, the selected sperm was used for evaluation.

2.3.3. *Swim-up*

For this method, 250 μ L spermatozoa were added at the bottom of a 15 mL tube, and 500 μ L MMM medium was carefully put on top. The tube was kept tilted for 15 min at a 45-degree angle in the incubator (38.5 °C, 6.5% CO₂). Subsequently, 100 μ L was removed from the top of the solution and centrifuged (300× g for 10 min, room temperature) to concentrate the sperm suspension. The separated sperm was resuspended in an MMM medium and evaluated (Banafshi et al., 2021).

2.4. *Experiment 1: Effects of selection methods on sperm morphology and functionality*

2.4.1. *Sperm kinetics parameters*

The epididymal sperm were analyzed using computer-assisted sperm analysis (CASA, IVOS 7.4G; Hamilton-Thorne Research, MA, USA) with settings previously described for red-rumped agouti: temperature, 37 °C; straightness threshold, 30%; minimum contrast, 45; low-velocity average pathway (VAP) cutoff of 10 m/s; and medium VAP cutoff of 30 m/s (Castelo et al., 2015). Five independent and nonconsecutive microscopic fields were selected for scanning. The following parameters were analyzed: total motility (TM, %), progressive motility

(PM, %), average pathway velocity (VAP, $\mu\text{m/s}$), velocity straight line (VSL, $\mu\text{m/s}$), curvilinear velocity (VCL, $\mu\text{m/s}$), amplitude lateral head (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %). The sperm population was subdivided into four categories: rapid, medium, slow, and static (%). Spermatozoa vigor was analyzed using light microscopy and rated on a scale of 0 to 5.

2.4.2. Sperm membrane functionality and morphology

A hypoosmotic swelling test (HOST) was conducted with a solution composed of distilled water (0 mOsm/L) with sodium citrate and fructose solution (50 mOsm/L). Aliquots with 5 μL containing epididymal sperm plus 45 μL of the solution were incubated in a dry bath for 40 min at 37 °C. They were evaluated using a phase-contrast light microscope ($\times 400$, 100 cells/group). Sperms with a swollen coiled tail were classified with a functional membrane (Dantas et al., 2022a).

For morphology parameters, a 10- μL aliquot of the sperm samples was stained with Bengal rose (Cromato[®]) and viewed under an optical microscope (1000 $\times$; 100 cells per slide). They were divided into two categories: normal or abnormal morphology. The abnormalities were also classified as head, middle piece, or tail defects (Silva et al., 2011).

2.4.3. Determination of intact DNA rates

Sperm samples were smeared and left to dry in the air. The slides were fixed in Carnoy's solution for 3 h, rinsed, dried at room temperature, 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. 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 fluorescence microscopy (400 $\times$; Olympus

BX51TF, Tokyo, Japan). The heads of the sperm cells with normal (double-stranded) DNA presented a green, fluorescent glow, and those with denatured or single-stranded DNA chains presented a yellow, orange, or red glow (indicating higher levels of damage, respectively) (Tomov et al., 2020).

2.4.4. Mitochondrial function activity and membrane integrity

A 10 μ L-aliquot of spermatozoa was incubated with 40 μ g/mL Hoechst 33342 at 37 °C for 5 min and with 0.5 mg/mL propidium iodide and 500 μ M chloromethyl-X-rosamine (Invitrogen, Carlsbad, CA, USA) for 8 min. In each group, one hundred cells were analyzed using fluorescence microscopy (400 $\times$). Sperm with a blue head and a midpiece red glow was considered to have an intact sperm membrane and normal mitochondrial activity (Santos et al., 2023).

2.5. Experiment 2: Effects of selection methods on oxidative status

Initially, intracellular ROS and glutathione (GSH) levels were quantified using 10 μ M 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Invitrogen, Carlsbad, CA, USA) and 10 μ M 7-amino-4-chloromethylcoumarin (CellTracker Blue; Invitrogen, Carlsbad, CA, USA), respectively. In a dry bath, spermatozoa were incubated in the dark with the different probes (1:1) for 30 min at 37 °C. The samples were centrifuged twice (500 $\times$ g, 5 min), resuspended in phosphate buffer solution (PBS), and analyzed by fluorescence microscopy (400 $\times$). To determine ROS and GSH levels, the image fluorescence intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The control group (WS) was used as a calibrator, and the measured value of each treatment was divided by the calibrator to generate relative expression levels (arbitrary fluorescence units, AFU) (Santos et al., 2023).

After collecting AFU data, a ROS/GSH proportion and Pearson's correlation were made to determine the oxidative status of the samples.

2.6. Experiment 3: Effects of selection methods on the fertilization capacity using he-IVF

2.6.1. Oocyte collection and in vitro maturation (IVM)

Twelve sexually mature female guinea pigs (three females/replicate) were anesthetized and euthanized with the same protocol as red-rumped agoutis. The ovaries were collected, stored in heated NaCl (0.15 M, 37 °C), and transported to the laboratory, where the follicles were sliced for oocyte recovery. Type I and II oocytes (with one to four *cumulus* cell layers) were selected for IVM in drops (100 µL) covered with mineral oil in a controlled atmosphere at 38.5 °C and 6.5% CO₂ for 24 h (Wang et al., 2019).

The oocyte maturation medium was composed of TCM-199 supplemented with 2.2 g/L sodium bicarbonate, 25 mM HEPES, 0.2 mM sodium pyruvate, 100 µM cysteamine, 50 ng/mL epidermal growth factor (EGF), 10 µg/mL follicle-stimulating hormone associated with luteinizing hormone (FSH/LH; Pluset[®], Hertape Calier, Juatuba, MG, Brazil), 10% fetal bovine serum (FBS), and 1% antibiotic–antimycotic solution (Wang et al., 2019).

2.6.2. Heterologous in vitro fertilization and morulae analysis

Matured oocytes were washed and pooled (10–20 oocytes) in 50 µL drops of Human Tubal Fluid medium (HTF; 101.6 mM NaCl, 4.7 mM KCl, 0.37 mM KH₂PO₄, 21.6 mM Na lactate, 2.78 mM glucose, 25 mM NaHCO₃, 2 mM CaCl₂, 0.20 mM MgSO₄, 25 mM HEPES, 4 mg/mL BSA, 0.33 mM sodium pyruvate, 10 µg/mL phenol red, and 1% antibiotic–antimycotic solution) for he-IVF. They were balanced at 38.5 °C in 6.5% CO₂ for approximately 30 min

before adding the prepared sperm with a final concentration of 1.0×10^6 sperm/mL to intact *cumulus*-enclosed oocytes (Cañón-Beltrán et al., 2021).

After 6 h of co-incubation, the structures were washed and pipetted to remove excess sperm and *cumulus* cells. They were transferred for *in vitro* culture (IVC) drops (50 μ L) with Potassium Simplex Optimized Medium (KSOM) medium (95 mM NaCl, 2.5 mM KCl, 0.35 mM KH_2PO_4 , 10 mM Na lactate, 0.2 mM glucose, 25 mM NaHCO_3 , 1.71 mM CaCl_2 , 0.20 mM MgSO_4 , 21 mM HEPES, 0.01 mM phenol red, 0.2 mM sodium pyruvate, 0.01 mM EDTA, 1 mM L-glutamine, 15 mM BSA, 1% antibiotic–antimycotic solution, 1% essential amino acid solution, and 0.5% non-essential amino acid solution) (Lawitts and Biggers 1993). After 48 h of culture (D2), 50% of the KSOM medium was changed. The non-cleaved cells were evaluated for oocyte–sperm interaction using Hoechst 33342 (10 μ g/mL, 30 min) under a fluorescence microscope, where the number of spermatozoa bound to the zona pellucida and the number associated with each oocyte were quantified, providing evidence for the occurrence of mono and polyspermy (Santos et al., 2023).

The cleaved heterologous zygotes were cultured for 120 h (Day 5) to evaluate development kinetics using an inverted microscope (Leipzig IMx 400, PhoenixOptics, Germany), where cell cleavage was assessed and classified as two, three to seven, or eight or more cells. The percentage of morulae formation was also evaluated on D5, in addition to the percentage of morulae/cleaved structures (Praxedes et al., 2023).

2.7. Statistical analysis

All data were expressed as mean $\pm$ standard error (one male/one replicate) and analyzed using the GraphPad software (Graph-Pad Software Incorporated, La Jolla, CA, USA). All results were

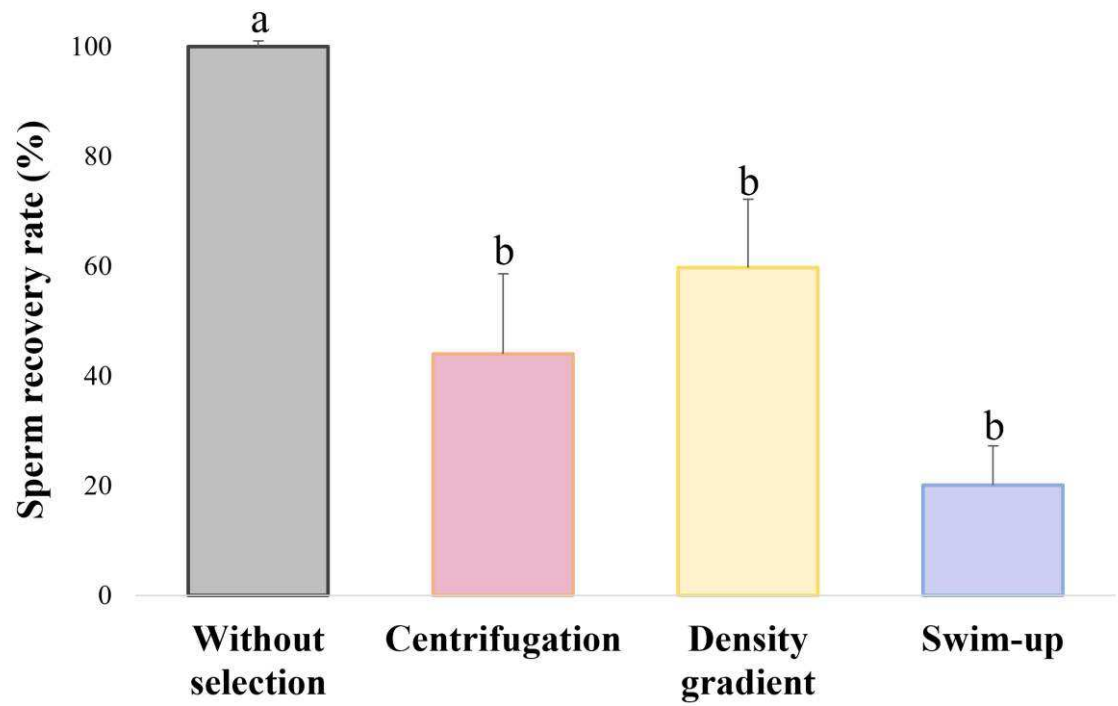
verified for normality by the Shapiro-Wilk test and homoscedasticity by Levene's test. Since data did not show a normal distribution, they were arcsine transformed and analyzed by ANOVA followed by the Tukey test. The Pearson correlation test was used to evaluate ROS/GSH correlation. All the other data were compared with a chi-squared test. Significance was set at $P < 0.05$.

3. Results

All epididymal flushed samples had a whitish color, with an average pH of 7.0 and 4.6 vigor score (0-5 scale). The average concentration was 567.1×10^6 sperm/mL, ranging from 210 to $1,320 \times 10^6$ sperm/mL. Only samples showing total motility higher than 80% were used for further evaluations.

3.1. Experiment 1: Effects of selection methods on sperm functionality and morphology

Sperm recovery rates were significantly lower in either centrifugation ($44.0\% \pm 14.6$), density gradient ($59.7\% \pm 12.5$), or swim-up ($20.1\% \pm 7.2$) groups when compared to without selection ($100.0\% \pm 0.0$) (Figure 2). Regarding sperm kinetics parameters (Table 1), there was no difference between total mobility, with all groups maintaining above 70% rates ($P > 0.05$). All treatments increased the values of VAP, VSL, VCL, and rapid and medium sperm subpopulations compared to the without selection group. However, the centrifugation method provided a greater progressive motility of more than 64.0% compared to other groups ($P < 0.05$).



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1729 **Figure 2.** Efficiency for red-rumped agouti epididymal sperm recovery after different sperm
1730 selection methods. Different subscript letters differ significantly ($P < 0.05$).

Table 1. Sperm kinetics parameters with CASA evaluation of red-rumped agouti epididymal sperm after three different selection methods.

CASA parameters	Without selection	Centrifugation	Density gradient	Swim-up
Total motility (%)	85.2 ± 2.15 ^a	86.2 ± 2.54 ^a	77.8 ± 8.46 ^a	77.8 ± 3.46 ^a
Progressive motility (%)	32.2 ± 7.03 ^b	64.6 ± 2.98 ^a	47.0 ± 7.54 ^b	48.4 ± 11.99 ^b
VAP (µm/s)	49.5 ± 7.44 ^b	125.1 ± 8.37 ^a	101.1 ± 14.9 ^a	126.2 ± 31.8 ^a
VSL (µm/s)	39.9 ± 7.14 ^b	115.4 ± 8.67 ^a	88.0 ± 14.71 ^a	105.7 ± 36.25 ^a
VCL (µm/s)	80.7 ± 7.36 ^b	159.4 ± 9.50 ^a	130.1 ± 15.99 ^a	137.8 ± 39.11 ^a
ALH (µm/s)	4.7 ± 0.16 ^b	5.5 ± 0.22 ^a	5.5 ± 0.16 ^a	4.5 ± 0.73 ^b
BCF (Hz)	37.8 ± 1.8 ^a	35.0 ± 1.40 ^a	33.1 ± 0.94 ^a	31.0 ± 2.09 ^a
STR (%)	72.8 ± 1.66 ^b	86.8 ± 1.85 ^a	78.2 ± 2.20 ^b	79.6 ± 6.58 ^{ab}
LIN (%)	43.4 ± 3.30 ^b	66.8 ± 2.85 ^a	57.4 ± 3.46 ^{ab}	60.6 ± 9.68 ^{ab}
Rapid (%)	37.0 ± 7.99 ^b	69.6 ± 3.33 ^a	55.6 ± 9.11 ^{ab}	45.8 ± 18.30 ^{ab}
Medium (%)	48.2 ± 6.51 ^b	14.4 ± 3.04 ^a	22.4 ± 6.29 ^a	11.8 ± 1.98 ^a
Slow (%)	0.8 ± 0.58 ^a	0.2 ± 0.20 ^a	0.2 ± 0.20 ^a	0.4 ± 0.24 ^a
Static (%)	14.2 ± 2.22 ^a	15.6 ± 4.13 ^a	22.0 ± 8.32 ^a	41.8 ± 17.00 ^a

^{a,b}: Values with different letters differ significantly (P<0.05). VAP, velocity average pathway; VSL, velocity straight line; VCL, curvilinear velocity;

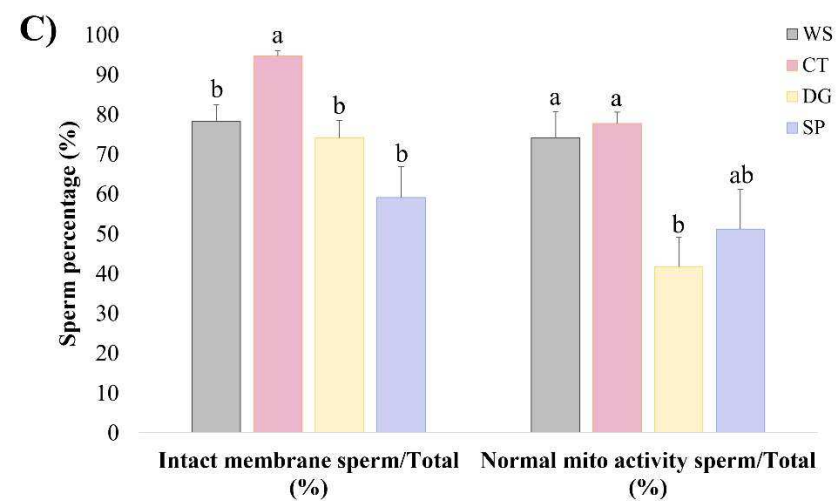
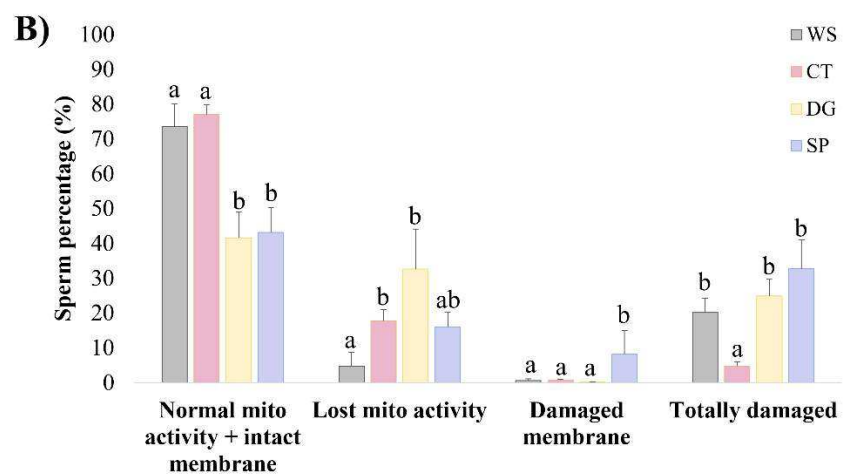
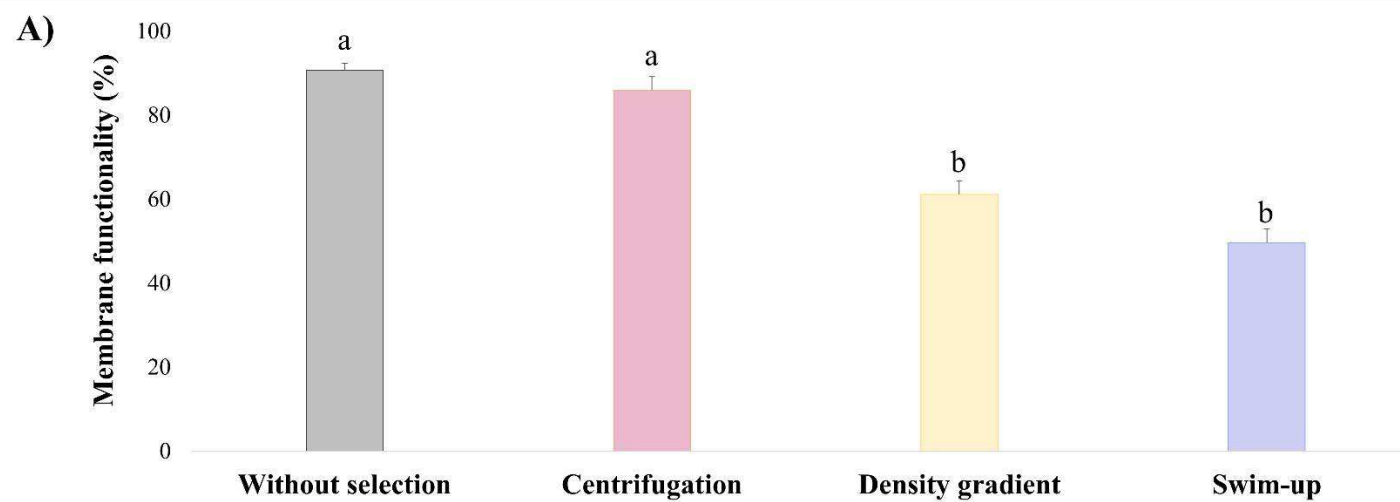
ALH, amplitude of lateral head; BCF, beat cross frequency; STR, straightness; LIN, linearity.

The centrifugation method successfully maintained membrane functionality ($85.9\% \pm 3.2$), performing similarly to the without selection group ($90.7\% \pm 1.6$) in contrast to the density gradient ($61.1\% \pm 3.2$) and swim-up ($49.6\% \pm 3.2$) (Figure 3-A). Additionally, a higher number of sperm with both normal mitochondrial activity and membrane integrity (Figure 3-B) were observed in the WS and CT groups ($P < 0.05$). Nonetheless, the centrifugation group showed the lowest rate of totally damaged cells (Figure 3-B, $P < 0.05$) and a membrane integrity percentage higher than 94.0% (Figure 3-C) ($P < 0.05$). The density gradient method significantly decreased the mitochondrial activity of red-rumped agouti spermatozoa, and the swim-up significantly increased the number of ruptured membranes (Figure 3-B, $P < 0.05$).

Finally, the three different separation methodologies showed no differences ($P > 0.05$) regarding intact DNA rates (Figure 4-A) compared to the control, with all values ranging between 68.8 and 87.5% (Figure 4-B). Among all groups, swim-up was the only one that increased denatured stranded DNA rates ($P < 0.05$) (Figure 4-B). All selection procedures maintained high rates of normal morphology sperm (Figure 4-C) similar to without selection ($P > 0.05$). However, the density gradient protocol presented a significant number of sperm cells with tail defects among the abnormalities (Figure 4-D) ($P < 0.05$).

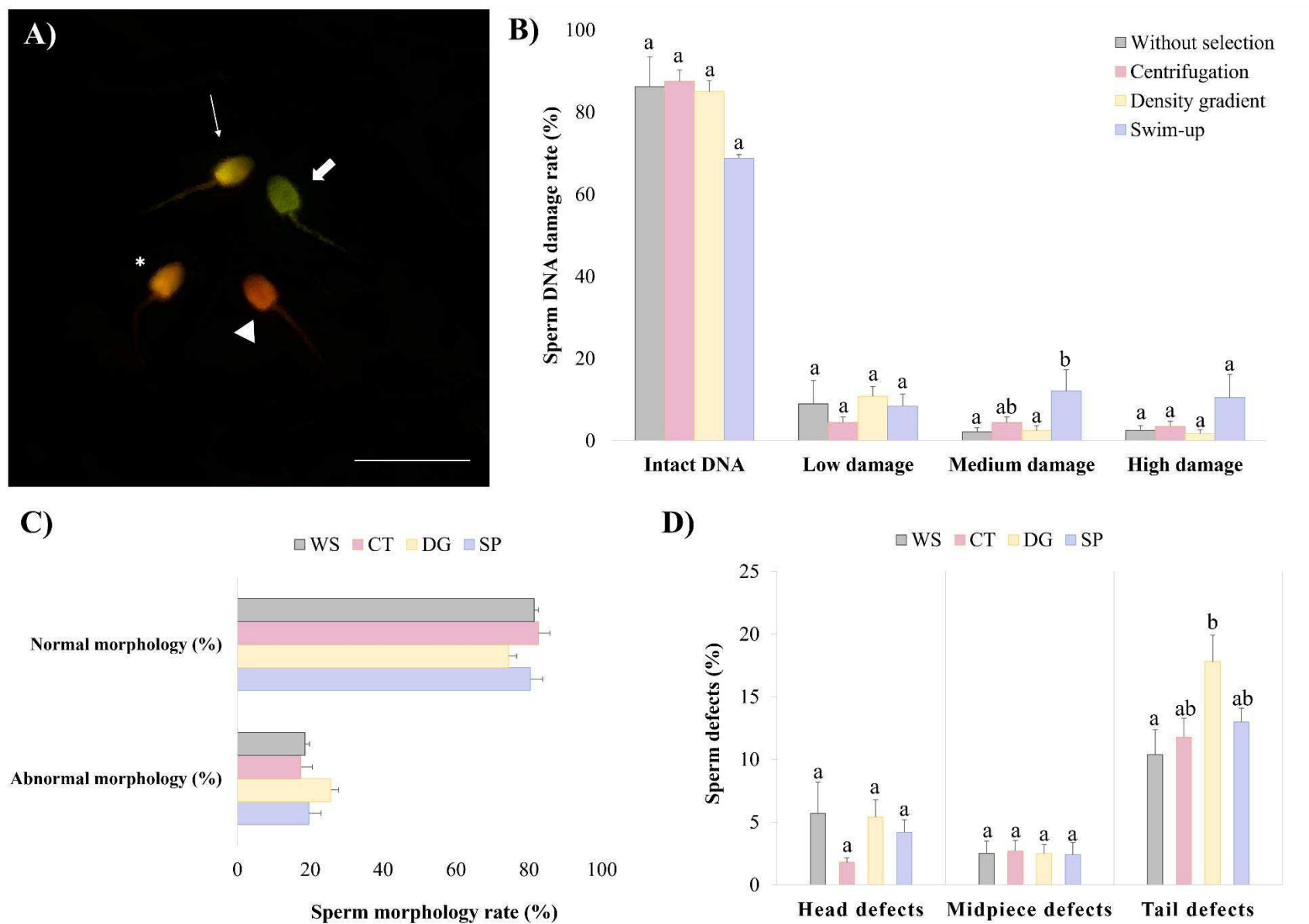
3.2. Experiment 2: Effects of selection methods on oxidative status

The oxidative status was defined by both ROS (Figure 5-A-D) and GSH (Figure 5-E-H) levels. Lower ROS levels (Figure 5-I) were detected in sperm cells after the centrifugation method was used (0.57 ± 0.1 AFU) in comparison with both density gradients (0.79 ± 0.1 AFU) and without selection (1.00 ± 0.4 AFU). At the same time, the swim-up treatment was the least efficient method, exponentially increasing ROS levels (1.40 ± 0.4 AFU) ($P < 0.05$). Similarly, GSH levels (Figure 5-I) were higher in cells selected by the centrifugation (1.82 ± 0.9 AFU) compared to



1771

1772 **Figure 3.** Effects of different selection methods in viability, mitochondrial activity, and sperm membrane functionality for red-rumped agouti. **(A)**
1773 Percentage of membrane functionally analyzed by the hypoosmotic swelling test (HOST). **(B)** Percentage of intact or damaged agouti sperm
1774 membrane and mitochondrial activity labeled with H33342, propidium iodide, and MitoTracker Red. **(C)** Percentage of selected sperm with intact
1775 membrane and normal mitochondrial activity for all counted cells. Values with different letters differ significantly ($P < 0.05$). WS: without selection,
1776 CT: centrifugation, DG: density gradient, SP: swim-up.



1778 **Figure 4.** DNA and morphology damage index of red-rumped agouti sperm after three different separation protocols. **(A)** Representative
1779 photomicrograph of acridine orange labeling in agouti spermatozoa, where the thick arrow indicates spermatozoa with intact DNA, the thin arrow
1780 exemplifies low DNA damage, the asterisk indicates medium damage, and the arrowhead represents high damage. **(B)** Percentage of intact, low,
1781 medium, and high damage on the DNA strand for agouti sperm using acridine orange labeling. **(C)** Percentage of normal and abnormal spermatozoa
1782 after selection ($P>0.05$). **(D)** Sperm defect rates are divided into head, midpiece, and tail abnormalities. Different letters (a,b) indicate a statistical
1783 difference ($P<0.05$). WS: without selection, CT: centrifugation, DG: density gradient, SP: swim-up. Scale Bar = 20 μm . 40 $\times$.

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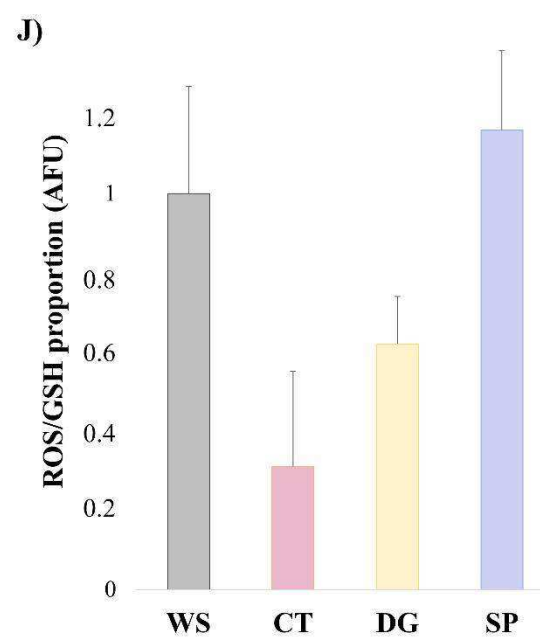
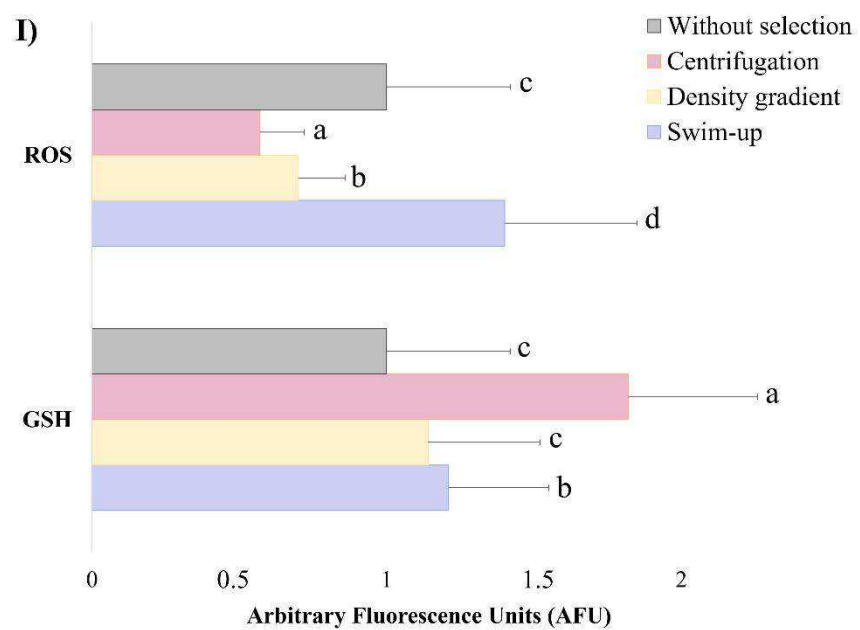
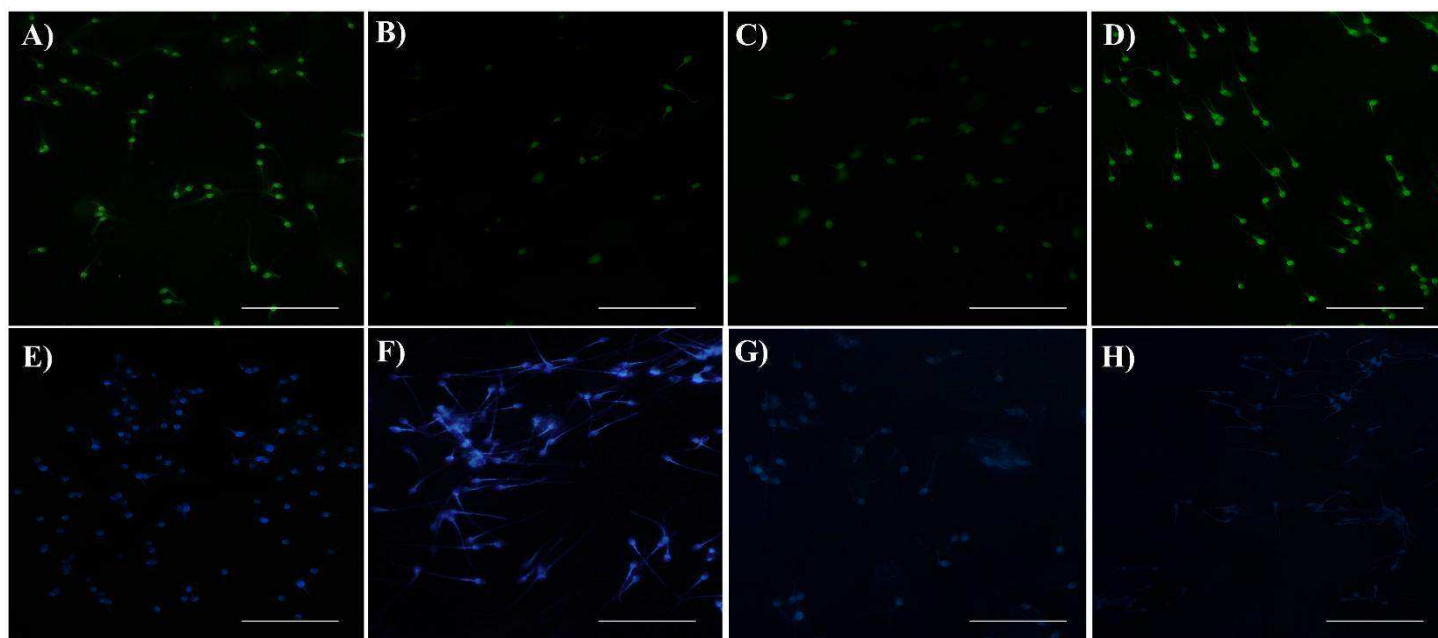
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1795 **Figure 5.** Representative images of fluorescently labeled agouti epididymal spermatozoa for evaluation of oxidative stress. **(A-D)** spermatozoa
1796 labeled with the H₂DCFDA probe for evaluating ROS in the WS, CT, DG, and SP groups, respectively; **(E-H)** spermatozoa labeled with the
1797 CellTracker Blue probe for evaluating intracellular GSH, respectively. **(I)** ROS and GSH levels in arbitrary fluorescence units. **(J)** Proportion of
1798 ROS/GSH in the different sperm samples ($P>0.05$). Different letters (^{a,b,c,d}) indicate a statistical difference ($P<0.05$). ROS: Reactive oxygen species;
1799 GSH: glutathione; WS: without selection; CT: centrifugation; DG: density gradient; SP: swim-up. Scale Bar = 20 μ m. 20 $\times$.

DG (1.14 ± 0.3 AFU), SP (1.21 ± 0.3 AFU), and WS (1.00 ± 0.4 AFU) ($P < 0.05$). There were no differences in the ROS/GSH ratio (Figure 5-J) between the methodologies used ($P > 0.05$). Meanwhile, all sperm selection groups presented a significant negative ROS/GSH correlation (Table 2), the strongest being centrifugation, followed by a moderate correlation in density gradient and swim-up. The without-selection sperms presented a positive correlation.

Table 2. Correlation analysis for intracellular ROS/GSH levels in red-rumped agouti sperm after different selection methods.

	Without selection (GSH levels, AFU)	Centrifugation (GSH levels, AFU)	Density gradient (GSH levels, AFU)	Swim-up (GSH levels, AFU)
ROS	<u>$r = 0.9233$</u> ;	<u>$r = -0.9274$</u> ;	<u>$r = -0.8302$</u> ;	<u>$r = -0.7966$</u> ;
levels	<u>$P = 0.0030$</u>	<u>$P = 0.0077$</u>	<u>$P = 0.0208$</u>	<u>$P = 0.0320$</u>

r = Pearson's correlation coefficient. P-values determined by two-tailed Student's t-test. Significant values are underlined ($P < 0.05$). AFU, arbitrary fluorescence units. ROS, reactive oxygen species. GSH, glutathione.

3.3. Experiment 3: Effects of selection methods on the fertilization capacity with he-IVF

A total of 24 ovaries and 167 viable immature oocytes were recovered (Figure 6-A), representing an average of 6.9 viable oocytes/ovary and 13.9 viable oocytes/female. These mature structures (Figure 6-B-C) were randomly assigned to he-IVF with differently selected sperm. After IVC, the number of sperm bound to oocytes and zones did not differ between groups, and all treatments maintained monospermic levels above 63% ($P > 0.05$) (Table 3).

Cleavage rates (Table 4) of fertilized oocytes (Figure 6-D) were more significant in the without selection, swim-up, and centrifugation groups, ranging from 32.0 to 54.1%. The density

gradient was the least effective method, the only one showing a lower cleavage rate ($P < 0.05$). All treatments presented the same results regarding the number of cells in cleavages (2, 3-7, or ≥ 8) after 120 h of IVC ($P > 0.05$). The selection methods and control group derivative structures were equally capable of reaching the morula stage (Figure 6-E-F), with maximum rates of 37.5% and 69.2% in morula/cleaved structures ($P > 0.05$).

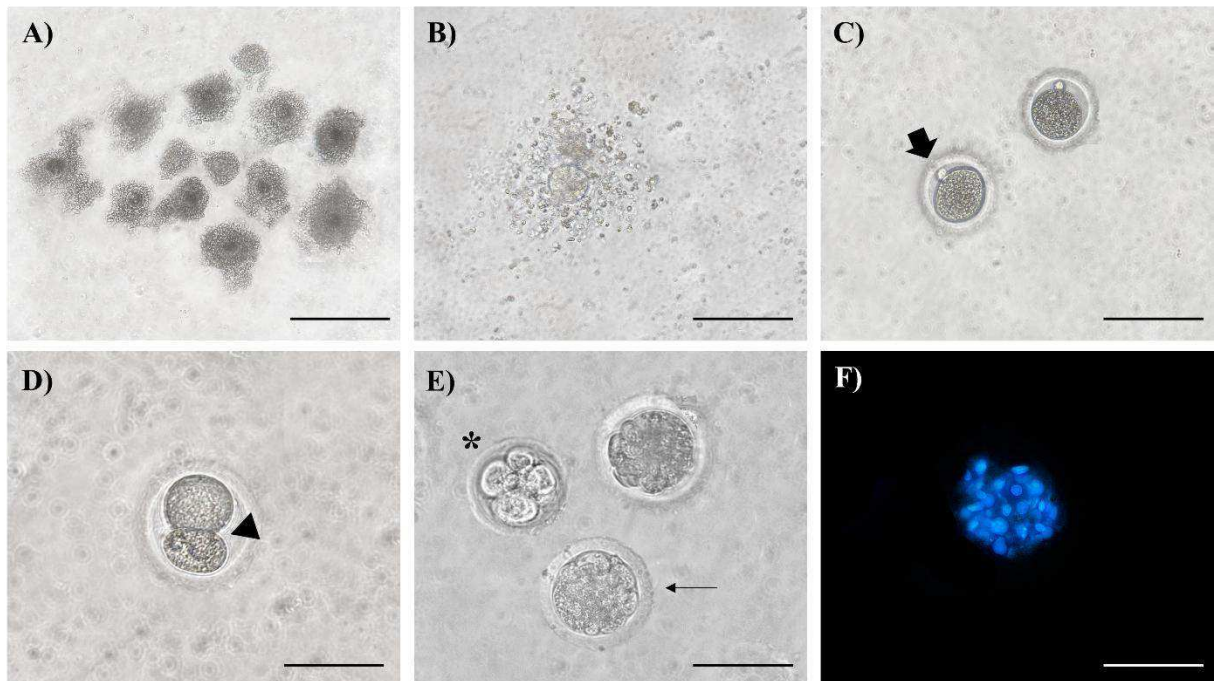


Figure 6. Representative images of guinea pig oocytes subjected to heterologous IVF with red-rumped sperm. (A) Immature oocytes after recovery; (B) matured oocyte 6h post-IVF; (C) structures after 7 h of IVF showing the first polar body (black arrow). (D) presumed zygote after 48 h at the 2-cell stage with the presence of pronuclei (arrowhead); (E) cleaved (asterisk) and morula stage embryos (thin arrow); (F) IVF morula labeled with H333342 for cell visualization. Scale bar: 100 μm (A and F); 50 μm (B, C, D and E). Magnification: 20 $\times$ (A); 40 $\times$ (B-F).

1837 **Table 3.** Sperm–oocyte interaction during he-IVF between red-rumped agouti and guinea pig.

Evaluations	Without selection	Centrifugation	Density gradient	Swim-up
Sperm/oocyte (mean ± SE)	1.0 ± 0.00 ^a	1.6 ± 0.58 ^a	1.6 ± 0.09 ^a	1.4 ± 0.12 ^a
Sperm/zone (mean ± SE)	28.7 ± 20.87 ^a	18.0 ± 5.51 ^a	36.3 ± 10.33 ^a	10.7 ± 3.76 ^a
Monospermy (%)	100.0 ± 0.00 ^a	83.3 ± 16.67 ^a	63.3 ± 6.94 ^a	66.7 ± 9.62 ^a
Polyspermy (%)	0.0 ± 0.00 ^a	16.7 ± 16.67 ^b	36.7 ± 6.94 ^b	33.3 ± 9.62 ^b

1838 Different subscript letters differ significantly (P<0.05). SE, standard error.

1839

1840 **Table 4.** Embryo kinetics after heterologous IVF with guinea pig oocytes and selected agouti
1841 sperm.

Variables	Without selection	Centrifugation	Density gradient	Swim-up
<i>Cleavage (120 h IVC)</i>				
Cleavage (%)	40.0 ± 4.8 (10/25) ^a	54.1 ± 2.4 (13/24) ^a	20.0 ± 2.4 (5/25) ^b	32.0 ± 9.6 (8/25) ^{ab}
2 cells (%)	12.0 ± 28.9 (3/25) ^a	0.0 ± 0.0 (0/24) ^a	4.0 ± 33.3 (1/25) ^a	0.0 ± 0.0 (0/25) ^a
3-7 cells (%)	20.0 ± 27.8 (5/25) ^a	33.3 ± 12.1 (8/24) ^a	12.0 ± 33.3 (3/25) ^a	28.0 ± 16.7 (7/25) ^a
≥ 8 cells (%)	8.0 ± 14.7 (2/25) ^a	20.0 ± 12.1 (5/24) ^a	4.0 ± 33.3 (1/25) ^a	4.0 ± 16.7 (1/25) ^a
<i>Morula (120 h IVC)</i>				
Total (%)	20.0 ± 12.6 (5/25) ^a	37.5 ± 7.1 (9/24) ^a	12.0 ± 5.3 (3/25) ^a	16.0 ± 3.4 (4/25) ^a
Total/Cleaved (%)	50.0 ± 27.7 (5/10) ^a	69.2 ± 16.6 (9/13) ^a	60.0 ± 22.2 (3/5) ^a	50.0 ± 18.5 (4/8) ^a

1842 Mean ± standard error. ^{a,b}: Values with different subscript letters differ significantly (P<0.05).

1843 IVC, *in vitro* culture.

4. Discussion

The results of this study showed that the centrifugation protocol in red-rumped agouti was the most effective in selecting spermatozoa with superior progressive motility, membrane functionality and viability, morphological integrity, normal mitochondrial activity, lower ROS levels, and higher intracellular GSH, strongly correlated. Likewise, the present study revealed the ability of selected epididymal red-rumped agouti spermatozoa to penetrate guinea pig oocytes and generate hybrid morulae, highlighting the conserved fertilization capacity of these sperm cells.

The simple centrifugation protocol was also successfully used in other rodents, such as rats (Kim et al., 2013), where the authors emphasized the method's ability to preserve motility, reduce basal intracellular ROS, and maintain mitochondrial function. This efficiency can be explained by combining low centrifugal forces with a shorter preparation time. An increase of $>400\times g$ has been proven to decrease rat sperm functionality and increase ROS levels (Kim et al., 2013). A time-dependent decrease in motility and plasma membrane integrity was shown when centrifugation protocols were extended from 5 min to 10 or 15 min (Varisli et al., 2009).

The collected epididymal spermatozoa's concentration and motility rate followed red-rumped agouti standards (Castelo et al., 2015). However, there was a significant concentration reduction after preparing these cells. This decrease is necessary to improve the sample quality (Vaughan and Sakkas, 2019). Otherwise, dead cells, immature spermatozoa, and cell debris may increase ROS production, leading to DNA damage (Mochida et al., 2019).

The increased values of VAP, VSL, and VCL observed in post-sperm preparation are important quality indicators, as high-velocity movement spermatozoa can swim fast enough to penetrate

the egg investments (*cumulus* cells and zona pellucida), increasing fertilization efficiency (Mochida et al., 2019). Additionally, higher progressive motility in the centrifugation group compared to others also contributes positively to the fertilization capacity of these samples. Similar results were observed in rats, where epididymal spermatozoa selected by centrifugation showed higher PM rates compared to density gradients prepared group (Kim et al., 2013).

Both without selection and centrifugation treatments succeeded in maintaining membrane functionality and integrity. This membrane status has already been correlated with sperm morphology, DNA fragmentation, decondensation, and parameters related to sperm nuclear architecture evaluation for early apoptosis (Bloch et al., 2021). Associations between the percentage of HOST-positive spermatozoa and reproductive outcomes have also been previously demonstrated (Jędrzejczak et al., 2005).

Although all treatments showed few abnormalities in sperm morphology, density gradient exhibited a higher incidence of tail abnormalities, predominantly characterized by structural breakage. These may be related to the method characteristics, where a higher density may have partially damaged the structure of these cells and the centrifugation required for a more extended period, which has already been able to double the number of tail defects in equine sperm (Morrell et al., 2009). These abnormalities in any part of the sperm tail can result in motility defects, including biochemical properties required for capacitation and hypermotility (Lehti and Sironen, 2017).

Throughout sperm selection, DNA damage may increase, which can be caused by multiple factors, including protocol errors, endogenous caspases, endonuclease activation, and increased oxidative stress (Li and Lloyd, 2020). This condition can generate negative impacts on

reproductive outcomes after ARTs, as normal embryonic development depends on the delivery of intact and complete genetic material from sperm to oocyte (Aitken et al., 2013), although sperm with minimal DNA damage can retain a lower fertilizing ability (Braga et al., 2023). In the present study, only swim-up increased denatured stranded DNA rates; this may be related to the method characteristics (double centrifugation and more extended periods in the incubator with non-viable sperm), as this situation is conducive to an intense increase in ROS levels (Younglai et al., 2001).

Normal mitochondrial activity in sperm is essential for their functionality, as this organelle has cellular functions besides ATP production, such as calcium homeostasis and generation of ROS to balance the sperm's oxidative status (Amaral et al., 2013). The efficiency of this structure is directly correlated with the motility, capacitation, acrosome reaction, and fertilization capacity of rodent sperm (Gu et al., 2019). Therefore, the centrifugation group presenting a higher percentage of epididymal spermatozoa with normal mitochondrial activity indicates their quality. In contrast, cells processed by density gradient showed higher loss of mitochondrial activity, contrary to what has already been observed in rat epididymal spermatozoa, where the authors observed an increase in mitochondrial function after sample processing (Kim et al., 2013).

Several critical biological processes of mammalian spermatozoa are redox-regulated, including capacitation, hyperactivation, and acrosomal exocytosis (Bennetts and Aitken 2005). However, when there is an imbalance in the intracellular antioxidants and ROS generated, spermatozoa can succumb to oxidative attacks, damaging the plasma membrane and DNA (Dutta et al., 2019). Regarding ROS production after sperm selection, the centrifugation method was the most efficient for red-rumped agouti. However, all separation treatments were superior to the

without selection group. This data can be explained by the method's capacity to remove excess inactive cells that increase the production of these radicals (Mochida et al., 2019). It is considered that all spermatozoa are highly susceptible to inducers of ROS production, and the antioxidant capacity is very low in rodent spermatozoa (Kim et al., 2013). Consequently, maintaining a higher number of cells with intracellular antioxidants, such as glutathione, could help with the viability parameters of these samples by regulating the oxidative balance. For red-rumped agouti sperm, higher levels of GSH were observed within the centrifugation group, confirming a strong negative correlation between both ROS and GSH parameters.

One of the best methods to analyze the selection damage caused to sperm is the fertilizing capacity evaluation, as it encompasses several physiological processes, such as oocyte-sperm interaction, pronuclear formation, embryo development, and quality (Cañón-Beltrán et al., 2023). Given the lack of available oocytes in wild species, such as red-rumped agouti, he-IVF with oocytes from phylogenetically close species (guinea pig) represents an effective approach to evaluate these parameters with red-rumped agouti sperm.

Guinea pig oocyte collection data was similar to that found within the species (Wang et al., 2019). After he-IVF, the guinea pig oocyte fertilized with all sperm groups was able to develop up to the cleavage and morula stages. Despite differing in previous results, fertilization capacity was not affected by different selection protocols. Similar results were found in collared peccary (*Pecari tajacu*) sperm, where the authors observed an equal production of morulae among different sperm selection protocols (Santos et al., 2023). These findings can be explained considering the *in vitro* environment, where spermatozoa do not need to travel long distances to interact with the matured oocyte, thus facilitating their interaction, unlike the *in vivo* environment (Coy et al., 2009).

1944

1945 **5. Conclusion**

1946 In conclusion, although the different selection methods were equally effective in selecting
1947 spermatozoa capable of fertilizing heterologous oocytes *in vitro*, we suggest the use of the
1948 centrifugation method for red-rumped agouti, which promotes the selection of gametes with
1949 better morphological and functional parameters and maintains a positive oxidative status in the
1950 *in vitro* environment. However, future studies should be conducted to highlight how these
1951 methods can impact the quality of these embryos. This was the first study to compare different
1952 sperm selection methods in red-rumped agouti and represents an important step towards better
1953 understanding the oocyte-sperm interaction in the species and contributing to the optimization
1954 of ARTs in wild rodents.

1955

1956 **Ethical statement**

1957 All experiments followed the Animal Ethics Committee of the Federal Rural University of
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1965

1966 **CRedit authorship contribution statement**

1967 **L.R.M. Oliveira:** Conceptualization, data curation, investigation, methodology, visualization,
1968 writing – original draft. **L.V.C. Aquino:** Investigation. **A.B.M. Pereira:** Investigation. **A.L.R.**

1969 **Rodrigues:** Investigation. **L.L.V. Rodrigues:** Investigation. **L.G.P. Bezerra:** Investigation.
 1970 **M.F. Oliveira:** Resources. **A.R. Silva:** Resources, writing – review & editing. **A.F. Pereira:**
 1971 Conceptualization, data curation, formal analysis, funding acquisition, project administration,
 1972 resources, supervision, writing – review & editing.

1973

1974 **Declaration of competing interest**

1975 None.

1976

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1980

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**CAPÍTULO 4 - TIME AND MEDIA-DEPENDENT MODULATION OF SPERM
CAPACITATION IN RED-RUMPED AGOUTI (*Dasyprocta leporina* LINNAEUS, 1758)**

ARTIGO EXPERIMENTAL 2

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Qualis 2017-2020: A2 (Medicina Veterinária)

Fator de impacto: 2.7

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Time and media-dependent modulation of sperm capacitation in red-rumped agouti
(*Dasyprocta leporina* Linnaeus, 1758)

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Abstract

The increasing focus on understanding spermatozoa mechanisms in rodents aims to enhance
 reproductive stability and support conservation efforts, particularly for ecologically significant
 and declining species like the red-rumped agouti. We aimed to evaluate the interaction between
 capacitation media and time, testing BSA concentrations —low (4 mg/mL) and high (15
 mg/mL) —with or without 2 mM calcium chloride (CaCl₂) across three time points. Epididymal
 sperm were distributed into five groups: without capacitation agent (WCA), high BSA (HBSA),
 high BSA with CaCl₂ (HBCa), low BSA (LBSA), and low BSA with CaCl₂ (LBCa), each

incubated for 1, 3, or 6 h. Total sperm motility was significantly higher only in media with CaCl_2 . However, only LBCa maintained high progressive motility. LBCa and HBCa maintained membrane integrity, mitochondrial functionality, and reduced reactive oxygen species levels, but only LBCa increased glutathione levels. HBSA, HBCa, LBSA, and LBCa improved the sperm capacitation rate, but LBCa yielded the highest proportion of capacitated sperm and acrosome-reacted cells after 6 h. Hyperactivation rates were higher in both LBCa and HBCa after 6 h. Therefore, the optimal condition for red-rumped agouti sperm capacitation is a low BSA concentration (4 mg/mL) supplemented with 2 mM calcium chloride following a 6 h incubation.

Keywords: Conservation biology; rodent reproduction; epididymal sperm; BSA supplementation; calcium chloride

1. Introduction

Efforts have been made to elucidate the complexities of spermatozoa in rodents, focusing on optimizing collection methods (Castelo et al. 2023), selecting viable cells (Banafshi et al. 2021), and enhancing their use in assisted reproductive techniques, such as *in vitro* fertilization (IVF) (Horie et al. 2015). A critical step in this process is ensuring that the male gametes are capacitated and capable of fertilizing the oocyte. Sperm capacitation involves intricate modifications, such as influx of bicarbonate (HCO_3^-) and calcium (Ca^{2+}) ions into the cell, efflux of cholesterol from the plasma membrane, activation of soluble adenylylase (sAC), increase in cyclic AMP (cAMP) concentrations, and kinase activation (Nicolli and Cesari 2023). These molecular cascades trigger changes in mitochondrial activity, necessary for altering motility patterns (flagellum hyperactivation), and prepare the sperm for the acrosome reaction (Balestrini et al. 2024).

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2201 Data on the specific requirements for sperm capacitation for wild rodents like the red-rumped
2202 agouti remain scarce. Optimizing this phase for these animals significantly improves their
2203 reproductive efficiency by enhancing their biological material for biotechnological
2204 applications, such as *in vitro* fertilization (IVF), and supporting conservation initiatives, given
2205 their role as keystone species and essential seed dispersers within ecosystems (Mittelman et al.
2206 2021). To overcome this challenge, phylogenetically proximate species to the red-rumped
2207 agouti, such as the guinea pig, can serve as viable models due to similarities in sperm
2208 morphophysiology (Mollineau et al. 2008; Gallardo et al. 2002).

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2210 Initial research in guinea pigs demonstrated that outcomes similar to those observed in the
2211 female reproductive tract can be achieved using minimal capacitating medium (MCM)
2212 supplemented with specific capacitation agents, primarily calcium chloride (CaCl_2) (Ramírez-
2213 Ramírez et al. 2020). Chen et al. (2009) and Ni et al. (2009) underscored the pivotal role of
2214 these ions (Ca^{2+} and Cl^-) in guinea pig epididymal sperm, asserting that they are indispensable
2215 for capacitation and subsequent acrosome reaction. In their initial experiment, they maintained
2216 a consistent concentration of bicarbonate ions (HCO_3^-) while excluding chloride ions (Cl^-),
2217 obtaining a notable 30% decrease in capacitation rates compared to the control (with Cl^-). In
2218 their subsequent trial, the omission of calcium ions (Ca^{2+}) from the medium led to a 15% decline
2219 in the rate of acrosomal reactions and hyperactivated motility. These results confirm the
2220 importance of Ca^{2+} and Cl^- . This was demonstrated in mouse sperm, where the ions regulate
2221 cAMP and tyrosine phosphorylation pathways during capacitation (Navarrete et al. 2015;
2222 Wertheimer et al. 2008).

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Bovine serum albumin (BSA) is another crucial modulatory agent for achieving optimal results in sperm capacitation in cavid rodents. This protein facilitates cholesterol extraction from the sperm plasma membrane, inducing phospholipid displacement and rearrangement, enhancing membrane fluidity, priming acrosome exocytosis, and preparing spermatozoa to penetrate oocyte before fusion (Mohanty et al. 2024). Despite its critical function, the optimal concentration of this compound remains variable across different rodents. In guinea pig, a low concentration of 4 mg/mL BSA was used in association with 2 mM Ca^{2+} , achieving a satisfactory capacitation rate of approximately 60% (Ni et al. 2009). In contrast, Hirose et al. (2020) used a higher profile of BSA (15 mg/mL) in association with 3.4 mM Ca^{2+} , obtaining excellent results in Syrian hamster (*Mesocricetus auratus*), reaching rates above 77%. Therefore, the requirement for this protein appears to be intrinsically linked to a species-specific determinant, requiring an evaluation to ascertain its efficacy and optimal activity in red-rumped agoutis.

The duration of exposure within the capacitating environment directly impacts the efficacy of this process in addition to the specific protein and ionic requirements essential for sperm capacitation. Sansegundo et al. (2022) compared the effectiveness of modified Tyrode's medium (mT-BH) with 15 mM NaHCO_3 , 4 mg/mL BSA, and 1.8 mM CaCl_2 in the capacitation of different species of the *Mus* family (*M. musculus* and *M. spretus*) at different times (0, 30, 60, and 90 min). *M. musculus* showed a lower percentage of sperm capacitated (41.6%) and became stable at 60 min, while *M. spretus* showed maximum value rate only at 90 min (61.2%). In *Cavia porcellus*, when using 1.71 mM CaCl_2 , different authors achieved rates from 34.2% (Delgado-Buenrostro et al. 2016) to 80% (Cordero-Martínez et al., 2018), increasing only the sperm exposure periods in the capacitation medium from 1 h to 2 h, respectively. Some authors even propose an extended capacitation period in conjunction with IVF to maximize the potential

of these spermatozoa, recommending durations of up to 6 h in guinea pigs (Chen et al. 2009; Ni et al. 2009).

Therefore, this study was designed to investigate the interaction between the capacitation medium and incubation time on the viability, functionality, and capacitation rate of red-rumped agouti epididymal sperm. This was achieved by comparing the MCM supplemented with differing concentrations of BSA—a higher (15 mg/mL) or lower (4 mg/mL) profile—with or without the addition of 2 mM CaCl_2 across varying exposure times (1, 3, or 6 h).

2. Materials and Methods

All experiments were approved by the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 20/2021) and Chico Mendes Biodiversity Conservation (ICMBio, no. 76655-1). The chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) except where mentioned otherwise.

2.1. Animals and epididymal sperm recovery

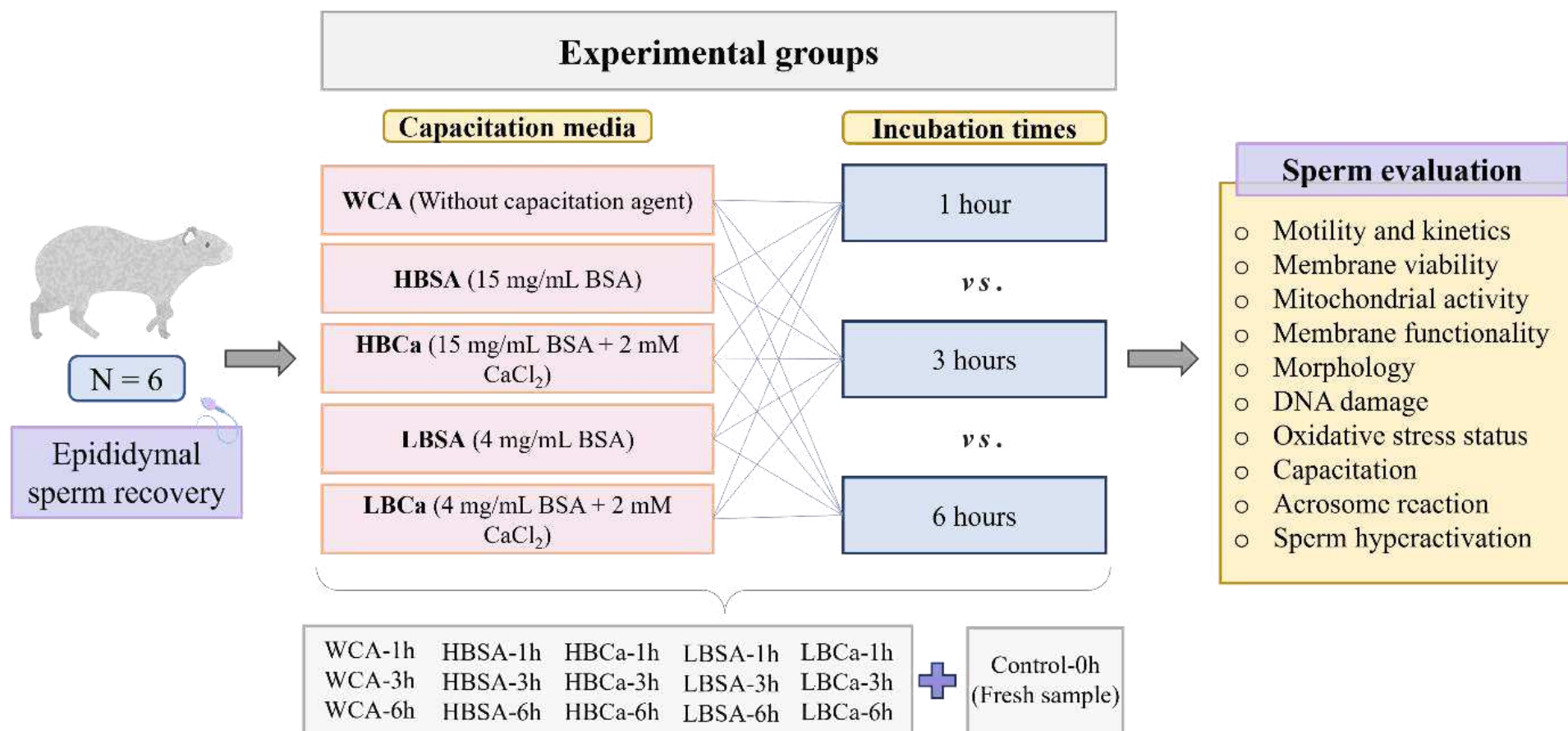
The males used in the study were kept at the Center of Multiplication of Wild Animals (CEMAS/UFERSA). They were fed commercial rabbit food and supplied with fresh drinking water *ad libitum*, using a 12-h natural photo period. Six sexually mature males were captured and pre-medicated with ketamine (15 mg/kg, Ketalar, Pfizer, SP, Brazil) and xylazine (1 mg/kg; Rompun, Bayer, SP, Brazil). Following a 15-min interval, anesthesia was induced via intramuscular administration of sodium thiopental at 50 mg/kg (Thiopentax; Cristalia, Sao Paulo, SP, Brazil). Subsequently, the animals were euthanized via intravenous administration of potassium chloride at 1 mL/kg (Equiplex, Goiania, GO, Brazil) (Castelo et al. 2015).

The testes-epididymis was recovered and transported to the laboratory in heated saline solution (37 °C, NaCl 0.15 M). The cauda region was excised and the epididymal spermatozoa were obtained by retrograde flushing with 1.0 mL of saline solution (Castelo et al. 2015). The recovered samples were kept in a water bath (37 °C), while the appearance, color, pH, and concentration (Neubauer chamber) were immediately evaluated. Only samples with over 80% motility were used.

2.2. Sperm processing and capacitation protocols

Spermatozoa were diluted to a concentration of 100×10^6 sperm/mL in minimum capacitation medium (MCM medium: 105.8 mM NaCl, 25 mM NaHCO₃, 5.56 mM glucose, 21.6 mM Na lactate, 25 mM HEPES, 0.25 mM sodium pyruvate, 10 µg/mL phenol red, and 1% antibiotic antimycotic solution). The most viable gametes were selected using a simple centrifugation protocol, where a 1:1 solution with spermatozoa and MCM was placed in a 15 mL plastic tube and centrifuged twice (300× g for 3 min, room temperature). The supernatant was discarded, and the cells were suspended in 200 µL MCM for analysis or capacitation (Cañón-Beltrán et al. 2021).

The samples were destined for capacitation at a concentration of 20×10^6 spermatozoa/mL in MCM medium added with supplements. The capacitation was conducted in a controlled atmosphere at 38.5 °C with 6.5% CO₂. The spermatozoa were incubated in microtubes (**Figure 1**) with five different MCM supplementations: without capacitation agents (WCA), 15 mg/mL BSA (HBSA), 15 mg/mL BSA with 2 mM CaCl₂ (HBCa), 4 mg/mL BSA (LBSA), and 4 mg/mL BSA with 2 mM CaCl₂ (LBCa). The influence of incubation time was evaluated in a controlled atmosphere for 1, 3, and 6 h for all treatments. A control group with fresh samples after collection (non-capacitated and 0 h of incubation) was analyzed to measure sperm health.



The impact of this process on the viability and functionality of these spermatozoa was evaluated by conducting a range of analyses, encompassing sperm kinetics and motility, membrane integrity, normal mitochondrial activity, membrane functionality, morphology, DNA integrity, oxidative stress status, capacitation, acrosome reaction, and flagellar hyperactivation.

2.3. Sperm motility and kinetics parameters

The different samples were analyzed using computer-assisted sperm analysis (CASA, IVOS 7.4G; Hamilton-Thorne Research, MA, USA) with settings previously described for red-rumped agouti: temperature, 37 °C; straightness threshold, 30%; minimum contrast, 45; low-velocity average pathway (VAP) cutoff of 10 m/s; and medium VAP cutoff of 30 m/s (Castelo et al. 2015). Five independent and nonconsecutive microscopic fields were selected for scanning. The parameters analyzed were total motility (%), progressive motility (%), average pathway velocity (VAP, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), curvilinear velocity (VCL, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), beat/cross-frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %). The sperm population was subdivided into rapid, medium, and static (%).

2.4. Spermatozoa membrane viability and mitochondrial activity

An aliquot of spermatozoa was incubated at 37 °C with a mixture of different fluorescent probes: 40 $\mu\text{g/mL}$ Hoechst 33342 (H342, Molecular Probes, Eugene, OR, USA) for 5 min and 0.5 mg/mL propidium iodide (Thermo Fisher Scientific, Waltham, MA, USA) and 500 nM CMXRos (Mito Tracker Red®, F-7512, Molecular Probes, Eugene, OR, US) for 8 min. For each experimental group per replica, 100 cells were analyzed using fluorescence microscopy (400 $\times$; Olympus BX51TF, Tokyo, Japan) (Celeghini et al. 2007). Sperms with a blue head and a

midpiece red glow were considered to have an intact sperm membrane and normal mitochondrial activity (Santos et al. 2023).

2.5. Sperm membrane functionality

A hypoosmotic swelling test (HOST) was performed using a solution composed of distilled water (0 mOsm/L) and a sodium citrate and fructose solution (50 mOsm/L). Aliquots of 5 μ L containing epididymal spermatozoa were mixed with 45 μ L of the hypoosmotic solution and incubated in a dry bath at 37 °C for 40 min. Subsequently, the samples were evaluated using a phase-contrast light microscope (400 $\times$ magnification, 100 cells per group). Spermatozoa exhibiting a swollen coiled tail were classified as possessing functional membranes (Dantas et al. 2022).

2.6. Morphology evaluation

For morphology parameters, a 10 μ L aliquot of the sperm samples was fixed and stained with a formaldehyde-Bengal rose solution (Cromato[®]) and viewed under a light microscope (1000 $\times$; 100 cells per slide). They were divided into two categories: normal or abnormal morphology. The abnormalities were also classified as presenting head, middle piece, or tail defects (Silva et al. 2011).

2.7. Spermatozoa DNA damage rates

Spermatozoa samples were smeared onto glass slides and air-dried for approximately 2 min. The slides were then fixed in Carnoy's solution for 3 h, rinsed, and air-dried at room temperature. Subsequently, the slides were incubated for 25 min in a buffer solution comprising 15 mM Na₂HPO₄ and 80 mM citric acid (pH 2.5) at 75 °C. The smears were stained with acridine orange (0.2 mg/mL) for 10 sec, washed with distilled water, and covered with a

coverslip while still wet. A total of 100 cells were evaluated using fluorescence microscopy (400× magnification; Olympus BX51TF, Tokyo, Japan). Sperm heads with intact (double-stranded) DNA exhibited green fluorescence. In contrast, those with denatured or single-stranded DNA displayed yellow, orange, or red fluorescence, indicative of progressively higher levels of damage (Tomov et al. 2020).

2.8. Sperm capacitation, Acrosome Reaction (AR), and hyperactivation (HYP) analysis

Sperm capacitation was evaluated using chlortetracycline (CTC) staining, according to the method described by Cordero-Martinez et al. (2018), 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 comprised 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 MCM medium (100× g for 3 min). Sperm suspension was stirred, and a coverslip was placed. The slides were scored with a fluorescence microscope (1000×). Samples were counted (100 cells per sample), and sperm status was characterized by the fluorescence pattern described. The F pattern (non-capacitated) displayed a pale fluorescence in the head, B pattern (capacitated) showed a strong fluorescence in the apical acrosome region, and for AR pattern (acrosome reacted), sperm exhibited no acrosome and showed intense fluorescence on the equatorial segment.

The percentage of spermatozoa showing signs of hyperactivation (HYP) was determined based on the methodology of Cañón-Beltrán et al. (2023) with minor alterations. The data obtained from the CASA analysis was restructured to identify the top 10% of spermatozoa exhibiting the highest VCL and ALH across all groups. The lowest VCL and ALH values were designated as non-hyperactivated within these groups. For this experiment with red-rumped agouti sperm the

values were: VCL = 65 $\mu\text{m/s}$ and ALH = 5.0 $\mu\text{m/s}$. Thus, we defined spermatozoon with VCL > 65 $\mu\text{m/s}$ and ALH > 5 $\mu\text{m/s}$ as showing hyperactive-like motility.

2.9. Oxidative stress evaluation

The levels of reactive oxygen species (ROS) and intracellular glutathione (GSH) were quantified using 10 μM 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Invitrogen, Carlsbad, CA, USA) and 10 μM 7-amino-4-chloromethylcoumarin (CellTracker Blue; Invitrogen, Carlsbad, CA, USA), respectively. Spermatozoa were incubated in the dark for 30 min at 37 °C in a dry bath. Subsequently, the samples underwent centrifugation twice (500× g, 5 min), were resuspended in phosphate-buffered saline (PBS), mounted in glass slides, and analyzed using fluorescence microscopy (400× magnification). Fluorescence intensity images were quantified with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) to determine ROS and GSH levels. The control group, consisting of fresh non-capacitated spermatozoa, served as a calibrator. The measured values of each treatment were normalized to the calibrator mean to generate relative expression levels (arbitrary fluorescence units, AFU) (Santos et al. 2023). Following the collection of AFU data, the ROS/GSH ratio was calculated to ascertain the oxidative status of the samples.

2.10. Statistical analysis

The results were expressed as mean $\pm$ standard error (one male/one replicate). Normality of residuals was assessed using the Shapiro-Wilk test, and variance homogeneity was evaluated via the Bartlett test. Percentile data were subjected to angular transformation [$\text{ARC-Sine}(\sqrt{x/100})$] to approximate a normal distribution and ensure stability. Due to repeated measures over time, the analyzed parameters (sperm kinetics and motility, membrane integrity, mitochondrial activity, membrane functionality, morphology, DNA integrity, oxidative stress

status, capacitation, acrosome reaction, and flagellar hyperactivation) were examined using a mixed linear model (mixed effect analysis procedure). This model included the main effects, different BSA profiles with or without CaCl_2 (HBSA *versus* HBCa *versus* LBSA *versus* LBCa), the incubation interval (1 h *versus* 3 h *versus* 6 h), and their interactions. The Tukey test was employed for mean comparison when significant effects were detected in the variance analysis (F-test). All statistical analyses were performed using GraphPad Prism[®] version 8 for Windows (GraphPad Software Inc., San Diego, CA, USA), with significance set at $p < 0.05$.

3. Results

All epididymal flushed samples exhibited a whitish color, with an average pH of 7.0 and a vigor score of 4.5 on a 0-5 scale. The mean sperm concentration was 252.3×10^6 spermatozoa/mL, with a range spanning from 179 to 290×10^6 spermatozoa/mL. Only samples demonstrating motility exceeding 80% were selected for subsequent analyses.

The statistic test for mixed effects showed a significant result of the main effects and the interaction (treatment $\times$ time) on the following sperm parameters: kinetics (BCF, STR, and LIN), normal morphology and tail defects, capacitation rate, acrosome reaction, ROS and GSH levels, ROS/GSH proportion, and hyperactivation analysis. Furthermore, the other variables exhibited no interaction effects, with observed differences attributable solely to the treatment conditions or the incubation time.

3.1. Non-capacitated spermatozoa characteristics

The results for the fresh (0 h) non-capacitated samples were as follows. Sperm kinetics are represented in **Table 1**. The data on mitochondrial activity and plasma membrane integrity revealed that $22.1\% \pm 13.1$ of spermatozoa exhibited an intact membrane and normal

mitochondrial activity, while $43.8\% \pm 13.7$ showed no mitochondrial activity. Additionally, $3.0\% \pm 1.5$ had compromised membrane integrity, and $31.0\% \pm 13.3$ were completely damaged. In summary, $66.0\% \pm 14.8$ of the sperm cells experienced mitochondrial activity loss, and $25.1\% \pm 12.5$ exhibited plasma membrane damage. Furthermore, the membrane functionality rate of these fresh samples was $48.6\% \pm 10.4$.

Table 1. Computer-aided sperm analysis of fresh (0 h) non-capacitated samples of red-rumped agouti epididymal sperm

CASA parameters	(mean $\pm$ SE)
Total motility (%)	91.8 ± 3.3
Progressive motility (%)	43.3 ± 12.6
VAP ($\mu\text{m/s}$)	80.8 ± 19.1
VSL ($\mu\text{m/s}$)	68.9 ± 18.0
VCL ($\mu\text{m/s}$)	111.6 ± 20.3
ALH ($\mu\text{m/s}$)	4.9 ± 0.1
BCF (Hz)	37.0 ± 1.1
STR (%)	76.1 ± 3.7
LIN (%)	51.8 ± 6.1
Rapid (%)	51.3 ± 14.8
Medium (%)	38.0 ± 11.3
Static (%)	10.6 ± 3.7

SE: standard error. VAP: velocity average pathway; VSL: velocity straight line; VCL: curvilinear velocity; ALH: amplitude of lateral head; BCF: beat cross frequency; STR: straightness; LIN: linearity. Sperm population: rapid, medium or static.

Regarding spermatozoa morphology, $89.8\% \pm 1.6$ presented normal characteristics, and the abnormal sperm presented defects in the head ($3.3\% \pm 6.0$), middle piece ($1.8\% \pm 0.8$), and tail ($5.0\% \pm 1.2$). The DNA damage rate results for the non-capacitated sperm: intact DNA ($90.0\% \pm 2.6$), low damage ($2.8\% \pm 1.0$), medium damage ($4.3\% \pm 1.5$), and high damage ($2.8\% \pm 1.1$). In addition, the ROS ($1.00 \text{ AFU} \pm 0.2$), GSH ($1.00 \text{ AFU} \pm 0.2$), and ROS/GSH proportion ($1.00 \text{ AFU} \pm 0.2$) were also normal.

Finally, for the capacitation analysis, only $20.2\% \pm 2.4$ sperm were spontaneously capacitated after recovery, and $16.3\% \pm 1.9$ had a spontaneous acrosome reaction. The percentage of fresh spermatozoa with hyperactive flagella was $33.0\% \pm 0.2$.

3.2. Effects of medium x incubation time on sperm morphology and functionality

Sperm kinetics (**Figure 2**) showed differences in all parameters ($P < 0.05$), except ALH ($P > 0.05$). The total motility was higher in media with CaCl_2 regardless of time and BSA profile ($P < 0.05$), ranging from $81.3\% \pm 4.1$ (1 h) to $50.0\% \pm 10.6$ (6 h) for the HBCa and $86.5\% \pm 4.4$ (1 h) to $74.8\% \pm 5.7$ (6 h) for LBCa (**Figure 2A**). The progressive motility results were similar, with better results only in CaCl_2 groups ($P < 0.05$); however, only LBCa maintained the progression high for 1 h ($49.8\% \pm 8.0$), 3 h ($41.8\% \pm 6.7$), and 6 h ($43.5\% \pm 4.2$) (**Figure 2B**, $P < 0.05$). For VAP, VSL, and VCL (**Figure 2C, 2D, 2E**), all treatments presented similar values at 1 h ($P > 0.05$), but HBCa and LBCa maintained the average velocity also at 3 h and 6 h ($P < 0.05$). BCF, STR, and LIN (**Figure 2G, 2H, 2I**) remained stable in all groups during the 6 h incubation ($P > 0.05$), except for HBSA, which showed low values after 1 h ($P < 0.05$). Finally, the rapid sperm population (**Figure 2J**) was higher in media with CaCl_2 regardless of incubation time ($P < 0.05$). Still, the static population (**Figure 2L**) was higher in HBCa compared to LBCa after 6 h ($P < 0.05$).

Sperm kinetics

1 h 3 h 6 h

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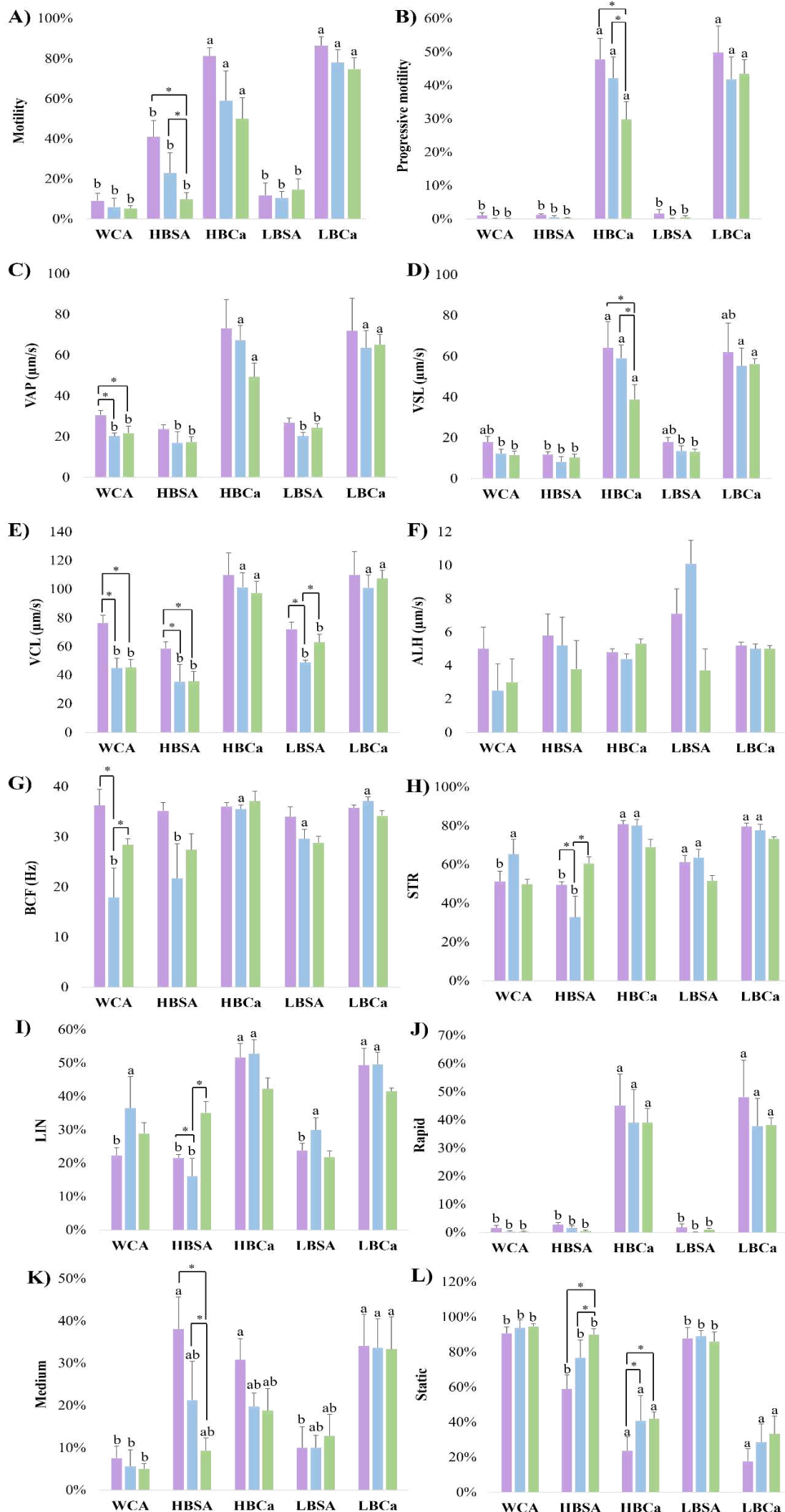


Figure 2. Efficiency of different capacitation protocols of epididymal sperm kinetic parameters of red-rumped agouti. **(A)** Total motility rate. **(B)** Progressive motility rate. **(C)** Average pathway velocity. **(D)** Straight-line velocity. **(E)** Curvilinear velocity. **(F)** Amplitude of lateral head displacement. **(G)** Beat/cross-frequency. **(H)** Straightness rate. **(I)** Linearity rate. **(J)** Rapid sperm population rate. **(K)** Medium sperm population rate. **(L)** Static sperm population rate. Letters indicate differences between treatments within the same time. Asterisk indicates differences between times within the same treatment. $P < 0.05$.

The incubation time did not affect sperm mitochondrial activity and plasma membrane integrity rates (**Table 2**). However, between treatments, both HBCa and LBCa maintained these parameters ($P < 0.05$). In addition, after 6 h of sperm capacitation, all treatments presented a higher number of sperm cells with absent mitochondrial activity, except HBCa and LBCa ($P < 0.05$). Either time or different capacitation media did not affect membrane functionality ($P > 0.05$) (**Table 3**).

Sperm cells presented around 90% normal morphology rates after capacitation (**Figure 3**) for all treatments for 1 h and 3 h ($P > 0.05$). However, the HBCa and LBSA groups showed a 10-15% reduction at 6 h (**Figure 3A**, $P < 0.05$). There were no differences in the head and middle piece ($P > 0.05$) among the morphological abnormalities found, but tail defects (**Figure 3D**) were more than triple after 6 h in the low BSA without CaCl_2 ($P < 0.05$).

Sperm capacitation conditions in the DNA damage rate affected none of the experimental groups (**Figure 4**, $P > 0.05$). Even after 6 h of incubation, the rates of intact DNA were above 90% while those of low, medium, and high damage were only 1-3% of the sperm population ($P > 0.05$).

Table 2. Effects of different capacitation conditions on mitochondrial activity and plasma membrane integrity of red-rumped agouti sperm.

Groups	Sperm mitochondrial activity and plasma membrane integrity		
	Capacitation times		
	1 h	3 h	6 h
<i>Intact membrane + functional mitochondria</i>			
WCA	20.5 ± 9.4 ^b	14.1 ± 10.6 ^b	29.5 ± 9.7 ^a
HBSA	45.1 ± 7.2 ^b	46.5 ± 7.2 ^{ab}	38.3 ± 3.5 ^a
HBCa	75.6 ± 4.0 ^a	76.8 ± 3.7 ^a	70.3 ± 13.3 ^a
LBSA	37.3 ± 7.5 ^b	33.5 ± 9.5 ^{ab}	37.0 ± 7.1 ^a
LBCa	76.8 ± 3.9 ^a	69.1 ± 3.7 ^a	65.6 ± 12.8 ^a
<i>Intact membrane + non-functional mitochondria</i>			
WCA	44.6 ± 13.2 ^a	24.5 ± 10.9 ^a	26.0 ± 10.0 ^b
HBSA	20.6 ± 11.2 ^a	23.3 ± 11.5 ^a	31.6 ± 6.5 ^b
HBCa	10.1 ± 5.3 ^a	7.3 ± 1.5 ^a	3.0 ± 1.0 ^a
LBSA	40.1 ± 11.0 ^a	33.1 ± 11.4 ^a	23.0 ± 5.7 ^b
LBCa	10.8 ± 4.8 ^a	9.8 ± 4.0 ^a	9.0 ± 4.4 ^a
<i>Ruptured membrane + functional mitochondria</i>			
WCA	8.6 ± 5.1 ^a	16.0 ± 9.2 ^a	0.1 ± 0.1 ^a
HBSA	0.5 ± 0.3 ^a	1.3 ± 0.6 ^a	1.5 ± 0.7 ^a
HBCa	0.0 ± 0.0 ^a	1.8 ± 1.6 ^a	11.1 ± 11.1 ^a
LBSA	1.6 ± 0.9 ^a	0.6 ± 0.3 ^a	2.1 ± 1.1 ^a
LBCa	0.3 ± 0.3 ^a	0.1 ± 0.1 ^a	8.0 ± 8.0 ^a
<i>Ruptured membrane + non-functional mitochondria</i>			
WCA	26.1 ± 7.2 ^a	45.3 ± 12.8 ^a	44.5 ± 16.4 ^a
HBSA	33.6 ± 7.0 ^a	28.8 ± 6.5 ^a	28.5 ± 6.3 ^a
HBCa	14.1 ± 3.7 ^a	14.0 ± 2.6 ^a	15.5 ± 2.9 ^a
LBSA	20.8 ± 8.0 ^a	32.6 ± 13.0 ^a	37.8 ± 8.7 ^a
LBCa	12.0 ± 1.3 ^a	20.8 ± 3.7 ^a	17.3 ± 6.3 ^a
<i>Intact membrane/total</i>			
WCA	65.1 ± 11.3 ^a	38.6 ± 16.3 ^a	55.5 ± 16.6 ^a
HBSA	65.8 ± 6.9 ^a	69.8 ± 6.8 ^a	70.0 ± 7.0 ^a
HBCa	85.8 ± 3.7 ^a	84.1 ± 3.6 ^a	73.3 ± 13.7 ^a
LBSA	77.5 ± 8.8 ^a	66.6 ± 13.2 ^a	60.0 ± 9.2 ^a
LBCa	87.6 ± 1.4 ^a	79.0 ± 3.8 ^a	74.6 ± 14.2 ^a
<i>Functional mitochondria /total</i>			
WCA	29.1 ± 8.4 ^b	30.1 ± 10.9 ^b	29.6 ± 9.6 ^b
HBSA	45.6 ± 6.9 ^b	47.8 ± 7.2 ^b	39.8 ± 3.3 ^b
HBCa	75.6 ± 4.0 ^a	78.6 ± 2.9 ^a	81.5 ± 2.6 ^a
LBSA	39.0 ± 7.3 ^b	34.1 ± 9.3 ^b	39.1 ± 7.3 ^b
LBCa	77.1 ± 4.0 ^a	69.3 ± 3.6 ^a	73.6 ± 5.5 ^a

mean ± standard error. Lowercase letters indicate differences between treatments within the same time ($P < 0.05$). WCA: without capacitation agent. HBSA: MCM + 15 mg/mL BSA. HBCa: MCM + 15 mg/mL BSA + 2 mM CaCl_2 . LBSA: MCM + 4 mg/mL BSA. LBCa: MCM + 4 mg/mL BSA + 2 mM CaCl_2 .

Table 3. Evaluation of epididymal sperm membrane functionality in red-rumped agouti after capacitation in different conditions.

HOST (% $\pm$ SE)				
Groups	Capacitation times			
	1 h	3 h	6 h	
WCA	70.8 $\pm$ 11.7	79.6 $\pm$ 3.7	75.8 $\pm$ 1.3	
HBSA	72.1 $\pm$ 6.0	67.0 $\pm$ 7.9	72.3 $\pm$ 5.0	
HBCa	88.1 $\pm$ 2.4	77.5 $\pm$ 5.8	78.5 $\pm$ 4.6	
LBSA	77.6 $\pm$ 6.1	69.0 $\pm$ 7.0	73.0 $\pm$ 7.1	
LBCa	88.6 $\pm$ 1.7	85.3 $\pm$ 2.9	84.3 $\pm$ 1.5	

SE: standard error. $P > 0.05$. HOST: hypoosmotic test. WCA: without capacitation agent.

HBSA: MCM + 15 mg/mL BSA. HBCa: MCM + 15 mg/mL BSA + 2 mM CaCl_2 . LBSA: MCM + 4 mg/mL BSA. LBCa: MCM + 4 mg/mL BSA + 2 mM CaCl_2 .

Sperm cells presented around 90% normal morphology rates after capacitation (**Figure 3**) for all treatments for 1 h and 3 h ($P > 0.05$). However, the HBCa and LBSA groups showed a 10-15% reduction at 6 h (**Figure 3A**, $P < 0.05$). There were no differences in the head and middle piece ($P > 0.05$) among the morphological abnormalities found, but tail defects (**Figure 3D**) were more than triple after 6 h in the low BSA without CaCl_2 ($P < 0.05$).

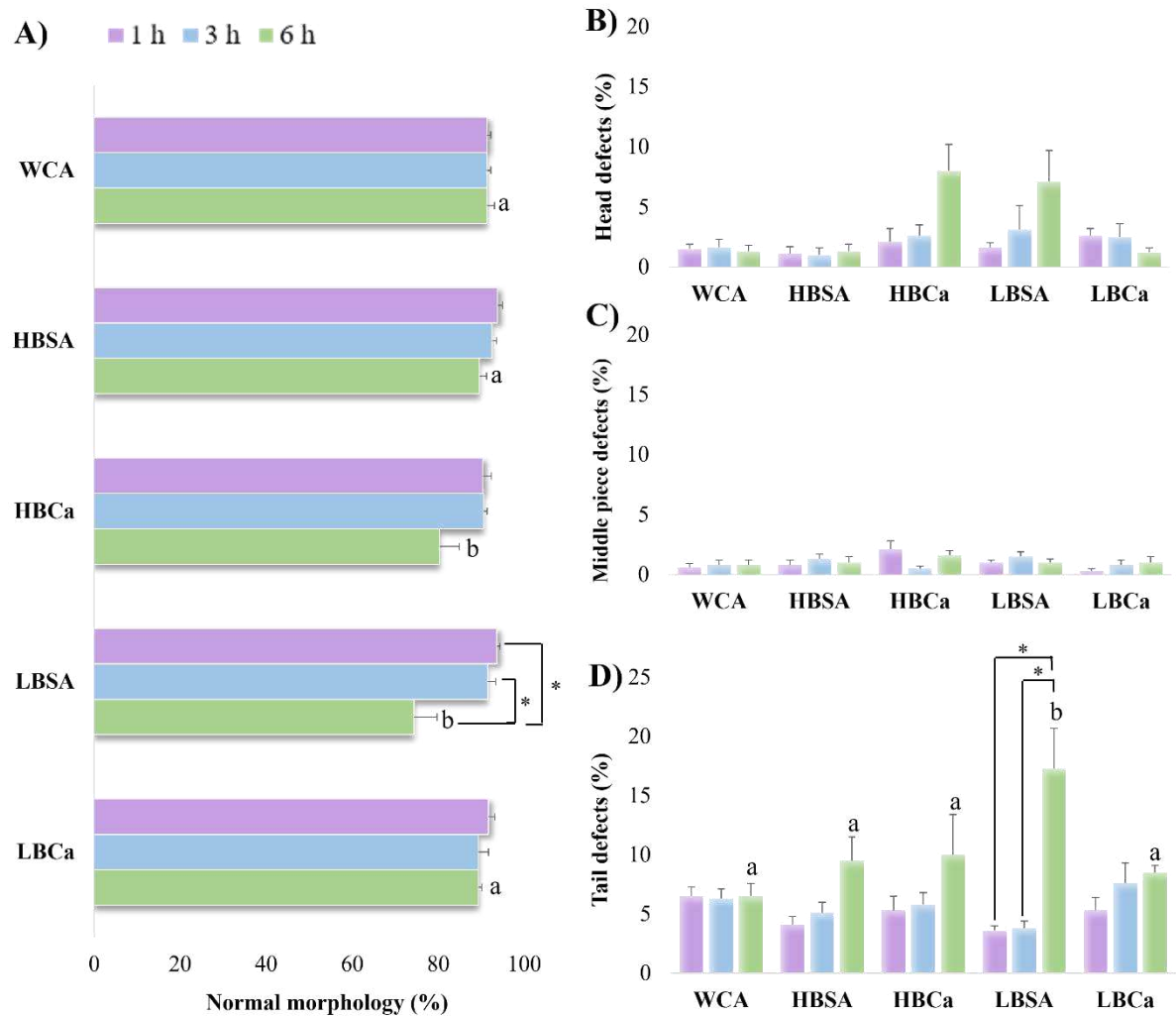


Figure 3. Effects of different capacitation media and incubation times for morphology characteristics of the sperm of red-rumped agouti. **(A)** Percentage of the sperm population with normal morphology after capacitation. **(B)** Rate of head defects in agouti sperm. **(C)** Middle piece defects rate. **(D)** Tail abnormalities rate in capacitated sperm. Letters indicate differences between treatments within the same time. Asterisk indicates differences between times within the same treatment. $P < 0.05$.

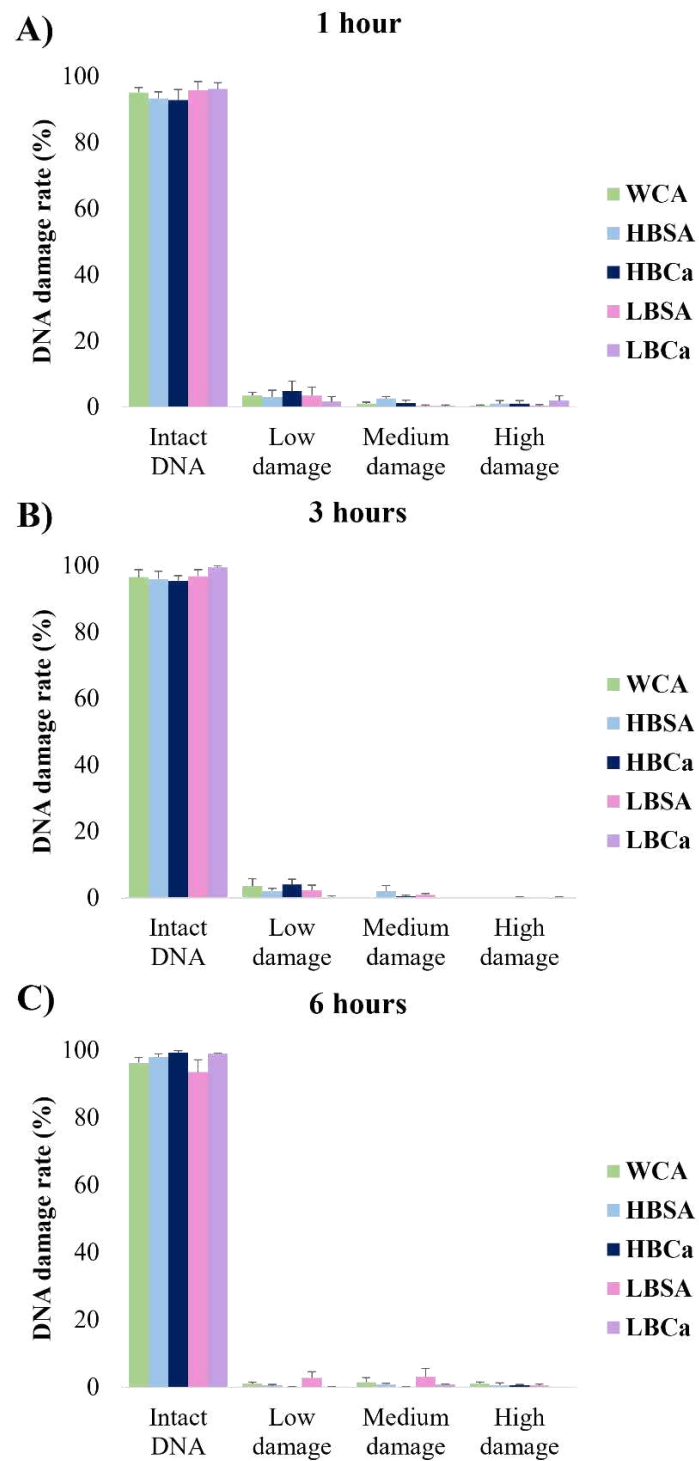


Figure 4. DNA damage index of the sperm of red-rumped agouti after different capacitation protocols. (A) Percentage of intact, low, medium, and high damage on the DNA strand after 1 h, (B) 3 h, and (C) 6 h of incubation. $P > 0.05$.

The oxidative stress status after capacitation was determined by the ROS and GSH levels, which showed significant differences between the capacitation media and the incubation times ($P < 0.05$). The sperm cells capacitated with different BSA profiles with CaCl_2 (HBCa and LBCa) maintained low levels of reactive oxygen species (**Figure 5A**) during 1 h, 3 h, or 6 h of incubation ($P < 0.05$). They were more efficient than all other groups ($P < 0.05$). WCA and HBSA showed higher ROS levels ($P < 0.05$) that increased significantly over 3 h and 6 h ($P < 0.05$). The LBSA showed higher ROS levels than HBCa and LBCa ($P < 0.05$), but this number did not increase as the hours passed ($P > 0.05$). For the intracellular glutathione (**Figure 5B**), LBCa presented the highest levels compared to the other treatments ($P < 0.05$), but this value decreased to 6 h ($P < 0.05$). The ROS/GSH proportion (**Table 4**) showed that LBSA, HBCa, and LBCa presented an almost zero proportion, indicating that low levels of ROS were always linked to high levels of GSH, while the WCA and HBSA showed significantly higher numbers for this proportion ($P < 0.05$). In addition, the WCA and HBSA proportion increased over time ($P < 0.05$), while the others managed to maintain their numbers even after 6 h of capacitation ($P > 0.05$).

All treatments, besides the WCA, improved sperm capacitation rate (**Figure 6A-B**) at 1 h ($P < 0.05$). After 3 h, only HBCa, LBSA, and LBCa maintained higher capacitation rates ($P < 0.05$). Finally, after 6 h, the best group for sperm cells was the LBCa, reaching around 90.7% of capacitation ($P < 0.05$). The data for acrosome-reacted sperm (**Figure 6C**) showed that the LBCa was superior to all treatments and the peak response occurred after 6 h ($P < 0.05$). In addition, the status of hyperactive sperm (**Figure 6D**) was higher for both CaCl_2 groups (HBCa and LBCa) after 6 h ($P < 0.05$), with similar data to both WCA and LBSA at 1 h ($P > 0.05$).

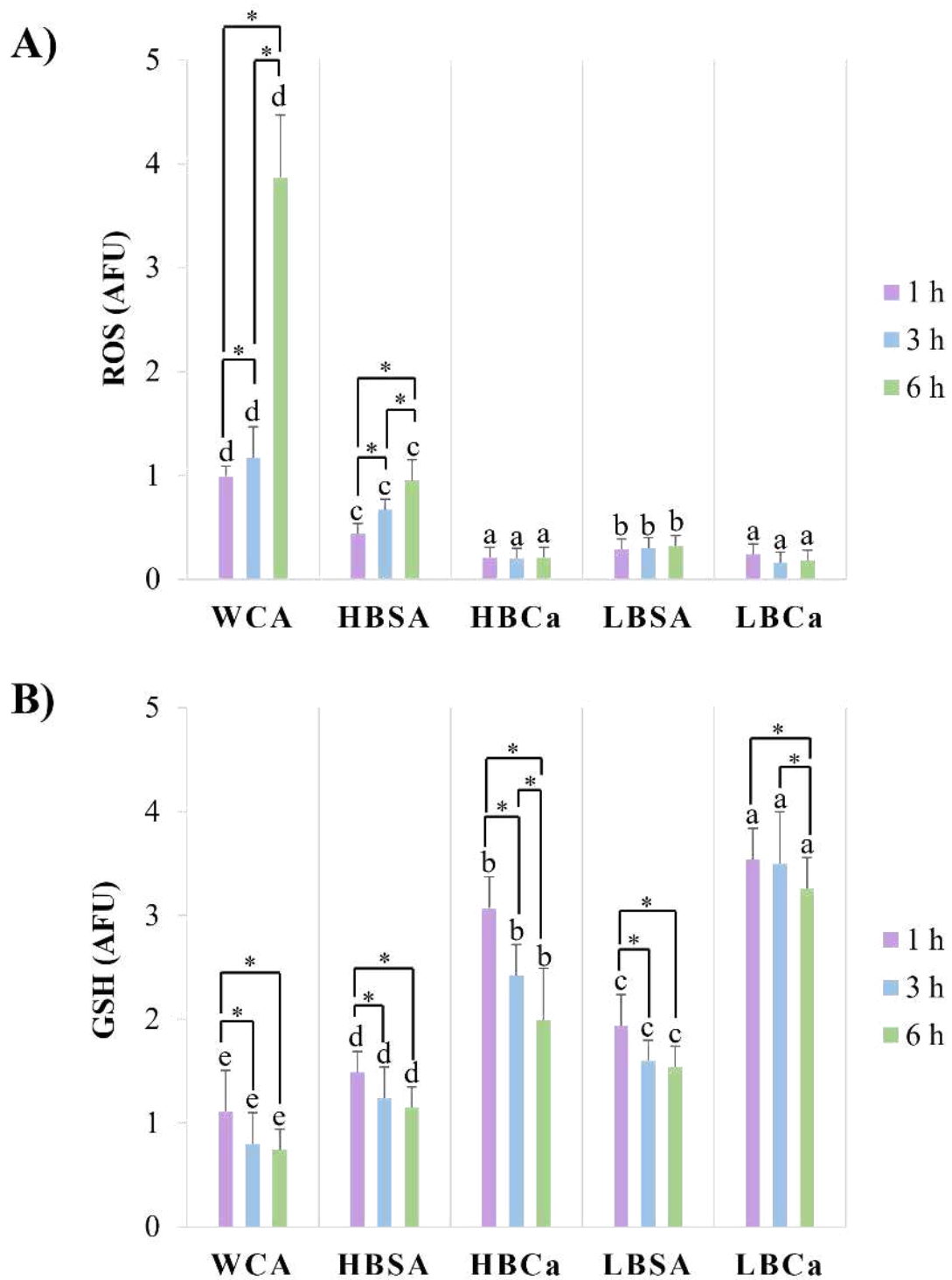


Figure 5. Evaluation of oxidative stress status after different sperm capacitation protocols in red-rumped agouti. (A) ROS levels in sperm capacitated in different media after 1, 3, and 6 h. (B) GSH levels in sperm were capacitated in different medias after 1, 3, and 6 h. Letters indicate differences between treatments within the same time. Asterisk indicates differences between times within the same treatment. $P < 0.05$.

Table 4. ROS/GSH proportion of red-rumped agouti spermatozoa cells after different capacitation treatments and incubation times

ROS/GSH proportion (mean $\pm$ SE)			
Groups	Capacitation times		
	1 h	3 h	6 h
WCA	0.93 $\pm$ 0.1 ^{cA}	1.50 $\pm$ 0.2 ^{cB}	5.41 $\pm$ 0.6 ^{cC}
HBSA	0.30 $\pm$ 0.1 ^{bA}	0.57 $\pm$ 0.1 ^{bA}	0.85 $\pm$ 0.1 ^{bB}
HBCa	0.07 $\pm$ 0.1 ^{aA}	0.09 $\pm$ 0.1 ^{aA}	0.11 $\pm$ 0.1 ^{aA}
LBSA	0.15 $\pm$ 0.1 ^{aA}	0.18 $\pm$ 0.1 ^{aA}	0.21 $\pm$ 0.1 ^{aA}
LBCa	0.07 $\pm$ 0.1 ^{aA}	0.05 $\pm$ 0.1 ^{aA}	0.05 $\pm$ 0.1 ^{aA}

SE: standard error. $P < 0.05$. Lowercase letters indicate differences between treatments within the same time. Uppercase letters indicate differences between times within the same treatment. WCA: without capacitation agent. HBSA: MCM + 15 mg/mL BSA. HBCa: MCM + 15 mg/mL BSA + 2 mM CaCl_2 . LBSA: MCM + 4 mg/mL BSA. LBCa: MCM + 4 mg/mL BSA + 2 mM CaCl_2 .

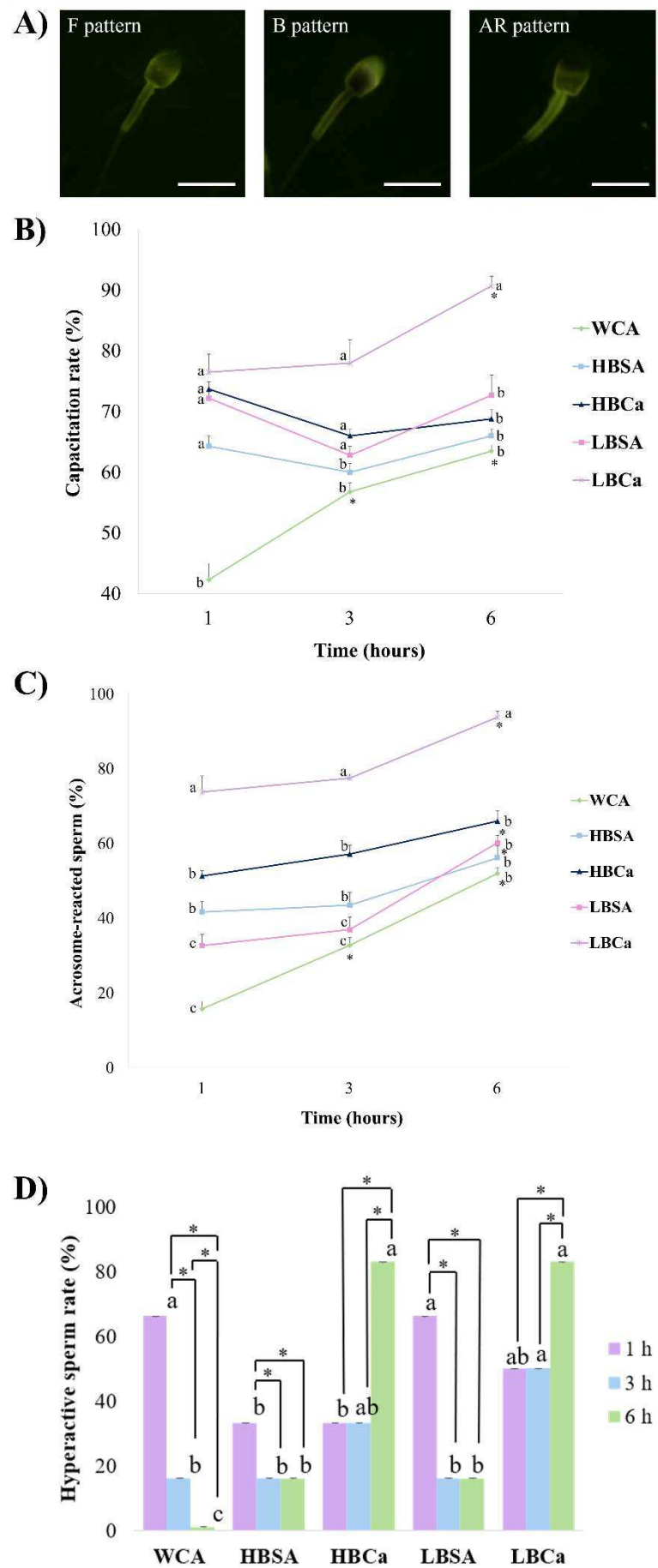


Figure 6. Capacitation, acrosome-reaction, and hyperactive rates after different treatments for the epididymal sperm of red-rumped agouti. **(A)** Representative image of CTC-stained sperm cells for capacitation and AR evaluation. F pattern (non-capacitated) displayed a pale fluorescence in the head, B pattern (capacitated) showed a strong fluorescence in the apical acrosome region, and for the AR pattern (acrosome reacted sperm), sperm exhibited no acrosome and showed intense fluorescence on the equatorial segment. Scale bar: 100 μ M. 100 $\times$ magnification. **(B)** Capacitation rates of agouti sperm after 1, 3, and 6 hours in different media. **(C)** AR rates after different protocols of capacitation in agouti. **(D)** Hyperactive sperm percentage after treatments and incubation times. Letters indicate differences between treatments within the same time. Asterisk indicates differences between times within the same treatment. $P < 0.05$.

4. Discussion

The results of this study showed that a lower BSA concentration (4 mg/mL) associated with 2 mM calcium chloride (LBCa group) during 6 h incubation is the most effective protocol for capacitating epididymal spermatozoa of red-rumped agouti. This protocol-maintained sperm progressive motility, membrane viability, morphological integrity, and normal mitochondrial activity while promoting reduced ROS production and elevated GSH levels. Additionally, it yielded a higher proportion of capacitated spermatozoa exhibiting acrosome reaction and hyperactive motility.

The optimal sperm kinetics parameters, including total motility, progressive motility, linearity, and curvilinear velocity, may serve as valuable prognostic indicators of sperm fertilization potential (Vallet-Buisan et al. 2023). The maintenance of these characteristics *in vitro* may be directly linked to the environmental conditions in which these cells are found, such as the

medium, temperature, and incubation time (Sansegundo et al. 2022). Both groups highlighted in our research (HBCa and LBCa) have two crucial agents for obtaining sperm movement: calcium and BSA.

Calcium (Ca^{2+}) is a fundamental messenger that contributes to sperm motility, related to crucial signaling pathways, such as cAMP/protein kinase A and phosphoinositide 3-kinase signaling (Dcunha et al. 2022). Likewise, Ca^{2+} has already been reported as essential for sperm motility initiation in mammals (Carroll 2001) and is directly linked to the functioning of the CatSper channels, which is most likely involved in the motility process since CatSper mutant mouse sperm cannot penetrate coat-intact eggs during IVF (Ren et al. 2001). The BSA modulates all these actions since it can induce Ca^{2+} influx in sperm (Xia et al. 2009).

Maintaining membrane integrity was more significant within the HBCa and LBCa groups, regardless of incubation time. This information is relevant because ruptured membranes have previously been associated with DNA fragmentation and chromatin decondensation, indicative of early apoptotic processes (Bloch et al. 2021). Therefore, BSA may have acted protectively, with a rapid membrane binding primarily aiding stabilization through interaction with phospholipids reducing membrane damage (Álvarez-Rodríguez et al. 2024).

In addition, the mitochondrial activity was also normal within the HBCa and LBCa groups. These results corroborate our findings, where the higher capacitation rates also occurred in the HBCa and LBCa groups, corroborating what has already been seen in previous studies that mitochondrial respiration increases during capacitation in mouse sperm (Balbach et al. 2020). However, after 6 h of incubation, all calcium-free groups significantly reduced mitochondrial activity. This result can be explained by the lack of calcium in the medium since the increase

in mitochondrial activity in capacitated sperm parallels the increase in mitochondrial calcium concentration during the capacitation of rodent sperm (Ferreira et al. 2021).

After 6 h of incubation, the morphology abnormality was increased in the group with high BSA concentration (HBCa) compared to the group with low concentration (LBCa). These results are probably related to the properties of this protein since it facilitates the extraction of cholesterol from the sperm plasma membrane, thereby inducing phospholipid displacement and rearrangement, enhancing membrane fluidity (Mohanty et al. 2024). Therefore, a higher exposure for many hours may be prejudicial for red-rumped agouti sperm. The higher rate of morphological defects in the tail may also be directly correlated with the lower sperm velocity results in the group with the highest BSA concentration (HBCa) since the sperm tail supports the flagellar motility (Yogo 2022).

Several critical biological processes of mammalian spermatozoa are redox regulated, including capacitation, hyperactivation, and acrosomal exocytosis (Bennetts and Aitken 2005). Sperm produce and export ROS to the extracellular environment, most of which are generated by the mitochondria, secondary to the flagellar activity of the cells. The loss of sperm function, i.e., the fertilization capacity, could result from the presence of high levels of ROS (Parodi 2021). Only the groups with calcium maintained the lowest ROS levels for 6 h. Since motility generates ROS, it is hypothesized that regulation by calcium reduces excess motility and the general metabolic state of the cells, leading to a reduction of oxidative stress (Parodi 2021). The low ROS production may also be related to higher levels of the cellular antioxidant present, such as intracellular GSH in the LBCa groups, which could have its production facilitated by the protection guaranteed by the adequate concentration of BSA and CaCl_2 in the medium.

Capacitation is crucial for the sperm to acquire its fertilizing capacity. In guinea pigs, Ca^{2+} Cl^- and BSA are essential to increase capacitation rates, acrosomal reactions, and hyperactivated motility (Chen et al. 2009; Ni et al. 2009). These substances were also essential for red-rumped agouti in our study. Using a low concentration of 4 mg/mL BSA in association with 2 mM Ca^{2+} , we achieved higher capacitation rates of 90.7%, compared with approximately 60% rate in guinea pig with the same media components (Ni et al. 2009).

All treatments except WCA improved capacitation rates after 1 h of incubation. This may be explained by the presence of BSA in the media and the minimal time for this agent to operate, since BSA induces tyrosine phosphorylation by the modulation of protein kinase A (PKA), and the tyrosine phosphorylation is a slow process starting at ~45 min of incubation in capacitation media in mouse sperm (Visconti 2009). However, the maximum capacitation rate for red-rumped agouti was seen after 6 h incubation with the LBCa. These results highlight the species-specific time necessary for better capacitation. This was previously demonstrated in guinea pig. When using 1.71 mM CaCl_2 , different authors achieved rates from 34.2% (Delgado-Buenrostro et al. 2016) to 80% (Cordero-Martínez et al. 2018), increasing the sperm exposure periods in the capacitation medium of choice from 1 h to 2 h, respectively.

Another significant result was the maximum acrosome-reacted sperm rate after 6 h in LBCa. This phenomenon is necessary to penetrate the zona pellucida so the sperm can fuse with the oocyte. The AR is activated by calcium ions, as demonstrated in mice, the AR and the ability to achieve fertilization do not occur in Ca^{2+} free capacitation media (Navarrete et al. 2015). In addition, a higher rate of AR before fertilization is desired in rodents, as seen by Jin et al. (2011) in mouse when investigating the effect of AR on IVF outcomes, finding that the spermatozoa that began the AR before reaching the zona penetrates the zona and fused with the oocyte's

plasma membrane. Also, the most fertile sperm cells underwent an AR before reaching the zona pellucida of *cumulus*-enclosed oocytes.

Finally, the higher hyperactivation motility rates in the LBCa and HBCa groups could be related to the calcium in the media. Since an increase in the intracellular calcium level (higher than 200 nM) is needed for the spermatozoa to attain hyperactivated motility in the female reproductive tract (Dcunha et al. 2022), the same must be true for the *in vitro* environment. Therefore, the 2 mM implement for red-rumped agouti sperm was enough to trigger a good HYP after capacitation.

5. Conclusion

The findings indicate that, during the *in vitro* capacitation of epididymal spermatozoa from the red-rumped agouti, the presence of CaCl₂ is indispensable for maintaining cell viability and functionality, while also reducing ROS production and achieving high rates of capacitation, acrosome reaction, and sperm hyperactivity. Moreover, a low concentration of BSA at 4 mg/mL proved to be the most effective in maintaining sperm morphology and enhancing capacitation outcomes. A 6-h incubation seems to be the most suitable for this species, particularly when combined with the LBCa medium, which promoted the highest capacitation and acrosome reaction rates. Further research is essential to determine whether these capacitated spermatozoa possess enhanced fertilizing capacity with oocytes during IVF. This study represents the first comparative analysis of *in vitro* sperm capacitation supplementations in the red-rumped agouti, marking a significant advance in understanding the species' sperm physiology and contributing to the refinement of ARTs in wild rodents.

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Author Contributions

LRMO: experimental design, data collection, data evaluation, statistical analysis, and writing. **LVCA:** data collection. **ABMP:** data collection. **ALRR:** data collection. **LLVR:** data collection. **LGPB:** animal manipulation and data collection. **RPS:** data evaluation and statistical analysis. **MFO:** animal management and manipulation. **ARS:** supervision and critical revision of the article. **AFP:** experimental design, data analysis, experiment supervision, secured funding, critical revision of the article, and manuscript drafting as the corresponding author. All authors reviewed and approved the final manuscript.

Conflict of interest

The authors have no conflict of interest to disclose.

Data availability statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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2908 **CAPÍTULO 5 - EFFECTS OF FERTILIZATION MEDIA ON *IN VITRO* EMBRYONIC**
2909 **DEVELOPMENT IN RED-RUMPED AGOUTI (*Dasyprocta leporina*) USING A**
2910 **HETEROLOGOUS SYSTEM WITH GUINEA PIG OOCYTES**

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2914 **ARTIGO EXPERIMENTAL 3**

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2916 **SUBMETIDO NA REPRODUCTION IN DOMESTIC ANIMALS**

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2919 **Qualis 2017-2020:** A3 (Medicina Veterinária)

2920 **Fator de impacto:** 1.6

2921 **Data de submissão:** 08/04/2025

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Effects of fertilization media on *in vitro* embryonic development in red-rumped agouti (*Dasyprocta leporina*) using a heterologous system with guinea pig oocytes

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Running title: Embryo development in agouti using heterologous fertilization

Contents

The red-rumped agouti is an ecologically and economically important wild rodent. Among efforts to aid in this species' reproduction, heterologous *in vitro* fertilization (he-IVF) serves as a powerful tool for investigating gamete interactions and early embryonic development under controlled conditions. However, the variability of he-IVF success emphasizes the need to optimize key parameters, especially the IVF medium, which plays a critical role in providing the necessary support to the gametes throughout the co-incubation. Therefore, this study aimed to investigate the impact of different he-IVF media on embryonic development outcomes of

red-rumped agoutis using guinea pig oocytes. Selected and capacitated epididymal sperm from red-rumped agoutis were incubated with matured guinea pig oocytes in three he-IVF media: human tubal fluid (HTF), Toyoda-Yokoyama-Hoshi (TYH), and Tyrode's albumin lactate pyruvate (TALP). Following a 6-hour fertilization, the resulting structures were assessed for morphology, oocyte-sperm interaction, developmental kinetics, and oxidative stress levels. The TALP medium significantly enhanced cleavage rates ($P < 0.05$) and yielded a higher proportion of embryos with ≥ 8 cells on both D2 and D5 of culture ($P < 0.05$). Additionally, embryos fertilized in TALP exhibited lower oxidative stress, indicated by reduced reactive oxygen species levels and mitochondrial membrane potential ($P < 0.05$). These findings suggest that TALP is the most effective medium for he-IVF in red-rumped agouti sperm and may hold potential for optimizing homologous IVF protocols to enhance embryonic development in this species.

Keywords: Wildlife, reproductive biology, gamete interaction, *in vitro* fertilization, epididymal sperm.

Introduction

The red-rumped agouti (*Dasyprocta leporina* Linnaeus, 1758) is a wild rodent species with a stable population (Patton and Emmons, 2015) that holds significant ecological importance (Hadler et al., 2016) as a seed disperser and plays an economic role in the South American commerce (Hosken, 2001). Consequently, ongoing efforts are focused on refining assisted reproductive strategies, including *in vitro* fertilization (IVF), to enhance reproductive efficiency and advance our understanding of reproductive physiology (Mochida et al., 2014).

Following the initial phase of obtaining sperm samples from wild animals for IVF studies, the subsequent challenge lies in collecting a sufficient number of viable oocytes. This remains a significant obstacle due to the limited research available on optimizing *in vivo* oocyte retrieval techniques in wild species (Swanson, 2023). In this context, heterologous *in vitro* fertilization (he-IVF) offers a promising alternative for studying *in vitro* interactions, using oocytes from domestic species and sperm cells of wild animals (Cañón-Beltran et al., 2023). The controlled environment of he-IVF allows us to assess valuable insights into cellular interactions during co-incubation and subsequent stages of embryonic development (Anifandis et al., 2014). Additionally, these findings could be applied to future homologous IVF in wild species.

Considering that the efficacy of he-IVF varies significantly among rodent species (Horie et al., 2015; Banafshi et al., 2021), it is essential to optimize key parameters, including sperm concentration, co-incubation duration, and, most importantly, the selection of an appropriate medium. These he-IVF media play a critical role in providing the necessary energy, protein, and ionic support to maintain gamete viability throughout co-incubation (Sciorio, & Rinaudo, 2023), thereby fostering an environment that facilitates the essential metabolic processes required for successful fertilization and embryonic development (Swain et al., 2016). Additionally, these media help mitigate oxidative stress, which could be primarily induced by the sperm, preventing potential damage that could adversely affect subsequent stages of embryonic development (Castro et al., 2016).

Given their diverse compositions, he-IVF media can fulfill distinct functions across different species. For mouse embryo production, different concentrations of glucose, amino acids, sodium, and growth factors were found to reduce the percentage of blastocyst formation (Chronopoulou, & Harper, 2015). This explains why variations in embryo outcomes are

observed even among the most used media—Human Tubal Fluid (HTF), Toyoda-Yokoyama-Hoshi (TYH), and Tyrode’s Albumin Lactate Pyruvate (TALP). Suzuki and Fujinoki (2023) used TALP medium for IVF in *Mus musculus*, resulting in a 67% cleavage rate at the two-cell stage, but with no live births. In contrast, Mochida et al. (2014) used HTF medium, reporting 97% cleavage and successful births after embryo transfer. Similarly, González et al. (2024) found that the TYH medium resulted in lower fertilization rates and fewer blastocysts compared to HTF in mouse strands. However, Toyoda and Yokoyama (2016) demonstrated that increasing the volume of TYH medium during gamete co-incubation significantly improved fertilization success in the same species.

Therefore, this study aimed to elucidate the impact of IVF medium on the embryonic development of the red-rumped agouti using he-IVF model with guinea pig oocytes. This research seeks to contribute to the understanding of the reproductive physiology of this species and generate data that could support the future development of homologous IVF techniques for this wild rodent species.

Materials and methods

The chemicals were procured from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified.

Bioethics and experimental design

All experimental procedures were conducted in compliance with the guidelines established by the Animal Ethics Committee of the Federal Rural Semi-Arid (no. 20/2021) and Institute for Biodiversity Conservation (ICMBio, no. 76655-1).

To determine the medium for he-IVF using red-rumped agouti spermatozoa and guinea pig oocytes, both gametes were assessed to ensure high quality. Epididymal spermatozoa were subjected to evaluation, including assessments of kinetic parameters, membrane functionality and viability, mitochondrial activity, DNA integrity, and morphological normality. The oocytes were confirmed to have reached maturation based on the expansion of *cumulus* cells and extrusion of the first polar body. Subsequently, gametes were co-incubated in microdroplets containing one of three distinct media formulations: Human Tubal Fluid (HTF), Toyoda-Yokoyama-Hoshi (TYH), and Tyrode's Albumin Lactate Pyruvate (TALP) medium. Following a 6-hour incubation, presumptive zygotes were examined for morphological normality, interactions between oocytes and spermatozoa, kinetic parameters during early embryonic development up to the morula stage, and levels of oxidative stress throughout *in vitro* development (IVD).

Animals and recovery procedures

The red-rumped agoutis and guinea pigs were kept at the Center of Multiplication of Wild Animals (CEMAS, UFERSA, Brazil). The animals were provided with *ad libitum* standardized commercial rabbit diet and fresh drinking water and maintained under a 12-hour natural photoperiod cycle.

For the recovery of spermatozoa, four sexually mature male red-rumped agouti specimens (one male/replicate) were captured and premedicated with a combination of ketamine (15 mg/kg; Ketalar, Pfizer, São Paulo, Brazil) and xylazine (1 mg/kg; Rompun, Bayer, São Paulo, Brazil). After a 15-minute interval, anesthesia was induced through intramuscular injection of sodium thiopental at a dosage of 50 mg/kg (Thiopentax; Cristalia, São Paulo, Brazil). Euthanasia was subsequently performed via intravenous administration of potassium chloride at a dosage of 1

mL/kg (Equiplex, Goiânia, Goiás, Brazil) (Castelo et al., 2015). For oocyte retrieval, twelve adult female guinea pigs (three females/replicate) were captured, anesthetized, and euthanized with the same protocol as the red-rumped agoutis.

Sperm preparation for he-IVF

The testes-epididymis complex was retrieved immediately following euthanasia and immediately transported to the laboratory in a pre-warmed saline solution (37 °C, 0.15 M NaCl). The cauda epididymis region was carefully dissected, and epididymal spermatozoa were extracted via retrograde flushing using 1.0 mL of saline solution (0.15 M NaCl) (Castelo et al., 2015). The collected sperm samples were maintained in a water bath at 37 °C, while initial assessments of appearance, color, pH, vigor, and concentration were conducted.

The spermatozoa concentration was adjusted to 100×10^6 sperm/mL using a minimum capacitation medium (MCM) composed of 105.8 mM NaCl, 25 mM NaHCO₃, 5.56 mM glucose, 21.6 mM sodium lactate, 25 mM HEPES, 0.25 mM sodium pyruvate, 10 µg/mL phenol red, and 1% antibiotic-antimycotic solution. A centrifugation protocol was employed to isolate the most viable gametes. A 1:1 mixture of spermatozoa and MCM was transferred to a 15 mL plastic tube and subjected to two centrifugation cycles (300× g for 3 min, room temperature). Following each centrifugation, the supernatant was carefully discarded, and the resulting pellet was resuspended for the he-IVF (Rodrigues et al., 2024a).

Additionally, sperm capacitation was conducted simultaneously with the 6-hour he-IVF process. The capacitation agents employed included 4 mg/mL bovine serum albumin (BSA) and 2 mM calcium chloride (CaCl₂), both of which were incorporated into the he-IVF media

within droplets overlaid with mineral oil (Rodrigues et al., 2024b). The procedure was performed under controlled environmental conditions of 38.5 °C and 6.5% CO₂.

Oocyte preparation for he-IVF

The ovaries were collected, stored in pre-warmed saline solution (0.15 M NaCl, 37 °C), and immediately transported to the laboratory. All follicles were sliced under stereomicroscopic guidance to retrieve oocytes. Type I and II oocytes with one to four *cumulus* cell layers and homogeneous cytoplasm were selected for *in vitro* maturation (IVM) and matured in drops (100 µL) covered with mineral oil in a controlled atmosphere at 38.5 °C and 6.5% CO₂ for 24 h (Wang et al., 2019).

The oocyte maturation medium was composed of TCM199 supplemented with 2.2 g/L sodium bicarbonate, 25 mM HEPES, 0.2 mM sodium pyruvate, 100 µM cysteamine, 50 ng/mL epidermal growth factor (EGF), 10 µg/mL follicle-stimulating hormone associated with luteinizing hormone (FSH/LH; Pluset[®], Hertape Calier, Juatuba, MG, Brazil), 10% fetal bovine serum (FBS), and 1% antibiotic–antimycotic solution (Wang et al., 2019). Following 24-hour maturation, matured oocytes were washed and grouped (10–20 oocytes per droplet) in preparation for he-IVF with the prepared spermatozoa.

he-IVF and *in vitro* development (IVD)

The he-IVF droplets (50 µL) were prepared with three different IVF media: HTF, TYH, and TALP. These media were composed as follow: HTF medium (101.6 mM NaCl, 4.7 mM KCl, 0.37 mM KH₂PO₄, 21.6 mM Na lactate, 5.56 mM glucose, 25 mM NaHCO₃, 2 mM CaCl₂, 0.20 mM MgSO₄, 25 mM HEPES, 4 mg/mL BSA, 0.33 mM sodium pyruvate, 10 µg/mL phenol red, and 1% antibiotic–antimycotic solution) (Wigger et al., 2023), TYH medium (119.3 mM NaCl,

3112 4.7 mM KCl, 1.19 mM KH₂PO₄, 2.78 mM glucose, 25 mM NaHCO₃, 2 mM CaCl₂, 1.19 mM
 3113 MgSO₄, 4 mg/mL BSA, 1 mM sodium pyruvate, 2 µg/mL phenol red, and 1% antibiotic–
 3114 antimycotic solution) (Toyoda, & Yokoyama, 2016), and TALP medium (114 mM NaCl, 3.2
 3115 mM KCl, 0.35 mM NaH₂PO₄, 10 mM Na lactate, 25 mM NaHCO₃, 2 mM CaCl₂, 0.50 mM
 3116 MgSO₄, 10 mM HEPES, 4 mg/mL BSA, 0.11 mM sodium pyruvate, 10 µg/mL phenol red, and
 3117 1% antibiotic–antimycotic solution) (Rath et al., 1999).

3118

3119 The droplets covered with mineral oil were balanced at 38.5 °C in 6.5% CO₂ for approximately
 3120 30 min before adding the prepared sperm with a final concentration of 1.0×10^6 sperm/mL to
 3121 10-20 intact *cumulus*-enclosed matured oocytes (Cañón-Beltrán et al., 2021). Following a 6-
 3122 hour co-incubation, structures were gently washed and pipetted to eliminate residual
 3123 spermatozoa and *cumulus* cells.

3124

3125 The structures were transferred for IVD medium drops (50 µL) at 38.5 °C in 6.5% CO₂ with
 3126 Potassium Simplex Optimized Medium (KSOM: 95 mM NaCl, 2.5 mM KCl, 0.35 mM
 3127 KH₂PO₄, 10 mM Na lactate, 0.2 mM glucose, 25 mM NaHCO₃, 1.71 mM CaCl₂, 0.20 mM
 3128 MgSO₄, 21 mM HEPES, 0.01 mM phenol red, 0.2 mM sodium pyruvate, 0.01 mM EDTA, 1
 3129 mM L-glutamine, 15 mM BSA, 1% antibiotic–antimycotic solution, 1% essential amino acid
 3130 solution, 0.5% non-essential amino acid solution, and 10% FBS) (Lawitts, & Biggers, 1993).
 3131 After 48 h of culture (D2), 50% of the KSOM medium was changed. The total culture time was
 3132 120 h (D5) to evaluate development kinetics (Praxedes et al., 2023).

3133

3134 **Sperm, oocytes and zygotes evaluations**

All gametes used for the he-IVF were systematically sampled and assessed to confirm their viability and suitability for the procedure. Following fertilization, the resulting structures were analyzed to identify the most effective experimental group.

Sperm evaluation

The hypoosmotic swelling test (HOST) was performed using a solution consisting of distilled water (0 mOsm/L) with a sodium citrate and fructose solution (50 mOsm/L). Aliquots of 5 μ L containing epididymal spermatozoa were combined with 45 μ L of the hypoosmotic solution and incubated in a dry bath for 40 min at 37 °C. The samples were then assessed under a phase-contrast light microscope at 400 $\times$ magnification, with 100 cells analyzed per group. Spermatozoa displaying a swollen and coiled tail were identified as possessing a functionally intact membrane (Dantas et al., 2022a).

A 10 μ L aliquot of spermatozoa was incubated with 40 μ g/mL Hoechst 33342 (Molecular Probes, Eugene, OR, USA) at 37 °C for 5 min, followed by incubation with 0.5 mg/mL propidium iodide (Thermo Fisher Scientific, Waltham, MA, USA) and 500 nM CMXRos (Mito Tracker Red[®], F-7512, Molecular Probes, Eugene, OR, US) for 8 min. One hundred cells were evaluated in each experimental group using fluorescence microscopy (Olympus BX51TF, Tokyo, Japan) at 400 $\times$ magnification. Spermatozoa exhibiting a blue-stained head and a red-glowing midpiece were classified as possessing an intact plasma membrane and normal mitochondrial function (Santos et al., 2023).

The epididymal spermatozoa were evaluated using a computer-assisted sperm analysis (CASA) system (IVOS 7.4G; Hamilton-Thorne Research, MA, USA) with parameters previously established for red-rumped agouti. The settings included a temperature of 37 °C, a straightness

threshold of 30%, a minimum contrast of 45, a low-velocity average pathway (VAP) cutoff of 10 $\mu\text{m/s}$, and a medium VAP cutoff of 30 $\mu\text{m/s}$. Five independent and nonconsecutive microscopic fields were systematically examined. The following kinetic parameters were assessed: total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), curvilinear velocity (VCL, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %). The sperm population was further categorized into four distinct groups: rapid, medium, slow, and static (%) (Castelo et al., 2015).

Sperm samples were prepared as smears and allowed to air-dry. The slides were subsequently fixed in Carnoy's solution for 3 h, rinsed, and dried again at room temperature. They were then incubated for 25 min in a buffer solution consisting of 15 mM Na_2HPO_4 and 80 mM citric acid (pH 2.5) at 75 °C. The smears were stained with acridine orange (0.2 mg/mL) for 10 s, rinsed with distilled water, and covered with coverslip. A total of 100 cells were examined using fluorescence microscopy (400 $\times$; Olympus BX51TF, Tokyo, Japan). Spermatozoa with normal (double-stranded) DNA exhibited a green, fluorescent emission, while those with denatured or single-stranded DNA displayed yellow, orange, or red fluorescence, indicating progressively higher levels of DNA damage (Tomov et al., 2020).

For morphological assessment, a 10 μL aliquot of the sperm samples was fixed and stained using a formaldehyde-Bengal rose solution (Cromato®). The samples were then examined under a light microscope at 1000 $\times$ magnification, with 100 cells evaluated per slide. The spermatozoa were categorized into two groups: normal morphology and abnormal morphology. Abnormalities were further classified as defects in the head, midpiece, or tail regions (Silva et al., 2011).

3185

3186 *Oocyte evaluation*

3187 Following 24-hour maturation, *cumulus*-oocyte complexes (COCs) were examined under a
3188 stereomicroscope to evaluate the extent of *cumulus* cell expansion. Structures demonstrating
3189 pronounced expansion and mucification were classified as mature. The presence or absence of
3190 the first polar body (1PB) was documented after removing excess *cumulus* cells through
3191 pipetting post-IVF. Oocytes exhibiting clear evidence of first polar body extrusion were
3192 identified as mature (Wang et al., 2019).

3193

3194 *Zygotes evaluation*

3195 After a 6-hour IVF, each presumptive zygote was examined using an inverted microscope
3196 (Leipzig IMx 400, PhoenixOptics, Germany). The zygotes were carefully rotated to allow a
3197 clear assessment of their morphological features and the extent of cytoplasmic fragmentation.
3198 Those displaying optimal or desirable characteristics were classified as normal according to
3199 established criteria (Hesters et al., 2008).

3200

3201 The cleaved heterologous zygotes were cultured for 120 h (up to Day 5) to assess developmental
3202 kinetics using an inverted microscope (Leipzig IMx 400, PhoenixOptics, Germany). Cell
3203 cleavage was evaluated and categorized into three groups: two cells, three to seven cells, or
3204 eight or more cells. Additionally, the percentage of morulae formation was quantified on Day
3205 5, along with the ratio of morulae to total cleaved structures (Praxedes et al., 2023).

3206

3207 The non-cleaved cells were analyzed for oocyte-sperm interaction by staining with Hoechst
3208 33342 (10 µg/mL, 30 min) and examined under a fluorescence microscope. The number of

spermatozoa attached to the zona pellucida, and the quantity associated with each oocyte were quantified, providing evidence of both monospermy and polyspermy (Santos et al., 2023).

To assess the oxidative stress response, reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi_m$) were quantified using 10 μ M 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Invitrogen, Carlsbad, CA, USA) and 500 nM MitoTracker Red[®] (Molecular Probes, Eugene, OR, USA), respectively. Both presumptive zygotes and morulae were incubated in the dark with the respective probes for 30 min at 38.5 °C under 6.5% CO₂. Subsequently, the samples were placed on glass slides in microdroplets, and images were captured using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). Fluorescence intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The control group served as the calibrator, and the fluorescence values of each treatment were normalized to the mean of the calibrator to generate relative expression levels, expressed in arbitrary fluorescence units (AFU) (Santos et al., 2023).

Statistical analysis

All data were expressed as mean $\pm$ standard error (one male/one replicate) and analyzed using the GraphPad software (GraphPad Software Inc., La Jolla, CA, USA). All results were verified for normality using the Shapiro-Wilk test and for homoscedasticity using Levene's test. Sperm interactions with oocytes and zygotes' oxidative stress evaluations did not show a normal distribution; therefore, they were arcsine transformed and analyzed by ANOVA, followed by the Tukey test. All other data were compared with a chi-squared test. Significance was set at $P < 0.05$.

Results

All flushed epididymal sperm samples exhibited a whitish color, with an average pH of 7.0 and a vigor score of 4.5 on a 0-5 scale. The mean concentration following selection was 53.0×10^6 sperm/mL. Furthermore, 24 ovaries were recovered, yielding 401 viable oocytes, which corresponds to an average of 16.7 ± 3.7 viable oocytes per ovary or 33.4 ± 7.5 viable oocytes per female.

Sperm and oocyte preparation for he-IVF

The CASA analysis demonstrated that the sperm used in he-IVF exhibited optimal and elevated rates across all evaluated parameters (**Table 1**), including total motility exceeding 96.0%, progressive motility surpassing 63.0%, and a substantial proportion of the sperm population classified as rapid (81.0%). The epididymal spermatozoa from red-rumped agouti demonstrated a high rate of membrane functionality (91.0%) by the HOS test following the selection process. The percentages of membrane integrity and mitochondrial activity (**Figure 1A**) were also notably high, with $68.0\% \pm 1.0$ of the samples exhibiting both an intact membrane and functional mitochondria (**Figure 1B**). Lower proportions were observed for spermatozoa displaying loss of mitochondrial function ($20.8\% \pm 4.7$), rupture of the plasma membrane ($0.5\% \pm 0.2$), and both loss of mitochondrial function and membrane rupture ($10.7\% \pm 5.4$). These findings indicate that approximately $88.8\% \pm 5.6$ of the spermatozoa used in he-IVF presented an intact plasma membrane, while $68.5\% \pm 0.8$ exhibited functional mitochondrial activity (**Figure 1C**).

Table 1. Computer-aided sperm analysis of red-rumped agouti epididymal sperm utilized for he-IVF

CASA parameters	(mean $\pm$ SE)
Total motility (%)	96.3 $\pm$ 1.8
Progressive motility (%)	63.0 $\pm$ 5.2
VAP ($\mu\text{m/s}$)	117.6 $\pm$ 16.8
VSL ($\mu\text{m/s}$)	98.4 $\pm$ 14.6
VCL ($\mu\text{m/s}$)	155.5 $\pm$ 11.5
ALH ($\mu\text{m/s}$)	5.7 $\pm$ 0.2
BCF (Hz)	29.5 $\pm$ 2.8
STR (%)	77.8 $\pm$ 1.6
LIN (%)	58.3 $\pm$ 5.1
Rapid (%)	81.0 $\pm$ 5.8
Medium (%)	15.5 $\pm$ 4.5
Slow (%)	0.0 $\pm$ 0.0
Static (%)	3.5 $\pm$ 1.8

SE: standard error. VAP: velocity average pathway; VSL: velocity straight line; VCL: curvilinear velocity; ALH: amplitude of lateral head; BCF: beat cross frequency; STR: straightness; LIN: linearity. Sperm population: rapid, medium, slow or static.

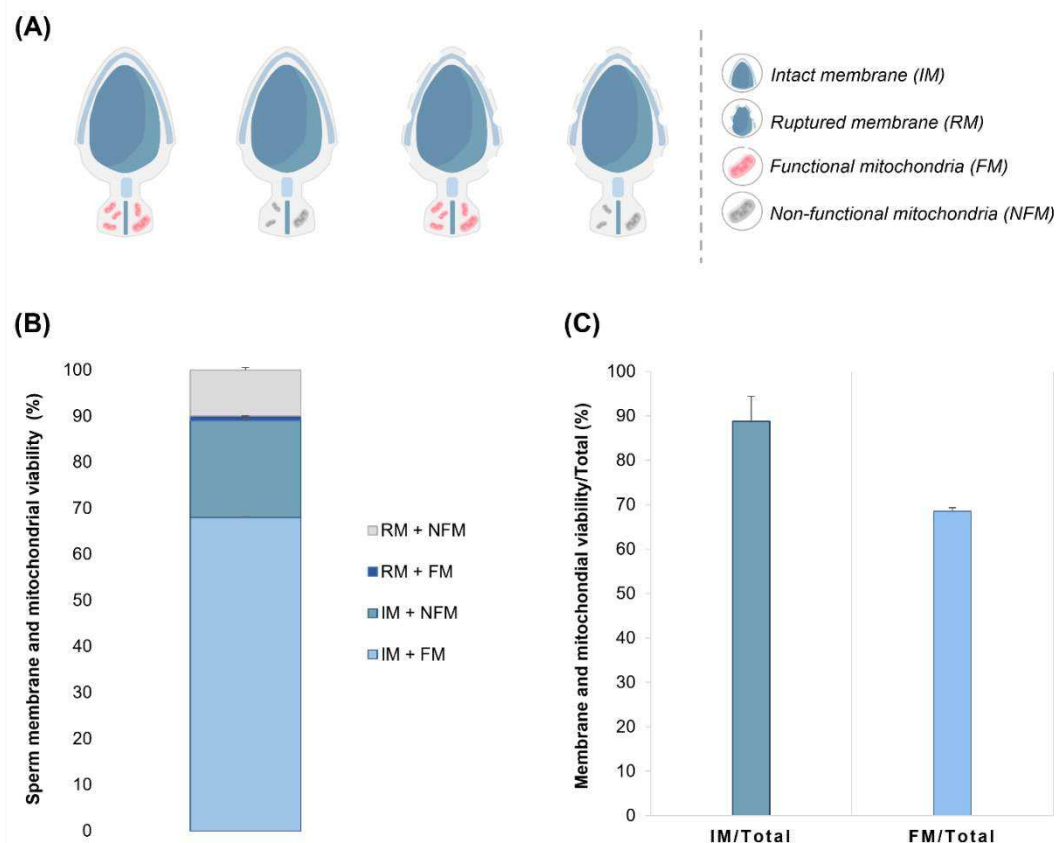


Figure 1. Sperm membrane viability and mitochondrial activity in red-rumped agoutis. **(A)** Representative illustration showing the parameters analyzed for sperm membrane viability and mitochondrial activity in red-rumped agouti. **(B)** The rate of intact or ruptured red-rumped agouti sperm membrane and mitochondrial activity labeled with H333342, propidium iodide and MitoTracker Red fluorescent probes. **(C)** The percentage of sperm cells with intact membranes and normal mitochondrial activity relative to the total number of counted cells.

No significant DNA damage was detected in the samples analyzed. Specifically, $87.5\% \pm 2.5$ of the sperm exhibited intact DNA, while $5.0\% \pm 1.4$ showed low DNA damage, $4.8\% \pm 0.8$ exhibited moderate DNA damage, and only $2.8\% \pm 1.3$ displayed high DNA damage. These findings were consistent with the sperm morphology parameters (**Table 2**), where more than 88.0% of the spermatozoa displayed normal morphology. The abnormalities observed were uniformly distributed across the three regions examined: head, middle piece, and tail.

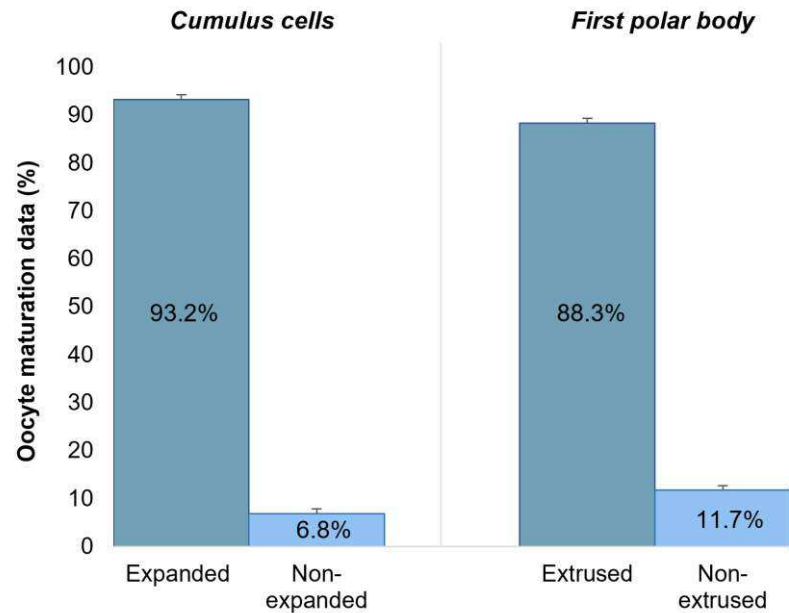
Table 2. Sperm morphology analysis from red-rumped agouti epididymal sperm samples for he-IVF

Sperm morphology	(% $\pm$ SE)
Normal	88.8 ± 2.9
Abnormal	11.2 ± 2.9
Abnormal morphology	(% $\pm$ SE)
Head defects	2.5 ± 0.6
Middle piece defects	5.3 ± 3.3
Tail defects	3.4 ± 0.2

SE: standard error.

Concerning the viability of guinea pig oocytes (**Figure 2**), more than 93.0% exhibited substantial *cumulus* cell expansion following 24 h of maturation. Additionally, more than

3298 88.0% of the oocytes exhibited 1PB extrusion, indicating nuclear maturity and suitability for
 3299 he-IVF.



3300

3301 **Figure 2.** Efficiency of oocyte *in vitro* maturation (IVM) from guinea pigs for he-IVF analyzed
 3302 by *cumulus* cells expansion and extrusion of the first polar body (1PB).

3303

3304 **he-IVF and *in vitro* development**

3305 The different he-IVF media significantly influenced the morphology of presumptive zygotes
 3306 (**Figure 3**). The TALP group ($85.0\% \pm 3.5$) exhibited a higher rate of normal morphology
 3307 compared to the HTF group ($67.0\% \pm 3.5$) ($P < 0.05$). The TYH group ($76.0\% \pm 6.4$) displayed
 3308 results comparable to those of both the TALP and HTF groups ($P < 0.05$).

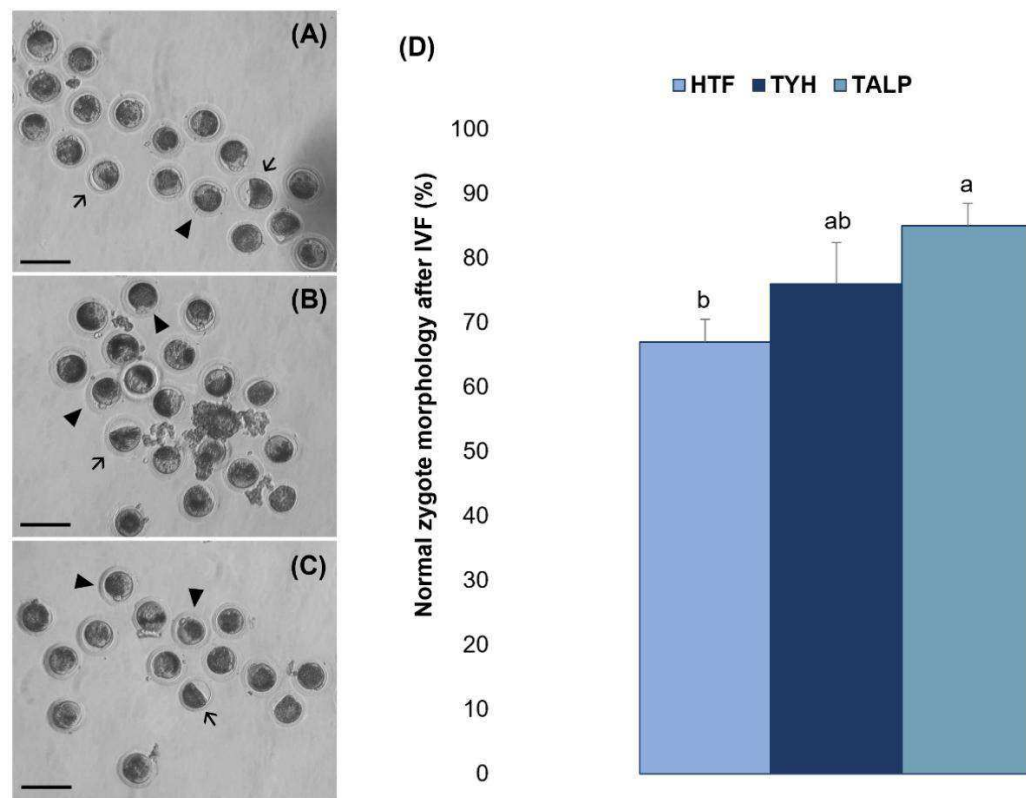


Figure 3. Impact of three distinct IVF media (HTF *vs.* TYH *vs.* TALP) on the morphological integrity of presumptive zygotes following a 6-h he-IVF protocol. **(A)** Representative images of presumptive zygotes fertilized in HTF, **(B)** TYH, and **(C)** TALP media. **(D)** Percentage of normal morphology zygotes after he-IVF. Arrows indicate zygotes with abnormal morphology; arrowheads represent zygotes exhibiting typical or desired morphological characteristics. Different letters (^{a,b}) indicate a statistical difference ($P < 0.05$). Scale Bar = 50 μ m. Magnification: 20 $\times$.

Comparable results were observed in embryo development kinetics (**Table 3**). On Day 2, the TALP group exhibited that more than 48.0% of the samples had cleaved after IVF, surpassing all other groups ($P < 0.05$). Among the cleaved structures, the TALP group also demonstrated the highest percentage of embryos, with more than eight cells ($P < 0.05$). On Day 5, the rate of cleaved structures was even higher in the TALP group ($P < 0.05$), with over 70% of cleavage

observed compared to 29-38% in the HTF and TYH groups. Similarly, the TALP group predominantly exhibited cleavage with more than eight cells, outpacing the other treatments ($P < 0.05$). Finally, no differences were observed between the groups regarding the percentage of morulae on Day 5 ($P > 0.05$), with the total percentage ranging from 9.2% to 14.4%, and the total/cleavage percentage varying between 20.2% and 30.8%.

Table 3. he-IVF efficiency using three different IVF media with prepared red-rumped agouti sperm and matured guinea pig oocytes

Groups	HTF	TYH	TALP
<i>Cleavage rate (D2)</i>			
Total cleavage (%)	25.3 ± 4.7 (22/87) ^b	19.4 ± 1.3 (21/108) ^b	48.6 ± 2.7 (54/111) ^a
2 cells (%)	16.1 ± 3.5 (14/87) ^a	10.2 ± 3.8 (11/108) ^a	18.0 ± 1.0 (20/111) ^a
3-7 cells (%)	9.2 ± 2.6 (8/87) ^{ab}	8.3 ± 2.5 (9/108) ^b	18.0 ± 2.1 (20/111) ^a
≥ 8 cells (%)	0.0 ± 0.0 (0/87) ^b	0.9 ± 0.6 (1/108) ^b	12.6 ± 1.2 (14/111) ^a
<i>Cleavage rate (D5)</i>			
Total cleavage (%)	29.9 ± 3.6 (26/87) ^b	38.0 ± 8.8 (41/108) ^b	71.2 ± 4.8 (79/111) ^a
2 cells (%)	6.9 ± 5.0 (6/87) ^a	12.0 ± 8.1 (13/108) ^a	13.5 ± 5.9 (15/111) ^a
3-7 cells (%)	10.3 ± 6.0 (9/87) ^b	13.9 ± 5.5 (15/108) ^{ab}	23.4 ± 2.1 (26/111) ^a
≥ 8 cells (%)	12.6 ± 4.7 (11/87) ^b	12.0 ± 3.4 (13/108) ^b	31.5 ± 2.5 (35/111) ^a
<i>Morulae (D5)</i>			
Total (%)	9.2 ± 3.2 (8/87) ^a	9.3 ± 3.3 (10/108) ^a	14.4 ± 1.8 (16/111) ^a
Total/Cleaved (%)	30.8 ± 10.2 (8/26) ^a	24.4 ± 3.8 (10/41) ^a	20.2 ± 3.1 (16/79) ^a

Mean ± standard error. ^{a,b}: Values with different subscript letters are significantly different between treatments ($P < 0.05$). HTF: Human Tubal Fluid medium. TYH: Toyoda-Yokoyama-Hoshi medium. TALP: Tyrode's Albumin Lactate Pyruvate medium. D2: day two of *in vitro* culture. D5: day five of *in vitro* culture.

No differences ($P > 0.05$) were observed in sperm-oocyte interaction following he-IVF (**Table 4**), with monospermy rates exceeding 93%. However, the evaluation of oxidative stress (**Figure 4**) revealed that zygotes fertilized with TALP media exhibited lower ROS production (0.66 ± 0.08 AFU) compared to those fertilized with HTF (1.24 ± 0.12 AFU) and TYH (1.10 ± 0.11 AFU) (**Figure 4D**, $P < 0.05$). Furthermore, the $\Delta\Psi_m$ was significantly lower in zygotes fertilized with TALP (0.69 ± 0.12 AFU) than in those fertilized with HTF (1.23 ± 0.22 AFU) and TYH (1.08 ± 0.24 AFU) (**Figure 4E**, $P < 0.05$). The morulae analysis indicated that both TYH (2.81 ± 0.14 AFU) and TALP (2.37 ± 0.29 AFU) media resulted in reduced ROS production compared to HTF (4.60 ± 0.36 AFU) (**Figure 4F**, $P < 0.05$). Similarly, $\Delta\Psi_m$ levels in morulae were lower in the TYH (3.34 ± 0.21 AFU) and TALP (3.37 ± 0.22 AFU) groups compared to HTF (5.52 ± 0.16 AFU) (**Figure 4G**, $P < 0.05$).

Table 4. Sperm–oocyte interaction during heterologous IVF between guinea pig and red-rumped agouti

Groups	HTF	TYH	TALP
Sperm/oocyte (mean $\pm$ SE)	1.1 ± 0.1	1.0 ± 0.0	1.1 ± 0.1
Sperm/zone (mean $\pm$ SE)	46.0 ± 14.2	48.0 ± 12.1	50.8 ± 15.0
Monospermy (%)	93.1 ± 4.7	98.2 ± 1.8	93.8 ± 6.3
Polyspermy (%)	6.9 ± 4.7	1.8 ± 1.8	6.3 ± 6.3

Mean $\pm$ standard error. $P > 0.05$. HTF: Human Tubal Fluid medium. TYH: Toyoda-Yokoyama-Hoshi medium. TALP: Tyrode's Albumin Lactate Pyruvate medium.

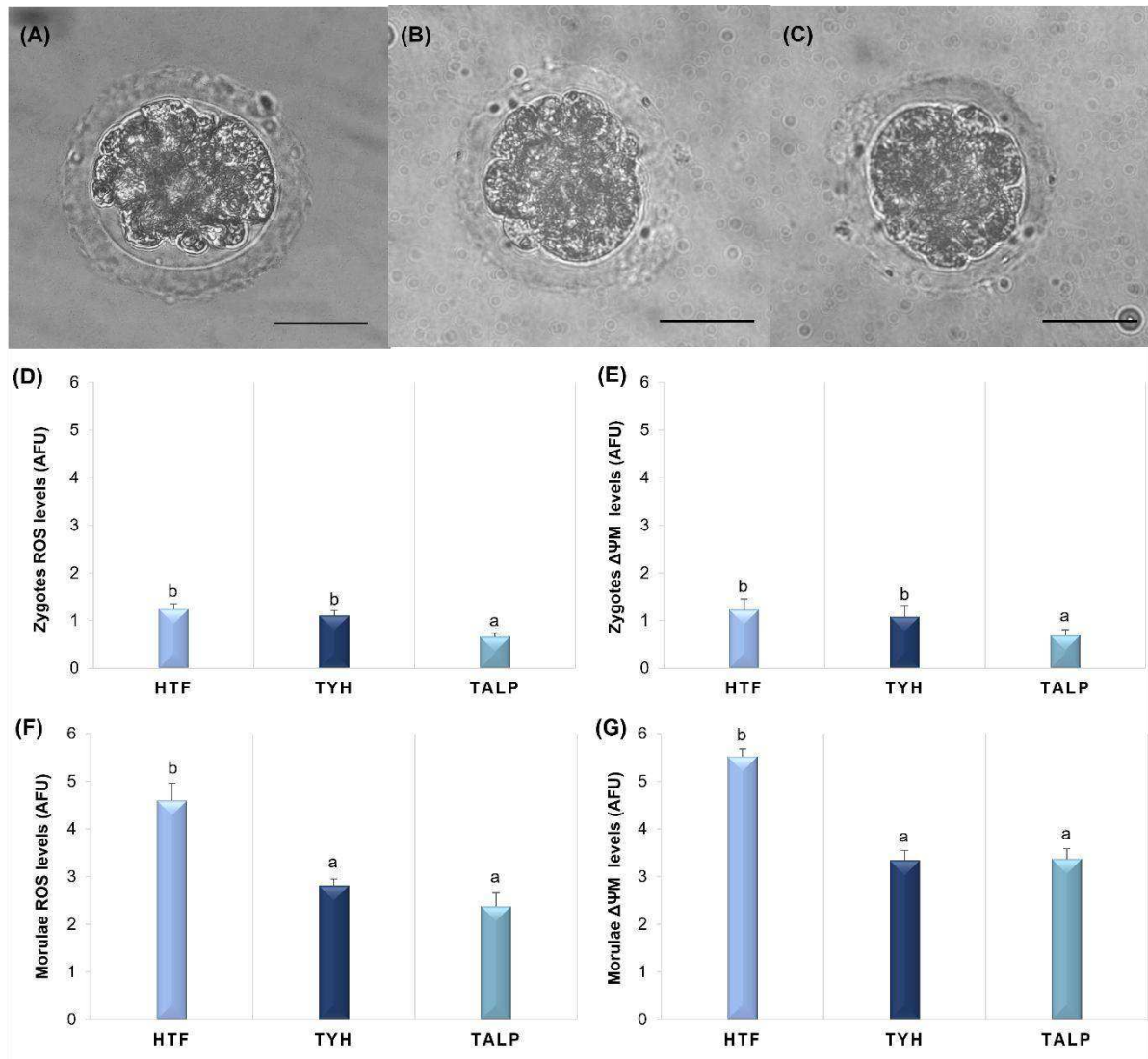


Figure 4. Effect of different he-IVF media on presumptive zygotes and morulae ROS production and mitochondrial membrane potential ($\Delta\Psi_m$). (A) Representative image of morulae on D5 of culture after being fertilized in HTF, (B) TYH, and (C) TALP media. (D) ROS levels in arbitrary fluorescence units (AFU) from labeled zygotes with the H₂DCFDA fluorescent probe. (E) $\Delta\Psi_m$ levels from labeled zygotes with MitoTracker Red fluorescent probe. (F) ROS levels from labeled morulae. (G) $\Delta\Psi_m$ levels from labeled morulae. Different letters (^{a,b}) indicate a statistical difference between treatments (P < 0.05). Scale Bar = 20 μm. Magnification: 40 ×.

Discussion

The findings of this study substantiate that TALP serves as the most effective medium for supporting the entire he-IVF process using red-rumped agouti sperm and guinea pig oocytes. This medium effectively mitigated oxidative stress, as indicated by reduced ROS levels and lower $\Delta\Psi_m$ production in zygotes, thereby minimizing morphological abnormalities in these structures. Moreover, the use of TALP during he-IVF significantly promoted high cellular cleavage during IVD on Days 2 and 5, supporting the progression of cleaved zygotes to the full morula stage.

Successful IVF requires the use of viable and functional gametes. The high functionality of the sperm membrane is particularly significant, as this membrane plays a crucial role in sperm capacitation, acrosome reaction, sperm-egg fusion, and regulating fluid influx into the cell, thereby maintaining osmotic balance (Neamah, 2022). Our findings, which show higher rates of functionality, align with those of Dantas et al. (2022ab, 2024), who reported membrane functionality rates of approximately 80% when conducting the hypo-osmotic swelling test on cauda epididymal sperm from red-rumped agoutis.

The minimal loss of mitochondrial function following selection and capacitation was also a significant factor in maintaining the viability of red-rumped agouti spermatozoa. Mitochondria are essential for sperm motility, glycolysis, and oxidative phosphorylation, and serve as the primary source of pro-oxidative agents. It is hypothesized that dysfunction of this organelle plays a critical role in oxidative imbalance, which can compromise sperm function (Barbagallo et al., 2020). Dantas et al. (2022ab, 2024) reported similarly high rates of normal mitochondrial activity in red-rumped agouti epididymal sperm, with approximately 80%, aligning closely with our findings.

Among all parameters, sperm motility is recognized as a highly reliable predictor of male fertility potential (Dcunha et al., 2022). Our findings demonstrate a total motility (TM) exceeding 96%, a progressive motility (PM) of 63%, and more than 80% of the sperm population was classified as rapid. These results represent an improvement in the red-rumped agouti, surpassing the findings of previous studies. For instance, Castelo et al. (2015) observed only 90% motility with epididymal sperm, whereas Dantas et al. (2022a) reported a TM of 79.8% and PM of 13.7%. Additionally, Castelo et al. (2023) found a TM of just 24.8%. This enhancement can be attributed to the optimization of novel protocols for sperm selection and capacitation developed by our team and implemented during this study.

In recent decades, sperm DNA integrity has become one of the most widely discussed biomarkers in basic andrology, driven by the growing need for a more comprehensive understanding of sperm physiology. DNA damage is known to negatively impact fertilization, embryo development, implantation, and pregnancy outcomes (Zhu et al., 2022). Therefore, the high levels of intact DNA observed following recovery in this study confirm the viability of these samples for he-IVF. A similar conclusion can be drawn from the elevated rates of normal sperm morphology, underscoring the potential positive implications of preserving these morphological characteristics, which may be crucial for egg recognition, enhanced motility, and improved fertilization potential of these samples (Pelzman & Sandlow, 2024).

Ensuring proper oocyte maturation is a crucial factor in optimizing IVF protocols and *in vitro* development outcomes, as it enables the necessary cytoplasmic and nuclear modifications required for successful sperm interaction during fertilization (Fair, & Lonergan, 2023). In our study, we achieved promising maturation rates exceeding 88.0%, aligning with previously

reported values for this species. For instance, Wang et al. (2019) observed a metaphase II (MII) rate of over 61.0% after a 24-hour *in vitro* maturation period for guinea pig oocytes.

Furthermore, our findings suggest a correlation between successful oocyte maturation and improved embryonic development rates. Even under less efficient IVF conditions, we observed 19.0% cleavage and 9.0% morula formation, which remain within acceptable standards for wild species IVF (Ferraz et al., 2020). Similar associations between oocyte maturation and subsequent embryonic development have been reported by Praxedes et al. (2023). Their study on red-rumped agouti oocytes demonstrated that the IVM medium yielding the highest maturation rate (10 ng/mL epidermal growth factor with 10 µg/mL FSH, 52.1% MII) also resulted in a superior cleavage index on Day 2 (43.2%) following oocyte chemical activation. In contrast, oocytes that exhibited inadequate maturation (100 ng/mL epidermal growth factor with 10 µg/mL FSH, 37.5% MII) displayed compromised developmental potential, with only 15.0% cleavage by Day 2.

Upon comparing the three different media following the 6-hour IVF, TALP was identified as the most suitable for this species, as it yielded a higher number of normally formed zygotes and demonstrated superior *in vitro* development. While the identification of embryos with the highest implantation potential remains an unresolved challenge in reproductive science, numerous approaches have been proposed in recent years to evaluate embryo viability (Stigliani et al., 2021). In our study, we employed a commonly used method of selection based on the analysis of morphological features, including size, shape, and degree of fragmentation. Our findings revealed that the group exhibiting the most normal morphological structures also showed the highest cleavage rate, providing evidence for the efficacy of the method applied in this research.

3445

3446 Due to the limited availability of oocytes from wild species, he-IVF using oocytes from
3447 phylogenetically related species, such as the guinea pig, is a valuable strategy for evaluating
3448 sperm parameters in the red-rumped agouti. Regardless of the IVF media used, we observed a
3449 satisfactory oocyte-sperm interaction, with a low incidence of polyspermy (~5%). This finding
3450 is particularly relevant, as polyspermy rates in IVF systems can exceed 10%, rendering the
3451 resulting embryos unsuitable for transplantation and compromising their subsequent
3452 development (Sun, & Zhu, 2023).

3453

3454 By Day 2 of embryo development, the TALP-derived structures exhibited the highest number
3455 of ≥ 8 cell cleavage stages, which is significant, as slower cleavage rates are generally
3456 considered to affect implantation potential negatively (Hesters et al., 2008). Studies have shown
3457 that the timing of the first cleavage of the zygote can serve as an important criterion for selecting
3458 embryos with the highest implantation potential (Lechniak et al., 2008). Furthermore, the
3459 transfer of embryos derived from early cleaved zygotes has been associated with higher
3460 pregnancy and implantation rates compared to the transfer of embryos from non-early cleaved
3461 zygotes (Almagor et al., 2015).

3462

3463 Following IVF, the three media appeared to exert distinct effects on *in vitro* embryonic
3464 development. The structures fertilized in HTF exhibited slower cleavage, with no structures
3465 surpassing eight cells by Day 2. It took a total of 120 hours for the structures to further develop;
3466 however, by Day 2, all cleaved structures appeared to have reached the morula stage, resulting
3467 in an increased morula/cleaved rate. Based on these findings, we hypothesize that, although
3468 HTF supported embryonic development in this species, the initial delay in cleavage may

adversely affect the quality of these structures, being regarded as the least effective medium among the three tested.

IVF using TYH appears to have facilitated development with intermediate potential, as structures with more than eight cells were formed by Day 2, albeit in smaller quantities. Cleavage rates increased throughout the IVD. However, these structures did not achieve a high number of morulae by Day 5 due to an initial disadvantage. While the medium was likely stable for the species under study, it appears that some factors were still lacking in optimizing its performance as the most effective medium.

Finally, the TALP medium appears to have positively influenced the development of these zygotes, as evidenced by the high percentage of cleaved structures with more than eight cells observed by Day 2. Furthermore, by Day 5, structures with 2-7 cells had progressed to eight cells or morulae, and even the delayed structures underwent cleavage, resulting in a high percentage of cleaved structures over the 120 hours. Additionally, the number of morulae generated was comparable to the percentage of more developed structures observed on Day 2. Based on these findings, we can hypothesize that the TALP medium is the most stable for red-rumped agouti he-IVF, providing an environment that supports the subsequent development of the structures within the embryonic medium.

The lack of stabilization in the HTF and TYH media during he-IVF, caused by excessive glucose, may have led to a further disruption in the metabolic balance of these structures, thereby exacerbating oxidative stress, as reflected in our findings of ROS production and mitochondrial membrane potential. This effect was especially evident at the morula stage, where embryos fertilized in HTF (which has a higher glucose concentration) demonstrated

increased ROS production and elevated mitochondrial membrane potential—both of which are recognized as detrimental to embryonic quality (Belli et al., 2019).

In summary, TALP medium demonstrated superior performance compared to HTF and TYH during he-IVF in red-rumped agouti and guinea pig, enhancing embryonic development rates following IVD. Moreover, TALP was the only medium that provided a lower oxidative stress environment for the embryos throughout the 6-hour he-IVF. This study represents a significant advancement in understanding the reproductive dynamics of these species, providing substantial data that could support the future development of homologous IVF techniques for this wild rodent.

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Conflict of interest

The authors declare that they have no conflict of interest.

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3689 **CAPÍTULO 6 - EFFECTS OF EMBRYO PRODUCTION METHOD AND CULTURE**
3690 **MEDIUM ON EMBRYONIC DEVELOPMENT AND QUALITY IN RED-RUMPED**
3691 **AGOUTI (*Dasyprocta leporina* LINNAEUS, 1758)**

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3695 **ARTIGO EXPERIMENTAL 4**

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3697 **SUBMETIDO NA THERIOGENOLOGY**

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3700 **Qualis 2017-2020:** A1 (Medicina Veterinária)

3701 **Fator de impacto:** 2.4

3702 **Data de submissão:** 13/04/2025

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Effects of embryo production method and culture medium on embryonic development and quality in red-rumped agouti (*Dasyprocta leporina* Linnaeus, 1758)

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Highlights

- IVF-KSOM group showed the highest cleavage rate and equal or more eight-cell structures in red-rumped agouti.
- KSOM medium improved morula formation in both IVF and AA groups in red-rumped agouti.
- Only the IVF-KSOM group achieved blastocyst formation in red-rumped agouti.
- IVF-derived structures presented superior normal zygote morphology in red-rumped agouti.

Abstract

Advances in assisted reproductive technologies help overcome reproductive barriers. However, a comprehensive understanding of embryonic development is crucial for their success. *In vitro* fertilization (IVF) and oocyte artificial activation (AA) are embryo production methods commonly used. Previous studies have reported low developmental success with these methods, possibly due to the culture medium used. Therefore, we aimed to evaluate the influence of production method and culture medium on embryonic development and quality in red-rumped agouti. Oocytes were matured *in vitro* and divided into two groups: IVF and AA. Structures were cultured in either KSOM or SOF medium after a 6-hour incubation. Morphology, oocyte-sperm interaction, developmental kinetics, and oxidative stress levels were assessed. IVF-derived structures exhibited a higher rate of normal morphology compared to AA-derived structures ($P < 0.05$). Embryo kinetics analysis showed that AA-KSOM and IVF-KSOM groups had a higher number of cleaved structures than the SOF groups ($P < 0.05$) on Day 2. On Day 5, the IVF-KSOM group presented the highest cleavage rate and the highest percentage of structures with eight or more cells ($P < 0.05$). Morula formation was significantly higher in IVF-KSOM and AA-KSOM ($P < 0.05$), highlighting KSOM's effectiveness. Notably, only the IVF-KSOM group produced blastocysts ($P < 0.05$). Oxidative stress assessment showed no differences between groups ($P > 0.05$). These findings confirm that IVF-KSOM is the most effective methodology for developing red-rumped agouti embryos, offering valuable insights into the reproductive biology of this species and aiding in the refinement of protocols for closely related rodents.

Keywords: caviid rodent; early cleavage dynamics; blastocyst development; artificial oocyte activation; *in vitro* fertilization; oxidative stress.

1. Introduction

Advances in assisted reproductive technologies (ARTs) have become crucial for the conservation of wildlife species, such as the red-rumped agouti (*Dasyprocta leporina* Linnaeus, 1758), a seed-scattering rodent of great importance on the South American continent [1]. The ARTs offer innovative solutions to overcome reproductive barriers, enabling the preservation of genetic diversity and facilitating the breeding of species in captivity [2]. These technologies allow the collection and storage of gametes, as well as the development of embryos under controlled conditions, thereby contributing to species recovery efforts [3].

However, it is essential to thoroughly understand the embryonic development of these species since the ability to sustain embryos without compromising their developmental potential is crucial for the success of these ARTs [4]. *In vitro* systems are extensively used to elucidate various aspects of reproductive physiology [5] due to the challenges associated with observing these phenomena *in vivo*, including gamete interactions [6], alterations in gene expression [7], and pre-implantation embryo kinetics [8]. Among the most frequently employed methods for embryo production in such studies are *in vitro* fertilization (IVF) and oocyte artificial activation (AA).

The AA facilitates the study of early developmental processes due to its resemblance to fertilized embryos in gene transcription patterns up to the blastocyst stage, resulting in initial cleavages that are identical to those of fertilized zygotes [9]. Additionally, AA requires a smaller number of animals, as only the mature oocyte is activated without requiring sperm, making it a valuable approach for studies in wild species [10]. However, embryos generated through AA may exhibit a higher rate of apoptosis and are incapable of developing to term [11],

highlighting the need also to investigate embryos with a complete genetic display, as obtained through IVF.

Embryo production via IVF provides deeper insights into gamete interactions, as its application needs optimal sperm selection and capacitation, as well as precise determination of the appropriate time and environment for co-incubation and development [8]. Furthermore, the inclusion of male genetic material enables the expression of Y chromosome-linked genes, which may influence developmental outcomes, particularly in the later stages of the pre-implantation period [12]. Therefore, it is possible to assess distinct physiological responses during the embryonic development of the red-rumped agouti by comparing these two methods of embryo production.

Additionally, research conducted by Praxedes et al. [13] on red-rumped agouti demonstrated that the AA protocol developed for this species was ineffective, with a developmental success rate of only 5–9%, as embryos failed to progress beyond the morula stage. This limited efficiency may be attributed to the *in vitro* development (IVD) medium used, the synthetic oviductal fluid (SOF), since potassium-enriched simplex optimized medium (KSOM) is commonly employed for rodent embryos [6,14]. The differences in protein and energy composition between these media may influence embryonic development, as each is designed to meet the specific requirements of the species [15].

Given this context, the study aimed to evaluate the *in vitro* developmental potential of red-rumped agouti embryos produced by two different methods, IVF and AA, and to assess the effects of IVD media on these structures. These findings will contribute to a deeper

understanding of the reproductive physiology of these species and facilitate the optimization of ARTs, ultimately supporting conservation efforts for red-rumped agoutis.

2. Materials and methods

Unless stated otherwise, the chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Experimental design

This study was divided into two comparisons to assess the *in vitro* embryo production (IVEP) potential of the red-rumped agouti (**Figure 1**). The first part aimed to compare the efficiency of two methods—IVF and AA—in supporting the embryonic development of red-rumped agouti, while the second part evaluated the impact of different IVD media compositions (KSOM vs. SOF) on the structures generated.

Oocytes were subjected to *in vitro* maturation (IVM) and assessed for nuclear maturation based on the extrusion of the first polar body. Moreover, sperm used for IVF were evaluated for viability through analyses of sperm kinetics, membrane functionality and integrity, mitochondrial activity, DNA damage levels, and morphology. Post-IVF, resulting structures were examined for oocyte-sperm interaction, while all presumptive zygotes from both methods were assessed for morphology, embryonic kinetics, and oxidative stress following IVD.

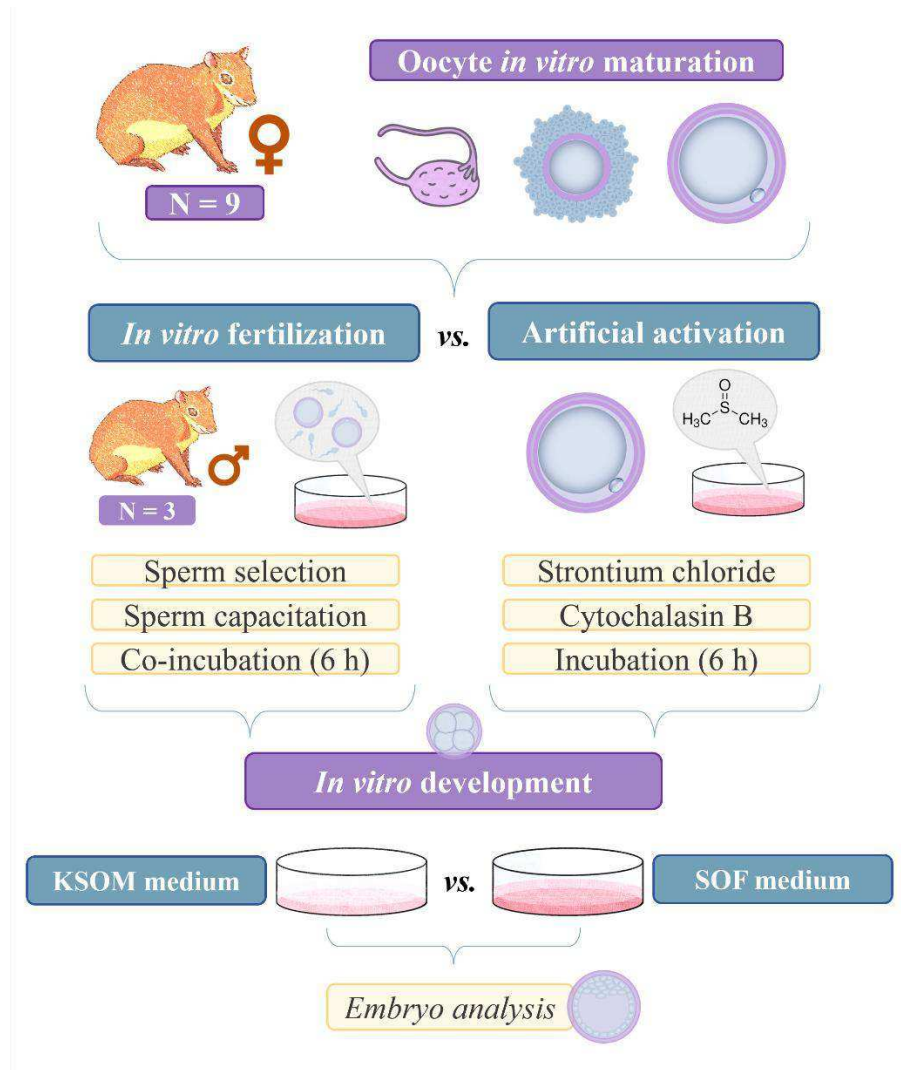


Figure 1. Experimental design: comparative analysis of two *in vitro* embryo production techniques (*in vitro* fertilization *versus* artificial oocyte activation) and optimization of the best *in vitro* development media in the red-rumped agouti.

2.2. Bioethics and animals

All procedures were conducted in strict accordance with the guidelines established by the Animal Ethics Committee of the Federal Rural University of Semi-Arid (Approval no. 20/2021) and the Chico Mendes Institute for Biodiversity Conservation (ICMBio, Approval no. 76655-1). The red-rumped agoutis were housed at the Center for Multiplication of Wild Animals (UFERSA, Brazil), where they were provided with *ad libitum* access to a standardized

commercial rabbit diet and fresh drinking water. The animals were maintained under a 12-hour natural photoperiod cycle to ensure optimal environmental conditions.

For spermatozoa recovery, three adult male red-rumped agouti specimens (one male per replicate) were premedicated with an intramuscular injection of ketamine (15 mg/kg; Ketalar, Pfizer, São Paulo, Brazil) combined with xylazine (1 mg/kg; Rompun, Bayer, São Paulo, Brazil). After a 15-minute interval, anesthesia was induced via intramuscular administration of sodium thiopental (50 mg/kg; Thiopentax, Cristalia, São Paulo, Brazil). Euthanasia was subsequently performed through intravenous administration of potassium chloride (1 mL/kg; Equiplex, Goiânia, Goiás, Brazil), following the protocol described by Castelo et al. [16]. For oocyte recovery, nine adult female red-rumped agoutis (three females/replicate) were anesthetized and euthanized with the same protocol as the male red-rumped agoutis.

2.3. Sperm preparations and analysis for IVF

The testes-vas deferens-epididymis complex was retrieved immediately following euthanasia and immediately transported to the laboratory in a pre-warmed saline solution (37 °C, 0.15 M NaCl). The cauda epididymis region was carefully dissected, and epididymal spermatozoa were collected via retrograde flushing using 1.0 mL of saline solution (0.15 M NaCl) [16]. The sperm samples were maintained in a water bath at 37 °C, while preliminary assessments of their appearance, pH, vigor, and concentration were conducted.

The spermatozoa concentration was adjusted to 100×10^6 sperm/mL using a minimum capacitation medium (MCM) composed of 105.8 mM NaCl, 25 mM NaHCO₃, 5.56 mM glucose, 21.6 mM sodium lactate, 25 mM HEPES, 0.25 mM sodium pyruvate, 10 µg/mL phenol red, and 1% antibiotic-antimycotic solution. A centrifugation protocol was employed to isolate

the most viable spermatozoa. A 1:1 mixture of spermatozoa and MCM was transferred to a 15 mL plastic tube and subjected to two centrifugation cycles (300× g for 3 min at room temperature). After each centrifugation, the supernatant was carefully discarded, and the resulting pellet was resuspended for IVF [17].

Furthermore, sperm capacitation was performed concurrently with the 6-hour IVF procedure. The capacitation agents used included 4 mg/mL bovine serum albumin (BSA) and 2 mM calcium chloride (CaCl₂), both of which were incorporated into the IVF medium in droplets overlaid with mineral oil [18]. The procedure was performed under controlled environmental conditions at 38.5 °C and 6.5% CO₂.

2.3.1. Sperm analysis

The epididymal spermatozoa were evaluated using a computer-assisted sperm analysis (CASA) system (IVOS 7.4G; Hamilton-Thorne Research, MA, USA) with parameters previously established for red-rumped agouti by Castelo et al. [16]. The settings included a temperature of 37 °C, a straightness threshold of 30%, a minimum contrast of 45, a low-velocity average pathway (VAP) cutoff of 10 µm/s, and a medium VAP cutoff of 30 µm/s. Five independent and nonconsecutive microscopic fields were systematically examined. The following kinetic parameters were assessed: total motility (TM, %), progressive motility (PM, %), average path 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, %), and linearity (LIN, %). The sperm population was further categorized into four distinct groups: rapid, medium, slow, and static (%).

The sperm membrane functionality was analyzed using the hypoosmotic swelling test (HOST), which involves a solution consisting of distilled water (0 mOsm/L) with a sodium citrate and fructose solution (50 mOsm/L), as defined for the red-rumped agouti by Dantas et al. [19]. Spermatozoa samples were combined with the hypoosmotic solution and incubated in a dry bath at 37 °C for 40 minutes. The samples were assessed under a phase-contrast light microscope at 400× magnification. The sperm were identified as possessing a functionally intact membrane when displaying a swollen and coiled tail.

For the membrane integrity and mitochondria functionality, a fluorescence microscopy analysis was performed combining 40 µg/mL Hoechst 33342 (Molecular Probes, Eugene, OR, USA) at 37 °C for 5 min, followed by 0.5 mg/mL propidium iodide (Thermo Fisher Scientific, Waltham, MA, USA) and 500 nM CMXRos (Mito Tracker Red®, F-7512, Molecular Probes, Eugene, OR, US) for 8 min. Samples were evaluated using fluorescence microscopy (Olympus BX51TF, Tokyo, Japan) at 400× magnification. Spermatozoa exhibiting a blue-stained head and a red-glowing midpiece were classified as possessing an intact plasma membrane and normal mitochondrial function [20].

The samples were prepared as smears and allowed to air-dry to detect the level of DNA damage in sperm samples. The slides were subsequently fixed in Carnoy's solution for 3 h and dried at room temperature. They were then incubated for 25 min in a buffer solution consisting of 15 mM Na₂HPO₄ and 80 mM citric acid (pH 2.5) at 75 °C. The smears were stained with acridine orange (0.2 mg/mL) for 10 seconds, rinsed with distilled water, and covered with a coverslip. A total of 100 cells were examined using fluorescence microscopy (400×; Olympus BX51TF, Tokyo, Japan). Spermatozoa with normal (double-stranded) DNA exhibited a green, fluorescent

emission, while those with denatured or single-stranded DNA displayed yellow, orange, or red fluorescence, indicating progressively higher levels of DNA damage [21].

For morphological assessment, an aliquot of the sperm samples was fixed and stained using a formaldehyde-Bengal rose solution (Cromato®). The samples were then examined under a light microscope at 1000× magnification, with 100 cells evaluated per slide. The spermatozoa were categorized into two groups: normal or abnormal morphology. Abnormalities were further classified as defects in the head, midpiece, or tail regions [22].

2.4. Oocyte IVM and evaluations

The IVM of red-rumped agouti oocytes was performed following the protocol previously described for this species by Praxedes et al. [13]. The ovaries were collected, stored in pre-warmed saline solution with antibiotics (0.15 M NaCl, 0.05 mg/mL penicillin, 37 °C), and immediately transported to the laboratory. Under stereomicroscopic guidance, all follicles were sliced using scalpel blades to retrieve immature *cumulus*-oocyte complexes (COCs). COCs with at least one layer of compacted *cumulus* cells and homologous or heterologous ooplasm were selected for IVM. The structures were matured in drops (100 µL, 10-20 oocytes) covered with mineral oil in a controlled atmosphere at 38.5 °C and 6.5% CO₂ for 24 h.

The oocyte maturation medium was composed of TCM199 supplemented with 2.2 g/L sodium bicarbonate, 25 mM HEPES, 0.23 mM sodium pyruvate, 100 µM cysteamine, 10 ng/mL epidermal growth factor (EGF), 10 mIU/mL follicle-stimulating hormone (FSH; Folltropin®, Vetoquinol, Brazil), 10% fetal bovine serum (FBS), and 1% antibiotic–antimycotic solution with a 7.2 pH [13].

Following the 24-h maturation, COCs were examined under a stereomicroscope to evaluate the extent of *cumulus* cell expansion. Structures demonstrating pronounced expansion and mucification were classified as mature. After the removal of excess *cumulus* cells through pipetting, the presence or absence of the first polar body (1PB) was documented using an inverted microscope (Leipzig IMx 400, PhoenixOptics, Germany). Oocytes exhibiting clear evidence of first polar body extrusion were identified as mature [13].

2.5. Red-rumped agouti embryo production: IVF vs. AA

For the IVF embryo production, 50 μ L droplets were prepared using Tyrode's albumin lactate pyruvate medium (114 mM NaCl, 3.2 mM KCl, 0.35 mM NaH₂PO₄, 10 mM Na lactate, 25 mM NaHCO₃, 2 mM CaCl₂, 0.50 mM MgSO₄, 10 mM HEPES, 4 mg/mL BSA, 0.11 mM sodium pyruvate, 10 μ g/mL phenol red, and 1% antibiotic–antimycotic solution, TALP) [23], which were then covered with mineral oil and balanced at 38.5 °C in 6.5% CO₂ for approximately 30 min. A final sperm concentration of 1.0×10^6 sperm/mL was added to 10-20 intact *cumulus*-enclosed matured oocytes per drop. The IVF procedure was performed for six hours at 38.5 °C in 6.5% CO₂. Subsequently, the resulting structures were gently washed and pipetted to remove residual spermatozoa and *cumulus* cells before proceeding with *in vitro* development [24].

The protocol described by Praxedes et al. [13] for the AA of red-rumped agoutis was used. Denuded mature oocytes were activated in calcium-free TALP medium (114 mM NaCl, 3.2 mM KCl, 0.35 mM NaH₂PO₄, 10 mM Na lactate, 25 mM NaHCO₃, 0.5 mM MgSO₄, 10 mM HEPES, 4 mg/mL BSA, 0.11 mM sodium pyruvate, 10 μ g/mL phenol red, and 1% antibiotic–antimycotic solution) with 10 mM strontium chloride (SrCl₂) and 5 μ g/mL cytochalasin B for 6 h at 38.5 °C and 6.5% CO₂. Subsequently, the activated structures were washed for preparation for IVD.

3973

3974 ***2.6. In vitro embryo development: KSOM vs. SOF***

3975 Structures derived from IVF and AA were divided between two IVD media drops (50 μ L) with
 3976 either Potassium Simplex Optimized Medium (KSOM) or Synthetic Oviduct Fluid medium
 3977 (SOF). The KSOM composition was 95 mM NaCl, 2.5 mM KCl, 0.35 mM KH_2PO_4 , 10 mM
 3978 Na lactate, 0.2 mM glucose, 25 mM NaHCO_3 , 1.71 mM CaCl_2 , 0.20 mM MgSO_4 , 21 mM
 3979 HEPES, 0.01 mM phenol red, 0.2 mM sodium pyruvate, 0.01 mM EDTA, 1 mM L-glutamine,
 3980 15 mM BSA, 1% antibiotic–antimycotic solution, 1% essential amino acid solution, 0.5% non-
 3981 essential amino acid solution, and 10% FBS [25]. SOF was composed of 0.2 mM sodium
 3982 pyruvate, 0.2 mM L-glutamine, 0.34 mM citrate sodium, 2.8 mM myo-inositol, 2% essential
 3983 amino acid solution, 1% non-essential amino acid solution, 1% antibiotic–antimycotic solution,
 3984 5 mg/mL BSA, and 2.5% fetal bovine serum [20]. IVD was conducted at a controlled
 3985 atmosphere at 38.5 °C and 6.5% CO_2 . After 48 h of culture (Day 2, D2), 50% of the media was
 3986 changed. The total culture time was 120 hours (Day 5, D5) [13].

3987

3988 ***2.6.1. Zygotes evaluation***

3989 Each presumptive zygote was analyzed under an inverted microscope (Leipzig IMx 400,
 3990 PhoenixOptics, Germany). The zygotes were carefully manipulated to allow a comprehensive
 3991 evaluation of their morphological traits and the degree of cytoplasmic fragmentation. Zygotes
 3992 displaying favorable characteristics were classified as normal, following the criteria established
 3993 by Hesters et al. [26].

3994

3995 The cleaved zygotes were cultured until D5 to evaluate their developmental kinetics using an
 3996 inverted microscope (Leipzig IMx 400, PhoenixOptics, Germany). Cell cleavage was
 3997 monitored and classified into three categories: two cells, three to seven cells, or eight or more

cells. Furthermore, the percentage of morula formation was determined on D5, along with the ratio of morulae and blastocysts to the total number of cleaved structures [13]. The expanded blastocyst was incubated with Hoechst 33342 for 15 minutes and then washed in phosphate-buffered saline/bovine serum albumin. Then, a digital image of the blastocyst was captured by fluorescence microscopy at 40× (370 nm).

The non-cleaved cells from IVF were analyzed for oocyte-sperm interaction by staining with Hoechst 33342 (10 µg/mL, 30 min) and examined under a fluorescence microscope. The number of spermatozoa attached to the zona pellucida and the quantity associated with each oocyte were quantified, providing evidence of both monospermy and polyspermy [20].

Presumptive zygotes and morulae were processed for scanning electron microscopy (SEM) to visualize their structure, following the protocol outlined by Vanroose et al. [27], with minor modifications. They were fixed for 2 h in 2.5% glutaraldehyde and 0.1 M cacodylate buffer (pH 7.2) at 4 °C, subsequently washed twice for 5 min in 2% cacodylate buffer, and post-fixed in 1% osmium tetroxide for 2 h at 25 °C. The structures were then washed in distilled water for 10 min, dehydrated through increasing concentrations of ethanol, and mounted on glass coverslips. After drying, the samples were gold-coated. Finally, the ultrastructure of the cells was examined using a scanning electron microscope (TESCAN VEGA3; Tescan Analytics, Fuveau, Bouches-du-Rhône, France).

Reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi_m$) were quantified using 10 µM 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Invitrogen, Carlsbad, CA, USA) and 500 nM MitoTracker Red[®] (Molecular Probes, Eugene, OR, USA), respectively, to evaluate the oxidative stress response. Both presumptive zygotes and morulae were incubated

in the dark with the respective probes for 30 minutes at 38.5 °C under 6.5% CO₂. Following incubation, the samples were placed on glass slides in microdroplets, and images were captured using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). Fluorescence intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The fluorescence values of each group were normalized to the mean of all groups to derive relative expression levels, which were expressed in arbitrary fluorescence units (AFU) [20].

2.7. Statistical analysis

All data are expressed as the mean $\pm$ standard error and were analyzed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA). Normality of all results was verified using the Shapiro-Wilk test, and homoscedasticity was confirmed with Levene's test. ROS and $\Delta\Psi_m$ were altered with arcsine and analyzed by variance analysis (ANOVA) followed by a Tukey test. All other data were compared using a chi-squared test. Statistical significance was set at $P < 0.05$.

3. Results

All epididymal sperm samples exhibited a whitish color, with an average pH of 7.0, and a vigor score of 5.0 on a 0-5 scale. The average concentration following selection was 75×10^6 sperm/mL. Additionally, a total of 18 ovaries were recovered from female red-rumped agoutis, yielding 343 viable oocytes, corresponding to an average of 19.1 viable oocytes per ovary and 38.1 viable oocytes per female.

3.1. Sperm and oocyte evaluations

The sperm kinetics assessed through CASA analysis (**Table 1**) revealed that the sperm utilized for IVF exhibited optimal characteristics across all evaluated parameters, with an average total

motility exceeding 94% and progressive motility surpassing 70%. Furthermore, the samples displayed a pronounced pattern of hyperactive-like motility, as indicated by elevated VCL and ALH values. The quality of these samples was further confirmed by a membrane functionality rate of 90%, as determined by the HOS test. Furthermore, the analysis of membrane integrity and mitochondrial activity (**Table 2**) revealed that approximately 70% of the sperm population exhibited an intact membrane and optimal mitochondrial function, while only ~6% of the samples demonstrated impairments in these parameters.

Table 1. Computer-aided sperm analysis of red-rumped agouti sperm used for IVF

CASA parameters	(mean $\pm$ SE)
Total motility (%)	94.3 $\pm$ 1.7
Progressive motility (%)	72.0 $\pm$ 1.0
VAP ($\mu\text{m/s}$)	160.3 $\pm$ 8.9
VSL ($\mu\text{m/s}$)	138.5 $\pm$ 7.7
VCL ($\mu\text{m/s}$)	187.9 $\pm$ 11.5
ALH ($\mu\text{m/s}$)	5.8 $\pm$ 0.3
BCF (Hz)	25.9 $\pm$ 1.4
STR (%)	83.3 $\pm$ 0.3
LIN (%)	71.0 $\pm$ 0.5
Rapid (%)	88.7 $\pm$ 1.8
Medium (%)	6.0 $\pm$ 0.5
Slow (%)	0.0 $\pm$ 0.0
Static (%)	5.3 $\pm$ 1.4

SE: standard error. VAP: velocity average pathway; VSL: velocity straight line; VCL: curvilinear velocity; ALH: amplitude of lateral head; BCF: beat cross frequency; STR: straightness; LIN: linearity. Sperm population: rapid, medium, slow or static.

The assessment of DNA damage levels in the sperm utilized for IVF revealed no abnormalities in the measured rates, with the following distribution: intact DNA (90.3 $\pm$ 1.2%), low DNA damage (3.7 $\pm$ 1.2%), moderate DNA damage (4.3 $\pm$ 1.2%), and high DNA damage (1.7 $\pm$ 1.2%). Comparable results in terms of sample normality were observed in the morphological

analysis (**Table 3**), where over 93% of the sperm population was classified as normal. The abnormalities identified were evenly distributed across the head, midpiece, and tail regions.

Table 2. Membrane integrity and mitochondrial activity rate in red-rumped agouti sperm used for IVF

IPM (+) MF (+)	IPM (+) MF (-)	IPM (-) MF (+)	IPM (-) MF (-)	IPM/TOTAL (%)	MF/TOTAL (%)
70.0 ± 1.1	22.7 ± 3.8	0.7 ± 0.6	6.7 ± 3.7	92.7 ± 4.3	70.7 ± 0.6

IPM: intact plasma membrane. MF: mitochondrial function. (+) presence. (-) absence. Mean ± standard error.

Table 3. Sperm morphology analysis from red-rumped agouti epididymal sperm for IVF

Sperm morphology	(% ± SE)
Normal	93.3 ± 2.7
Abnormal	6.7 ± 2.7
Abnormal morphology	(% ± SE)
Head defects	2.0 ± 1.1
Middle piece defects	3.0 ± 1.7
Tail defects	1.7 ± 0.8

SE: standard error.

Following the IVM of red-rumped agouti oocytes (**Figure 2**), *cumulus* cell expansion was observed at a rate of 95.0% ± 1.0. Moreover, the extrusion of the 1PB occurred in approximately 68.2% ± 0.9 of the oocytes.

3.2. Embryo production and IVD

The different embryo production methods had a significant impact on the morphology of presumptive zygotes (**Figure 3A-D**). IVF-derived structures (**Figure 4A-D**) exhibited higher

rates of normal morphological characteristics, an average of $84.5\% \pm 1.7$, whereas AA-derived structures showed only $63.3\% \pm 2.5$ ($P < 0.05$). No differences ($P > 0.05$) were observed in sperm-oocyte interaction following IVF (**Table 4**), with monospermy rates exceeding 93% in both the SOF and KSOM groups.

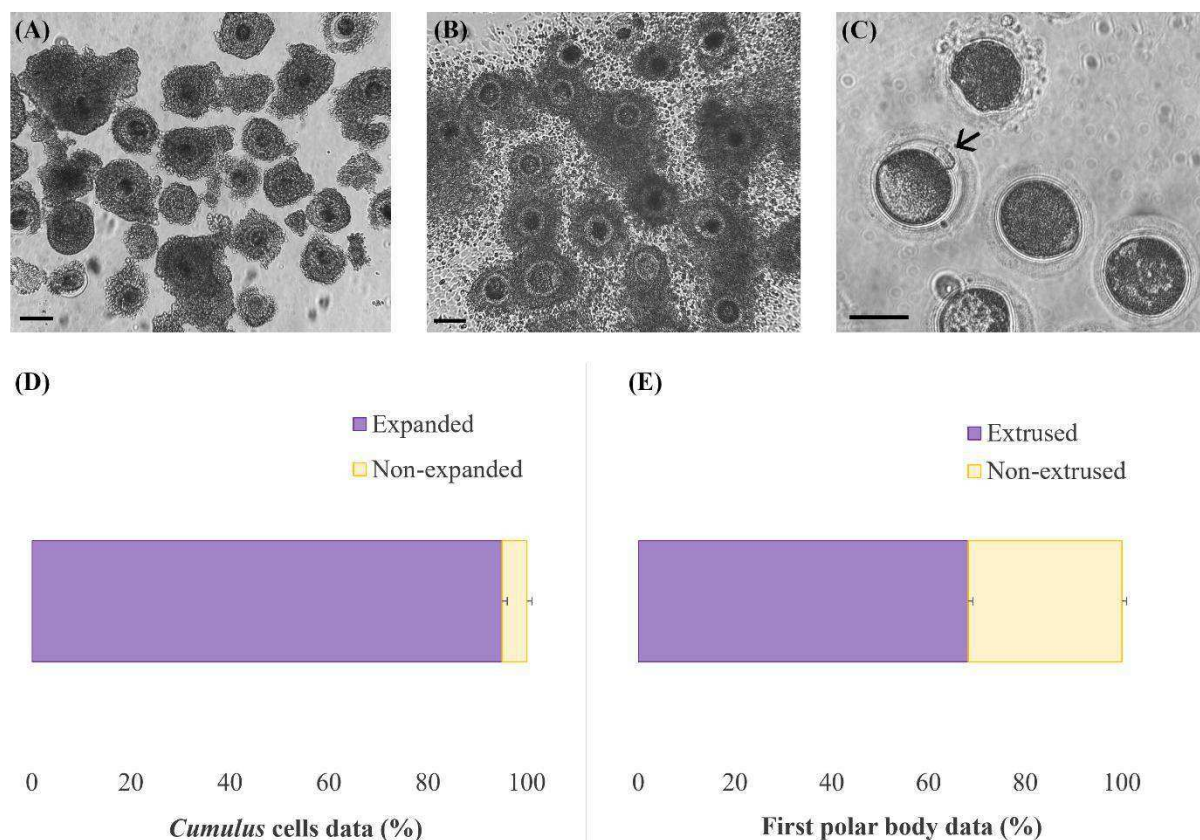


Figure 2. Efficiency of oocyte *in vitro* maturation of red-rumped agouti. **(A)** Representative photomicrographs of immature red-rumped agouti oocytes, **(B)** matured oocytes exhibiting *cumulus* cell expansion, and **(C)** nuclear-matured oocytes with an extruded first polar body (1PB). **(D)** *Cumulus* cell expansion rate following IVM. **(E)** 1PB extrusion rate post-IVM. Arrow indicates extruded 1PB. Scale bar: 100 μ m (A–B) and 50 μ m (C). Magnification: 10 $\times$ (A–B) and 20 $\times$ (C).

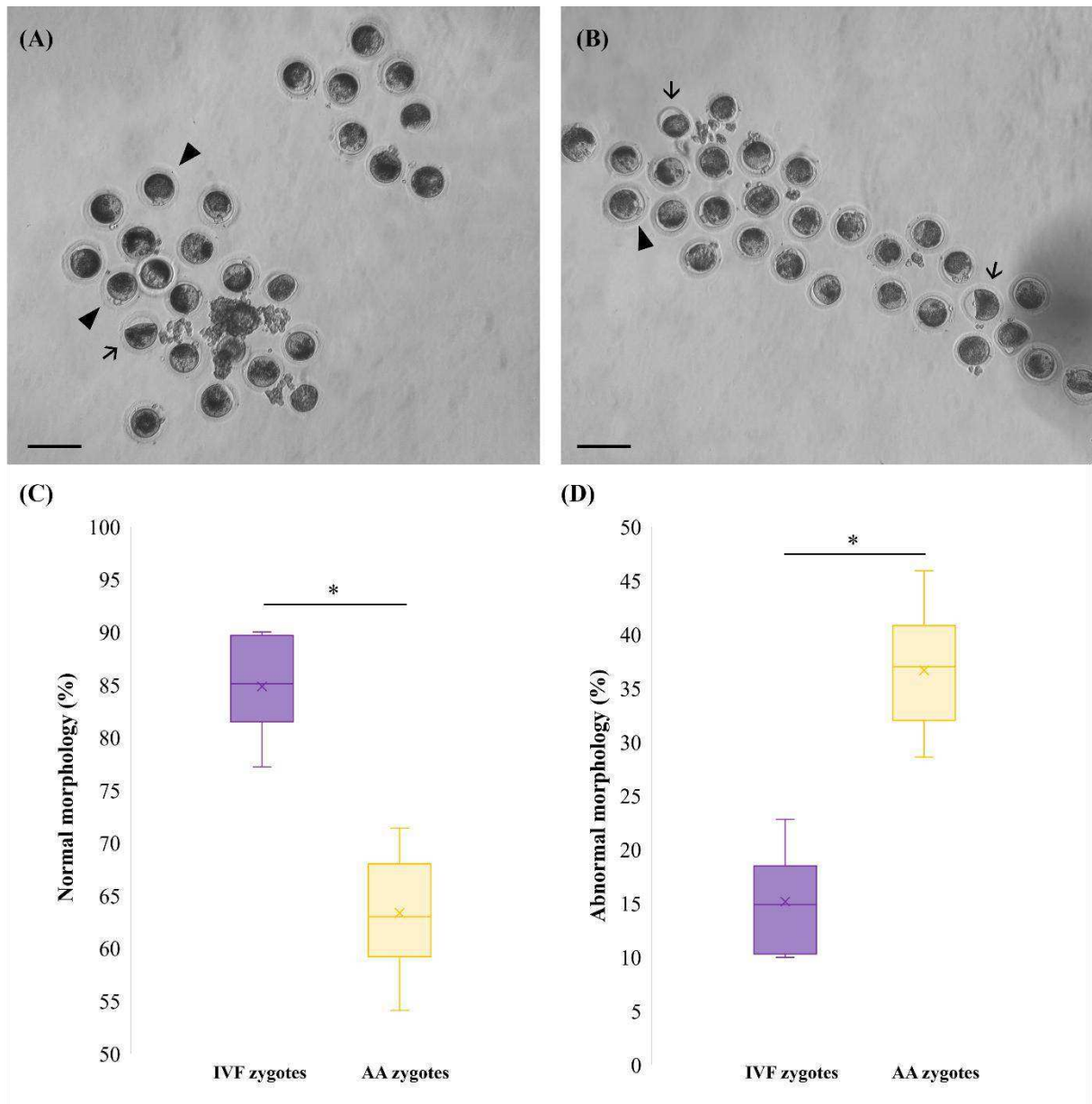


Figure 3. Efficiency of presumptive zygote production using different techniques (*in vitro* fertilization vs. oocyte artificial activation) assessed through morphological analysis. (A) Representative image of presumptive zygotes derived from IVF and (B) AA. (C) Proportion of zygotes exhibiting normal morphology and (D) those with abnormal morphology following IVF and AA. The arrowhead denotes zygotes with normal morphology, while the arrow indicates those with abnormal morphology. Asterisks indicate statistically significant differences between groups ($P < 0.05$). Scale bar: 100 μm . Magnification: 10 $\times$.

Table 4. Red-rumped agouti sperm interaction with oocyte after IVF when cultured in two different embryo media

Groups	Sperm/oocyte (mean $\pm$ SE)	Sperm/zone (mean $\pm$ SE)	Monospermy (%)	Polyspermy (%)
KSOM	1.1 $\pm$ 0.1 ^a	52.0 $\pm$ 4.7 ^a	93.3 $\pm$ 6.7 ^a	6.7 $\pm$ 8.2 ^a
SOF	1.0 $\pm$ 0.0 ^a	62.3 $\pm$ 4.4 ^a	100.0 $\pm$ 0.0 ^a	0.0 $\pm$ 0.0 ^a

Mean $\pm$ standard error. $P > 0.05$.

Embryo kinetics analysis (**Table 5**) revealed significant differences between the techniques and media employed. On D2, both AA-KSOM and IVF-KSOM yielded a higher number of cleaved structures compared to the groups cultured in SOF ($P < 0.05$). However, the cleavage rate of AA-KSOM was comparable to that of the SOF groups ($P > 0.05$).

Regarding the cleavage rates on D2, presumptive zygotes cultured in SOF (AA-SOF and IVF-SOF) exhibited a higher proportion of structures with fewer cells, as compared to those cultured in KSOM ($P < 0.05$). In contrast, both AA-KSOM and IVF-KSOM groups demonstrated a higher incidence of structures with ≥ 8 cells, with rates exceeding 55% ($P < 0.05$).

On D5, the IVF-KSOM group exhibited the highest percentage of cleaved structures (78.4%) compared to all other groups ($P < 0.05$, **Figure 4E-H**), underscoring the impact of both the technique and the IVD medium used. This group also showed the highest percentage of structures with ≥ 8 cells, with values exceeding 85% ($P < 0.05$).

Furthermore, the total percentage of morulae on D5 was significantly higher in the IVF-KSOM group (50.0%, **Figure 4I-J**) compared to the other groups ($P < 0.05$). Additionally, AA-KSOM outperformed both IVF-SOF and AA-SOF groups ($P < 0.05$), demonstrating the effectiveness of this culture medium for the species, regardless of the method employed. The superiority of KSOM was further evidenced by the morulae/cleaved ratio, where both AA-KSOM and IVF-

KSOM outperformed the SOF-cultured groups ($P < 0.05$). Notably, only the IVF-KSOM group produced blastocysts (1.3%, **Figure 4K-L**), making it the most efficient among all evaluated groups ($P < 0.05$).

Table 5. *In vitro* development of presumptive zygotes from red-rumped agouti derived from both IVF and AA cultured with different embryo culture media

<i>Cleavage rate (D2)</i>				
Groups	Total cleavage (%)	2 cells (%)	3-7 cells (%)	≥ 8 cells (%)
IVF-KSOM	70.2 ± 3.8 (52/74) ^a	7.7 ± 3.2 (4/52) ^b	28.8 ± 5.9 (15/52) ^b	63.5 ± 5.0 (33/52) ^a
IVF-SOF	42.5 ± 4.1 (31/73) ^b	9.7 ± 2.2 (3/31) ^{ab}	74.2 ± 6.6 (23/31) ^a	16.1 ± 3.8 (5/31) ^b
AA-KSOM	52.8 ± 1.5 (28/53) ^{ab}	10.7 ± 0.4 (3/28) ^{ab}	32.1 ± 3.3 (9/28) ^b	57.1 ± 3.4 (16/28) ^a
AA-SOF	37.0 ± 2.3 (20/54) ^b	30.0 ± 4.0 (6/20) ^a	55.0 ± 4.9 (11/20) ^{ab}	15.0 ± 2.8 (3/20) ^b
<i>Cleavage rate (D5)</i>				
Groups	Total cleavage (%)	2 cells (%)	3-7 cells (%)	≥ 8 cells (%)
IVF-KSOM	78.4 ± 5.8 (58/74) ^a	6.9 ± 1.6 (4/58) ^a	5.2 ± 2.1 (3/58) ^b	87.9 ± 3.4 (51/58) ^a
IVF-SOF	45.2 ± 4.0 (33/73) ^b	15.2 ± 3.7 (5/33) ^a	30.3 ± 10.5 (10/33) ^a	54.5 ± 7.8 (18/33) ^b
AA-KSOM	58.5 ± 2.2 (31/53) ^b	3.2 ± 2.1 (1/31) ^a	32.3 ± 0.7 (10/31) ^a	64.5 ± 1.8 (20/31) ^b
AA-SOF	40.7 ± 4.1 (22/54) ^b	13.6 ± 0.4 (3/22) ^a	45.5 ± 10.1 (10/22) ^a	40.9 ± 6.7 (9/22) ^b
<i>Morulae and blastocyst rate (D5)</i>				
Groups	Total morulae (%)	Morulae/Cleaved (%)	Total blastocyst (%)	Blastocyst/Cleaved (%)
IVF-KSOM	50.0 ± 9.5 (37/74) ^a	63.8 ± 10.6 (37/58) ^a	1.3 ± 1.2 (1/74) ^a	1.7 ± 1.4 (1/58) ^a
IVF-SOF	10.9 ± 1.1 (8/73) ^c	24.2 ± 4.1 (8/33) ^b	0.0 ± 0.0 (0/73) ^b	0.0 ± 0.0 (0/33) ^b
AA-KSOM	30.2 ± 3.0 (16/53) ^b	51.6 ± 4.3 (16/31) ^a	0.0 ± 0.0 (0/53) ^b	0.0 ± 0.0 (0/31) ^b
AA-SOF	9.3 ± 1.6 (5/54) ^c	22.7 ± 4.3 (5/22) ^b	0.0 ± 0.0 (0/54) ^b	0.0 ± 0.0 (0/22) ^b

Mean $\pm$ standard error. ^{a,b,c}: values with different subscript letters in columns are significantly different ($P < 0.05$). D2: day two of *in vitro* development. D5: day five of *in vitro* development.

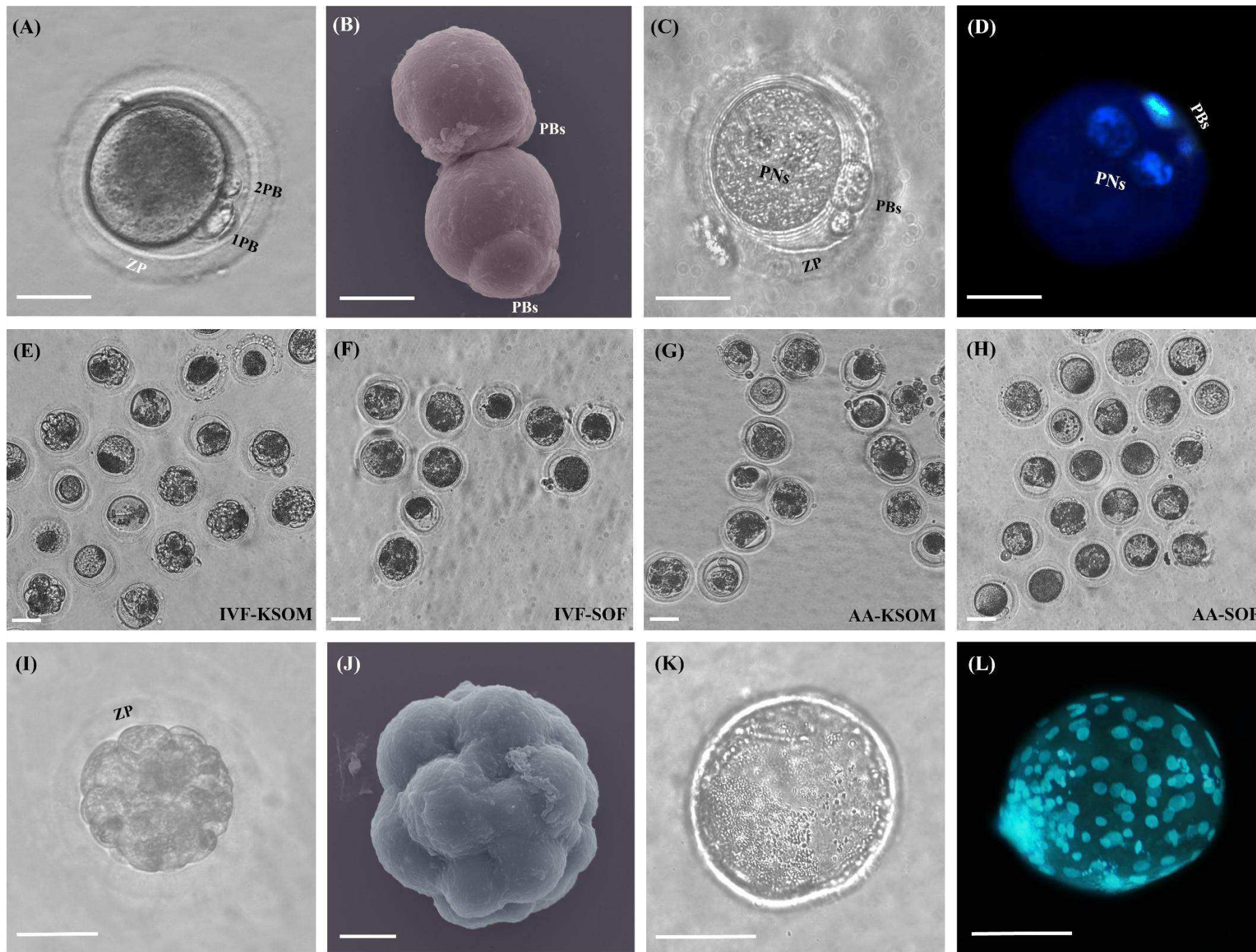


Figure 4. Embryo development kinetics following *in vitro* fertilization and oocyte artificial activation in the red-rumped agouti. **(A)** Representative image of a presumptive zygote displaying the first and second polar bodies. **(B)** Colored scanning electron microscopy (SEM) image of a presumptive zygote. **(C)** Visualization of female and male pronuclei after IVF using an inverted microscope. **(D)** H33342 staining of both pronuclei in a post-IVF structure. **(E–H)** Representative images of IVD plates on Day 5, illustrating cleavage in structures generated through IVF and AA and cultured in KSOM or SOF media. **(I)** Morula produced via IVF on D5 in KSOM medium, exhibiting well-defined cellular cleavage. **(J)** Colored SEM image of a red-rumped agouti morula. **(K)** Expanded blastocyst generated by the IVF-KSOM group, visualized under an inverted microscope. **(L)** Expanded blastocyst of red-rumped agouti stained with H33342 to identify blastomeres. ZP: zona pellucida. 1PB: first polar body. 2PB: second polar body. PBs: polar bodies. PNs: pronuclei. Scale bar: 10 μm (J), 20 μm (A–D), 50 μm (E–I; L), 60 μm (K). Magnification: 20 $\times$ (E–H), 40 $\times$ (A; C–D; I; K–L). SEM magnification: (J) 3.65 kx and (B) 2.27 kx.

Finally, no differences were observed among the groups ($P > 0.05$) in the assessment of oxidative stress (**Figure 5**). However, the levels of ROS and mitochondrial membrane potential were higher in the morulae compared to the presumptive zygotes ($P < 0.05$).

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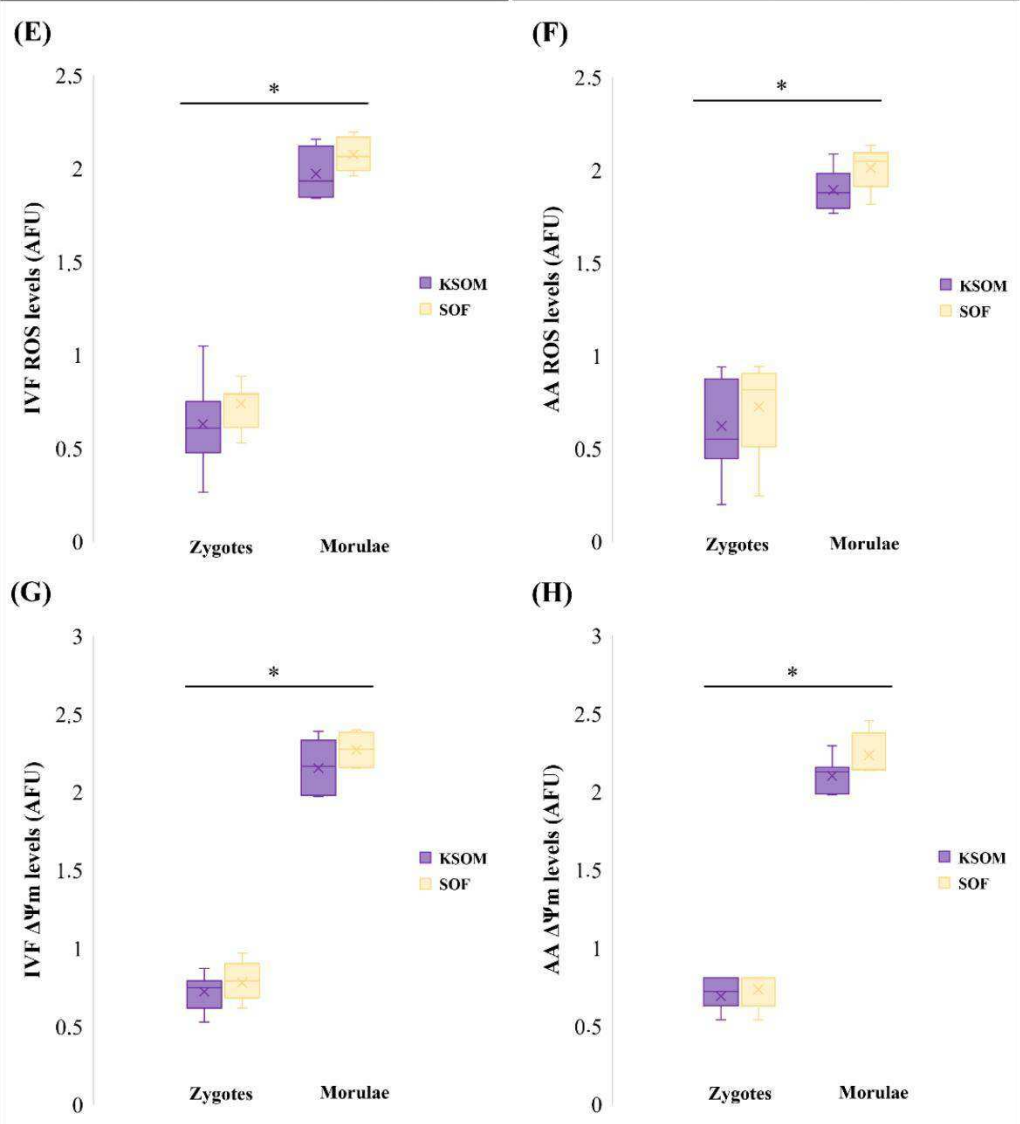
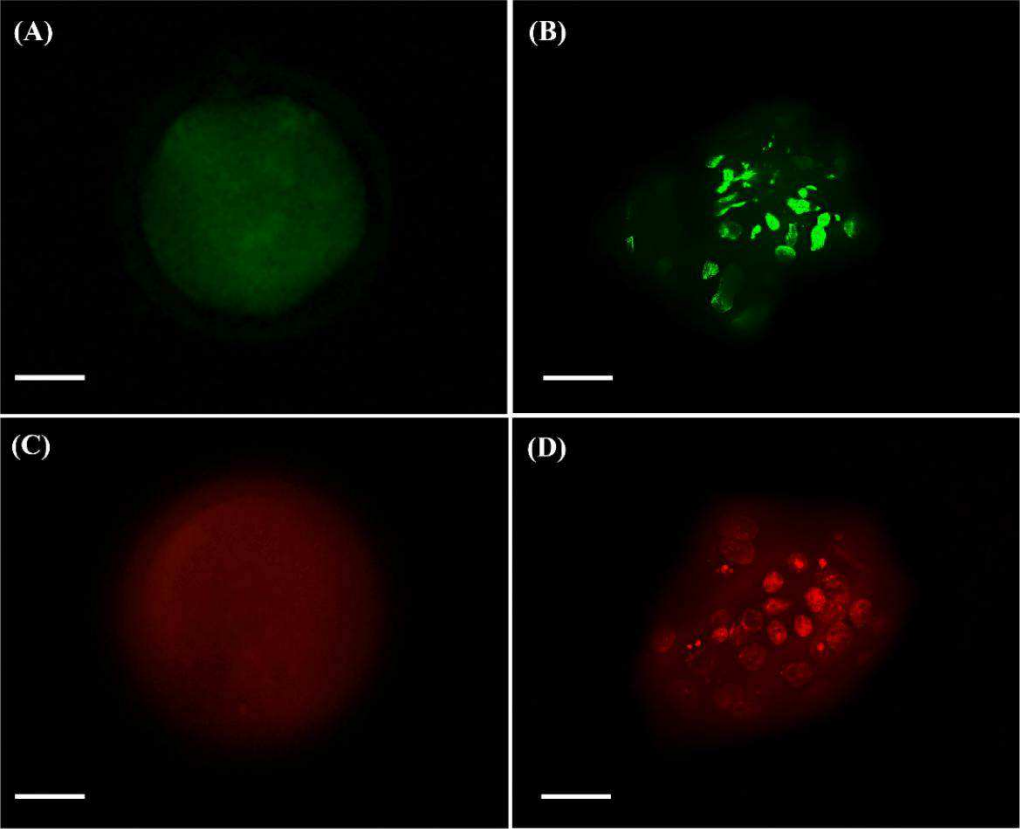


Figure 5. Effect of different embryo production techniques (IVF vs. AA) and embryo culture media (KSOM vs. SOF) on presumptive zygotes and morulae ROS production and mitochondrial membrane potential ($\Delta\Psi_m$). **(A)** Representative image of a zygote and **(B)** morulae stained with the H₂DCFDA fluorescent probe for the assessment of reactive oxygen species (ROS) levels. **(C)** Photomicrograph of a zygote and **(D)** morulae labeled with the MitoTracker Red fluorescent probe for the evaluation of mitochondrial membrane potential ($\Delta\Psi_m$) levels. **(E)** ROS levels in IVF-derived structures cultured in KSOM or SOF. **(F)** ROS levels in AA-derived structures cultured in KSOM or SOF. **(G)** $\Delta\Psi_m$ levels in labeled IVF-derived structures. **(H)** $\Delta\Psi_m$ levels in labeled AA-derived structures. Asterisks indicate statistically significant differences between structure types ($P < 0.05$). Scale bar: 20 μm . Magnification: 40 $\times$.

4. Discussion

This study demonstrated that IVF using KSOM medium is currently the most effective approach for the *in vitro* production of red-rumped agouti embryos. The optimal performance of IVF-KSOM was evidenced by a lower incidence of zygotes with morphological abnormalities, higher cleavage rates up to D5, increased morula formation, and its distinction as the only group to surpass the IVD block in this species, successfully reaching the blastocyst stage for the first time. Despite the lower developmental rates observed with AA, the use of the KSOM medium for the first time in this species improved the technique's efficiency.

Successful IVF in rodents relies on sperm samples exhibiting high motility, normal morphology, and intact DNA, as these parameters are strong predictors of male reproductive success in ARTs [28]. We achieved exceptional results through optimizations in sperm selection and capacitation protocols for the red-rumped agouti, conducted by our research team

[17-18], with total motility (TM) exceeding 94.0%. This represents a significant improvement compared to the findings of Castelo et al. [29], who reported a TM of only 24.8% in the same species. Additionally, our study demonstrated an increase in progressive motility (PM), reaching 72.0%, whereas Dantas et al. [19] observed a PM of just 13.7%. Moreover, the sperm samples used in IVF exhibited hyperactivated motility, a critical attribute that facilitates detachment from the isthmus reservoir and successful penetration of the surrounding oocyte envelopes [30].

Sperm membrane integrity is fundamental for key reproductive processes, including capacitation, acrosome reaction, sperm-egg fusion, and the regulation of fluid influx to maintain osmotic balance [31]. In the present study, we observed high membrane functionality rates (90%), which are consistent with the findings of Dantas et al. [19,32-33], who reported functionality rates of approximately 80% when evaluating cauda epididymal sperm from red-rumped agoutis using the hypo-osmotic swelling test.

Sperm quality is intrinsically associated with mitochondrial functionality, as this organelle plays a crucial role in regulating various metabolic processes, including ATP production and sperm motility [34]. The results obtained in this study are consistent with existing data for the species, as reported by Dantas et al. [19,32-33], who observed approximately 80% normal mitochondrial function.

Furthermore, the normal sperm morphology observed in the samples used for IVF may have positively influenced the higher IVD rates. In mice, Kwai et al. [35] have demonstrated that morphological abnormalities, such as tail breakage and defects in the midpiece, can reduce fertilization potential by up to 40%. The high proportion of intact DNA in our sperm samples

further corroborates the efficiency of our IVF outcomes, as sperm DNA integrity has become one of the most extensively investigated biomarkers in reproductive biology. It is well established that DNA damage negatively impacts fertilization, embryonic development, implantation, and pregnancy success rates [36].

The IVD outcomes may be directly associated with proper oocyte maturation, as the cytoplasmic and nuclear modifications within the oocyte are essential for successful sperm interaction during fertilization [37]. Our findings, which include a 95.0% *cumulus* cell expansion rate and 68.2% 1PB extrusion, exceed those reported by Ferraz et al. [38], who achieved only 27% after 24 hours of IVM in their study of black-rumped agouti (*Dasyprocta prymnolopha*). However, more recent research by Praxedes et al. [13] on red-rumped agouti (*Dasyprocta leporina*), aimed at optimizing the maturation medium for this species, yielded results comparable to ours, with a maturation rate of 52.0% and 100.0% *cumulus* cell expansion.

Upon comparing the outcomes of IVF and AA in the development of red-rumped agouti embryos, it was observed that, after 6 hours of incubation, IVF yielded a higher number of morphologically normal zygotes. Although identifying embryos with the most significant implantation potential remains a persistent challenge in reproductive science, various strategies have been proposed in recent years to assess embryo viability, including the morphological assessment of zygotes [39]. The poorer results observed in the AA groups may be attributed to hypothesized developmental failures resulting from improper genome imprinting and inadequate parthenogenetic activation, which may lead to irregular cytoplasmic reorganization of these embryos [40].

Regardless of the IVD medium used, our observations revealed a favorable interaction between oocytes and sperm following IVF, with polyspermy occurring at a rate of approximately 6% or less. This is an important finding, as higher polyspermy rates, often surpassing 10% in IVF systems, can result in embryos that are unsuitable for transplantation and hinder their further development [41].

On D2 of IVD, the first cleavages revealed that embryos from both techniques cultured in KSOM medium displayed the highest frequencies of total cleavage and $\geq$ reached or exceeded the eight-cell stage. This finding is noteworthy, as previous studies have shown that the timing of initial cleavage can be a crucial factor in identifying embryos with greater implantation potential [42]. Additionally, transferring embryos from early cleaved zygotes has been associated with improved pregnancy and implantation rates compared to transferring embryos from zygotes that cleave later [43].

The key factor contributing to the success of this study was the selection of KSOM as the IVD medium. Mammalian preimplantation embryos are highly sensitive to their surrounding environment, pivotal in influencing their developmental capacity by modulating key metabolic processes [44,15]. Consequently, it is crucial to provide a culture medium tailored to the specific needs of each species. KSOM is a well-established culture medium for mouse embryos, which has notably advanced the field of IVEP for rodents by enabling the successful progression of embryos from the two-cell stage blockage to blastocysts for the first time [45].

The superior performance of KSOM over SOF in red-rumped agoutis can be attributed to the medium's unique composition, which includes a blend of essential amino acids, high glucose concentration (5.56 mmol/L), and bovine serum albumin (4 mg/mL). Research has

demonstrated that these components are vital for supporting rodent embryo development, resulting in higher cleavage rates and increased blastocyst production across various mouse strains [46].

Starting from D5, the IVF-KSOM group exhibited superior cleavage rates and cell numbers compared to the AA-KSOM group, highlighting a distinct difference in embryonic development beyond the medium composition. One potential explanation for this outcome could be the enhanced fertilization success observed in the IVF group, where maternal and paternal gene expressions are crucial for the proper progression of embryonic development [47]. The limited developmental potential of activated embryos in some mammalian species remains largely unexplained. Still, one hypothesis is that the absence of paternal genetic material may prolong the cell cycle, slowing down the developmental process [48]. This is consistent with the observation that embryos produced through artificial activation tend to develop more slowly than those generated through IVF, particularly during preimplantation [49]. Another plausible explanation is that the reduced DNA content in AA embryos may be insufficient to fully regulate the gene expression network, leading to increased apoptosis or failure in critical developmental stages [47].

To refine the AA procedure for red-rumped agouti, incorporating KSOM as the IVD medium demonstrated favorable outcomes, with a total cleavage rate of 58.5% and 30.2% morulae, exceeding the results obtained for other agouti species. In comparison, the activation in black-rumped agouti, as outlined by Ferraz et al. [38], achieved a cleavage rate of 63.6% on D2 and 15.1% morulae on D4. For red-rumped agouti, Praxedes et al. [13] reported a cleavage rate of 43.2% on D2 and 8.3% morulae production on D5, in the best-performing experimental group. These variations can likely be attributed to the use of SOF medium in both studies, which could

have affected the overall success of their IVEP. Our results support this hypothesis, as the AA-SOF group exhibited lower cleavage (37.0%) and morulae rates (9.3%) compared to the AA-KSOM group.

We observed an improvement in the method when comparing our results to those of Praxedes et al. [13], using the same AA protocol but with KSOM instead of SOF, reinforcing the superiority of KSOM as the medium for red-rumped agouti. Despite this optimization, further adjustments are necessary in the pre-IVD stages of artificial activation, particularly in fine-tuning incubation times and identifying the most suitable protein activators or inhibitors to increase the success rate of *in vitro* embryo production.

This study represents the first successful report of IVF in red-rumped agouti. Prior research by Ferraz et al. [38] had already demonstrated the application of this reproductive biotechnology in black-rumped agouti. They used a conventional bovine IVF protocol with a commercial medium, employing non-capacitated epididymal sperm and a high sperm concentration of 25×10^6 sperm/mL for oocyte co-incubation. Their protocol involved a prolonged IVF duration of 15 h and used SOF as the IVD medium. Despite these efforts, the success rates were relatively low, with only 8% cleavage on D2 and 2.9% morulae on D5.

The better results observed in our study with red-rumped agouti may be attributed to several key methodological differences. Specifically, we focused on optimizing the IVF protocol based on characteristics more closely aligned with rodent species. This included refined sperm selection and capacitation techniques [17-18], the use of a lower sperm concentration, a shorter IVF incubation period, and the selection of an IVD medium that is widely employed in rodent IVF protocols. We aimed to maximize the efficiency of each step by carefully evaluating and

fine-tuning each aspect of the process. As a result, this study marks the first successful production of a blastocyst via IVF in caviid rodents, demonstrating significant advancements in embryo development outcomes.

Finally, no differences were observed in $\Delta\Psi_m$ levels or ROS production across the evaluated groups, indicating the stability of the culture environment regardless of the method or medium used. However, elevated levels were detected when comparing structures at the 6-hour incubation mark to morulae on D5. This increase may be associated with the transient rise in $\Delta\Psi_m$ observed in late-stage embryos, coinciding with embryonic genome activation, as previously reported in mouse IVF embryos [50]. Similarly, the increase in ROS production aligns with its essential role in regulating gamete function and embryonic development, where a higher cell count requires greater ROS levels than zygotes [51].

5. Conclusions

The combination of IVF and KSOM medium has proven to be the most effective approach for IVEP in red-rumped agoutis, promoting superior zygote morphology and improved embryonic development rates. Moreover, applying KSOM in AA contributed to refining this method for this species.

This study represents the first documented case of *in vitro* blastocyst production in red-rumped agouti. It offers valuable insights into this species' reproductive biology, aids in the refinement of protocols for closely related rodents, and ultimately supports conservation efforts for the agoutis.

CRediT authorship contribution statement

L.R.M. Oliveira: Conceptualization, data curation, investigation, methodology, visualization, writing – original draft. **L.V.C. Aquino:** Investigation. **L.G.P. Bezerra:** Investigation. **M.F. Oliveira:** Resources. **A.R. Silva:** Investigation, resources, writing – review & editing. **A.F. Pereira:** Conceptualization, investigation, data curation, formal analysis, funding acquisition, project administration, resources, supervision, writing – review & editing.

Availability of data and materials

The dataset analyzed during the current study is available from the corresponding author on reasonable request.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use artificial intelligence-assisted technologies in the writing process.

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Declaration of interest

The authors declare no conflict of interest.

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6 CONSIDERAÇÕES FINAIS

Os resultados obtidos nesta tese avançaram significativamente na compreensão dos fatores que influenciam a eficiência das técnicas de fertilização *in vitro* e desenvolvimento embrionário em cutias-vermelhas, oferecendo bases sólidas para a aplicação em protocolos reprodutivos da espécie e de outros roedores neotropicais.

ETAPA 1 – Seleção espermática: a escolha do método de seleção espermática impactou diretamente na qualidade funcional e no equilíbrio redox dos espermatozoides. Dentre os métodos avaliados, a centrifugação simples destacou-se por preservar a integridade das membranas, a funcionalidade mitocondrial e por promover um ambiente intracelular com menores níveis de EROs e maior presença de glutatona. Apesar das diferenças observadas na qualidade espermática, todos os métodos avaliados mantiveram a capacidade de fertilização na FIV heteróloga, resultando na formação de embriões até o estágio de mórula.

ETAPA 2 – Capacitação espermática: a otimização das condições de capacitação espermática permitiu identificar que a combinação de menor concentração de BSA (4 mg/mL) com a adição de cálcio (2 mM) e um tempo de incubação de 6 h proporcionou o melhor desempenho. Esse protocolo favoreceu a motilidade progressiva, preservou a integridade estrutural das células e potencializou os mecanismos antioxidantes, resultando em maior taxa de espermatozoides capacitados, com reação acrossômica e hiperatividade, elementos cruciais para o sucesso da fertilização.

ETAPA 3 – Meio para fertilização *in vitro*: o meio TALP mostrou-se o mais promissor, promovendo taxas superiores de clivagem e desenvolvimento de embriões mais avançados até o quinto dia de cultivo após FIV heteróloga. Além disso, os embriões gerados neste meio apresentaram menores níveis de estresse oxidativo, fator determinante para a qualidade embrionária e para a progressão saudável do desenvolvimento inicial.

ETAPA 4 – Técnica de PIVE e meio de desenvolvimento embrionário: ao comparar diferentes meios DIV e métodos de produção *in vitro* de embriões, foi comprovada que a combinação da fertilização *in vitro* homóloga com cultivo em meio KSOM foi a mais eficiente para o desenvolvimento embrionário em *D. leporina*. Este protocolo não apenas favoreceu a formação de mórulas, mas também foi o único a alcançar o estágio de blastocisto no gênero *Dasyprocta*, etapa crucial para aplicações em transferência de embriões e conservação da espécie. Importante destacar que, independentemente do método utilizado, o cultivo em KSOM

consistentemente promoveu melhores resultados, consolidando-se como a melhor escolha para o desenvolvimento embrionário da espécie, inclusive na ativação oocitária artificial.

Em conjunto, os resultados desta tese elucidam aspectos importantes da biologia reprodutiva da *D. leporina*, oferecendo subsídios valiosos para o aprimoramento de técnicas de reprodução assistida voltadas à conservação da espécie. As descobertas não apenas contribuem para a eficiência dos protocolos existentes, mas também ampliam as possibilidades de aplicação prática, aproximando cada vez mais a biotecnologia da reprodução de estratégias efetivas de preservação da biodiversidade.

7 PERSPECTIVAS

Os resultados obtidos nesta tese estabelecem bases consistentes para o avanço e aplicação de biotecnologias reprodutivas voltadas à conservação da *D. leporina*. A elucidação de aspectos fundamentais relacionados à seleção espermática, capacitação, condições de fertilização e desenvolvimento embrionário *in vitro* permite novas possibilidades para a ampliação do conhecimento e o desenvolvimento de protocolos otimizados para esta espécie, até então pouco estudada nesse contexto.

Apesar da otimização dos protocolos de seleção espermática, permanece a necessidade de investigar as implicações do uso de espermatozoides de menor viabilidade nos procedimentos de fertilização *in vitro*, bem como compreender os mecanismos pelos quais sua capacidade fecundante não foi significativamente afetada após seleção.

Com a definição do meio de capacitação mais eficiente para a cutia-vermelha, torna-se necessário investigar de que forma a taxa de capacitação obtida se reflete na eficiência da produção *in vitro* de embriões, bem como elucidar os mecanismos moleculares envolvidos no processo de capacitação espermática desta espécie, especialmente frente à utilização de BSA e cloreto de cálcio ao longo do tempo.

Os avanços obtidos na compreensão do melhor meio para co-incubação na FIV sugerem oportunidades para o desenvolvimento de meios de cultivo mais fisiológicos. A investigação de formulações sintéticas enriquecidas e de combinações específicas de suplementos com base no ambiente *in vivo* pode ampliar as taxas de sucesso da fertilização e do desenvolvimento embrionário inicial. Adicionalmente, a otimização do desenvolvimento embrionário permite espaço para a aplicação de técnicas complementares, como a avaliação da competência epigenética dos embriões, análise transcriptômica e proteômica, buscando aprofundar o entendimento sobre os mecanismos que sustentam o desenvolvimento embrionário inicial da *D. leporina*.

Finalmente, os resultados obtidos nesta pesquisa acerca do desenvolvimento embrionário *in vitro*, apesar de pioneira, ainda requer mais avanços. Portanto, a otimização de protocolos para a formação de blastocistos viáveis ainda é necessária, por meio da investigação de diferentes concentrações espermáticas durante a co-incubação, tempo de FIV, condições atmosféricas na DIV, otimizações na maturação oocitária e estudos moleculares acerca da cinética embrionária dessa espécie.

4638 ANEXO A – CERTIFICADO DE AUTORIZAÇÃO DO PROJETO PELA COMISSÃO
4639 DE ÉTICA NO USO DE ANIMAIS DA UFERSA (CEUA/UFERSA)



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

Mossoró, 08 de junho de 2022.

PARECER 20/2021

*Certificamos que o projeto intitulado “Avaliação das etapas da fecundação in vitro homóloga e heteróloga em *Dasyprocta leporina* (Linnaeus, 1758)” 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 UFERSA em reunião no dia 02 de maio de 2022.*

Vigência do projeto	setembro/2022; janeiro-julho/2023, 2024
Espécie/linhagem	1) <i>Dasyprocta leporina</i> ; 2) <i>Cavia porcellus</i>
N. de Animais	1) 12; 2) 8
Peso/idade	Adultos
Sexo	1) Machos (6) e fêmeas (6); 2) fêmeas
Origem	CEMÁS

SIDNEI
MIYOSHI
SAKAMOTO
11203780877
Coordenador CEUA-UFERSA

4641 ANEXO B – AUTORIZAÇÃO DO PROJETO PELO SISTEMA DE AUTORIZAÇÃO
4642 E INFORMAÇÃO EM BIODIVERSIDADE – SISBIO



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: 71837-1	Data da Emissão: 23/09/2019 12:19:00	Data da Revalidação*: 23/09/2020
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: Alexandra Fernandes Pereira	CPF: 913.071.983-68
Título do Projeto: OBTENÇÃO DE CARIOPLASTOS E CITOPLASTOS DE <i>Dasyprocta leporina</i> (LINNAEUS, 1758) VISANDO A TRANSFERÊNCIA NUCLEAR DE CÉLULAS SOMÁTICAS	
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	Colheita de ovários e processamento de oócitos	02/2020	02/2024
2	Análises in vitro das amostras somáticas	02/2020	02/2022
3	Processamento e análise de amostras somáticas	02/2020	02/2020
4	Colheita de amostras somáticas	11/2019	01/2020

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
3	Erika Almeida Praxedes	Colaborador	017.722.684-61	Brasileira

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Dados do titular

Nome: Alexsandra Fernandes Pereira	CPF: 913.071.983-68
Título do Projeto: OBTENÇÃO DE CARIOPLASTOS E CITOPLASTOS DE <i>Dasyprocta leporina</i> (LINNAEUS, 1758) VISANDO A TRANSFERÊNCIA NUCLEAR DE CÉLULAS SOMÁTICAS	
Nome da Instituição: UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO	CNPJ: 24.529.265/0001-40

Observações e ressalvas

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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 X Táxons

#	Atividade	Táxon	Qtde.
1	Coleta/transporte de amostras biológicas ex situ	<i>Dasyprocta leporina</i>	-

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

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Dados do titular		
Nome: Alessandra Fernandes Pereira		CPF: 913.071.983-68
Título do Projeto: OBTENÇÃO DE CARIOPLASTOS E CITOPLASTOS DE <i>Dasyprocta leporina</i> (LINNAEUS, 1758) VISANDO A TRANSFERÊNCIA NUCLEAR DE CÉLULAS SOMÁTICAS		
Nome da Instituição: UNIVERSIDADE FEDERAL RURAL DO SEMI-ÁRIDO		CNPJ: 24.529.265/0001-40

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[illegible]

* Identificar o espécime do nível taxonômico possível

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ANEXO C – VERSÃO DO ARTIGO PUBLICADO NA LABORATORY ANIMALS

**CAPÍTULO 2 - INSIGHTS AND IMPLICATIONS FROM A COMPARATIVE
ANALYSIS OF *IN VITRO* FERTILIZATION SUCCESS IN CAVIOIDEA AND
MUROIDEA RODENTS**

Qualis 2017-2020: A2 (Medicina Veterinária)

Fator de impacto: 1.3

Doi: <http://doi.org/10.1177/00236772251331683>

Insights and implications from a comparative analysis of in vitro fertilization success in Caviioidea and Muroidea rodents

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Alexsandra Fernandes Pereira 

Laboratory Animals
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Abstract

In vitro fertilization (IVF) is an assisted reproduction technique widely used due to its capacity to generate more offspring within a brief timeframe while simultaneously providing fundamental physiological data through gamete interactions. However, its suboptimal development in most species is challenging when IVF is applied to rodents. Despite numerous studies focusing on the Caviioidea and Muroidea superfamilies, some species struggle to achieve satisfactory cleavage rates, whereas others obtain over 90% efficiency with live offspring. Efforts to enhance these rates include adopting methodologies commonly used in other mammals or developing entirely new protocols. Nonetheless, the distinct morphophysiological differences among these animals necessitate careful consideration to avoid overgeneralization during optimization. Therefore, we aimed to review what has been established for the two most researched rodent superfamilies, Caviioidea and Muroidea, regarding the steps of IVF, such as gamete manipulation and embryonic development, to understand better the protocol diversification in the rodent order and how it affects IVF efficiency. After an extensive analysis of data accumulated over the years, it becomes evident that the Muroidea superfamily is used more extensively in reproductive studies than the Caviioidea. Furthermore, IVF procedures achieve total efficiency in only one rodent species, the laboratory mouse (*Mus musculus*). Consequently, developing a successful IVF technique across rodent species requires substantial modifications to optimize species-specific early steps – a challenge complicated by difficulties in obtaining viable gametes during the initial stages and the limited research interest in species without significant commercial value, such as wild rodents.

Keywords

Genetic diversity preservation, assisted reproductive technologies, wild rodent conservation, in vitro embryo production, oocyte maturation and epididymal sperm capacitation

Date received: 16 December 2024; accepted: 11 February 2025

Introduction

In vitro fertilization (IVF) of rodents serves two primary goals: (a) elucidating the reproductive physiology of different species and (b) increasing the number of offspring.¹ Initially, the study of physiology is possible by observing the behavior of these gametes in a controlled environment that emulates in vivo conditions, providing information from the interactions of these cells during co-incubation to the embryonic development phases up to the blastocyst stage.² Moreover, as a tool for reproductive enhancement in rodents, IVF is used to sustain populations in the laboratory, such as

Mus musculus, and thus more easily meet the population numbers required for research using these animals.¹ Furthermore, it can be used to conserve

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endangered wild species, being an option to increase the number of descendants quickly.³

Most of the studies aimed at optimizing this biotechnology are concentrated in just two superfamilies: Cavoidea and Muroidea (Figure 1). This is because they represent the most used species in laboratory studies, such as the laboratory mouse (Muroidea; *Mus musculus*), rat (Muroidea; *Rattus norvegicus*), hamster (Muroidea; *Mesocricetus auratus*), and guinea pig (Cavoidea; *Cavia porcellus*).⁴ The importance of these animals lies in their essential role in the ecosystem, acting as seed dispersers and forming a crucial foundation for various food chains.⁵ They have also been extensively involved in scientific research for logistical reasons, such as low feeding and maintenance costs, easy breeding, large litter sizes, and a short generation turnover.⁶ However, even within these superfamilies, the small murids are the only species in which IVF is regarded as a routine and efficient technique.⁷

These success gaps between the species may be linked to a long history of clashing approaches, with authors either elucidating all the initial steps or using previously established protocols as a basis. Horie et al.⁸ promoted an efficient protocol for prairie voles (*Microtus ochrogaster*), in which, initially, superovulation and collection of matured oocytes in females was conducted, followed by collection and capacitation of epididymal sperm, gamete incubation, and in vitro culture in different embryonic development medium. Approximately 32.6% of blastocysts were obtained in this study, and two offspring were born after embryo transfer. In contrast, Ferraz et al.⁹ performed an IVF in

black-rumped agouti (*Dasyprocta prymnolopha*) using a protocol for cattle, in which immature oocytes were obtained, followed by in vitro maturation for 24 h, collection of epididymal sperm, gamete co-incubation for 15 h, and embryo development in a commercial embryo medium. The authors reported a 2.9% morula rate; no embryos or offspring were generated. Presumably, this phenomenon occurs due to the significant morphophysiological differences presented by these species that prevent the optimal application of fertilization among rodents of these superfamilies when combined with a low number of research for reproductive characteristics, such as follicular fluid composition, estrus duration and synchronization, gamete characteristics and the mechanisms necessary for their interaction, and dynamics for embryonic development.

Therefore, this review aimed to discern the current phase of IVF optimization in rodents from the Cavoidea and Muroidea superfamilies. By scrutinizing the various stages of IVF, this review seeks to understand why this technique has not yet attained consistency among these animals, drawing on observations from studies conducted over the past several years.

Navigating the complexities of sperm preparation for IVF

Fundamentals of sperm collection and selection

The intricacies of instituting an effective IVF procedure in Cavoidea and Muroidea species (Figure 2) become

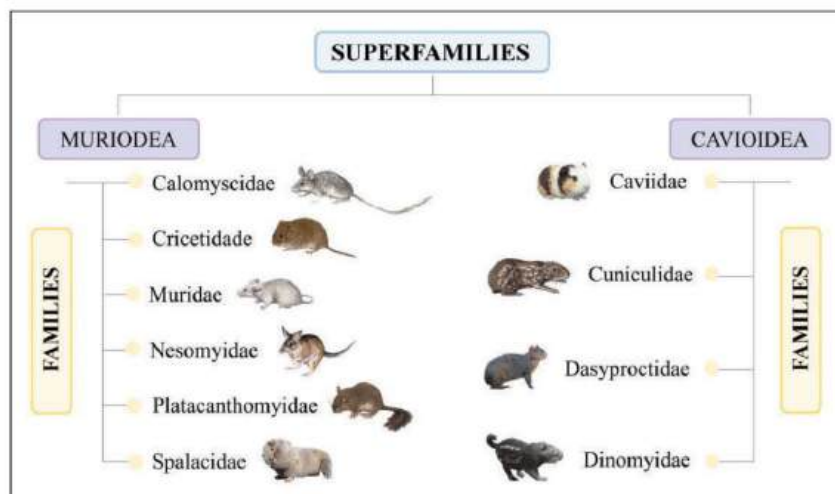


Figure 1. Representative image of the families that comprise the two large rodent superfamilies (Cavoidea and Muroidea) included in the optimization of IVF protocols.

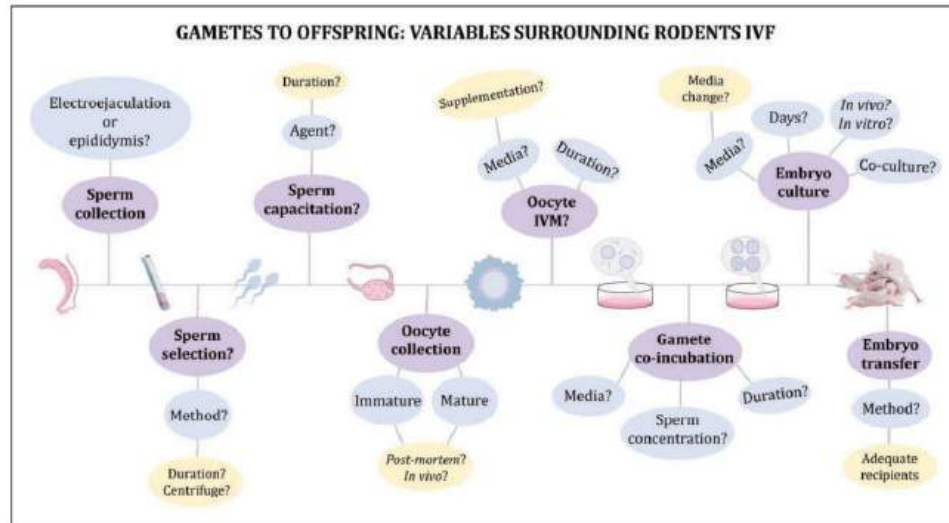


Figure 2. Representative scheme highlighting the steps necessary to optimize an IVF protocol in rodents.

apparent before sperm manipulation as there are disparities even in the methodologies employed for acquiring sperm cells. Different authors obtained varied results in both rodents' superfamilies when applying electroejaculation or epididymal sperm recovery.¹⁰ Although there are reports of success with electroejaculation in mice (*Mus musculus*), the technique is considered more difficult and expensive than obtaining cauda epididymal sperm.^{11,12} As a result, smaller species, such as Muroidea rodents, have their spermatozoa preferably collected after anesthesia/euthanasia, followed by recovery of the vasal-epididymal complex with associated testicles.¹³ There is a high success rate linked to this type of collection, with sperm motility above 80% in different species, such as the Cairo spiny mouse (*Acomys cahirinus*),³ large Japanese field mouse (*Apodemus speciosus*),¹⁴ Japanese grass vole (*Microtus montebelli*),¹⁵ East European vole (*Microtus rossiaemeridionalis*)¹⁵ and common vole (*Microtus arvalis*).¹⁵

In contrast, despite electroejaculation being the most advocated technique for spermatozoa recovery in Caviioidea rodents due to the larger size of the animals, efficiency has varied within the superfamily and even within the same species. Benavides et al.¹⁶ successfully performed electroejaculation in guinea pigs (*Cavia porcellus*) and obtained an ejaculate with 95% spermatozoa with a motility of 90.8%. On the other hand, with the first collections in red-rumped agouti (*Dasyprocta leporina*), the authors observed an efficiency of only 30–41% of ejaculates containing spermatozoa with 50% motility.^{17,18} Castelo et al.¹⁹ raised these numbers in *D. leporina* to 57% ejaculate-containing

cells, with motility above 60%. Currently, there is still no proved efficiency using this method in *D. leporina*, as the recovered samples only presented half the volume and concentration compared with samples obtained from the epididymis by retrograde flushing, making the latter the method of choice for these animals.²⁰ The necessity of euthanizing these animals for sperm collection could be considered a significant limitation in procuring samples, contributing to the sluggish advancement of the IVF steps for these rodents.

In addition to optimal collection, it is necessary to examine fundamental characteristics of the cells, such as sperm morphology. This is particularly challenging because species within the Caviioidea and Muroidea superfamilies exhibit substantial differences in their sperm cells. For Caviioidea, among 10 widely studied species, such as guinea pigs (*Cavia porcellus*), yellow-toothed cavies (*Galea musteloides*), chacoan mara (*Dolichotis salinicola*),²¹ and red-rumped agoutis (*D. leporina*),¹⁷ we observed a predominance of oval and paddle-like heads, with no hooks, ranging from 5 to 14 μm . Their midpiece and tail length are noticeably smaller than Muroidea, ranging from 3 to 12 μm and 33 to 92 μm , respectively. In contrast, a study conducted with about 170 species from three families and 10 sub-families of Muroidea rodents showed predominantly flattened sperm heads with a long apical hook and a size that was generally smaller (3–6 μm) than Caviioidea. On the other hand, their midpiece (17–63 μm) and tail length (75–175 μm) were longer than the other rodent superfamily.^{22,23}

These small morphology discrepancies can be key aspects for IVF improvement, influencing the choice of sperm selection methods as morphological patterns are closely linked to the success of spermatozoa cells in the female tract *in vivo*.²⁴ For Muroidea rodents, the swim-up (SW) method seems to be the most efficient (Table 1), as seen by Banafshi et al.⁷ comparing two selection methods in mouse (*Mus musculus*) epididymal sperm, in which half of the samples were selected by a density gradient (DG, 45/90%), followed by incubation for 15 min at 37°C and centrifugation at 300 × *g* for 10 min, twice. The other half were selected by the SW protocol, in which the cells were kept tilted for 15 min at 37°C and centrifuged at 300 × *g* for 5 min. After selection, a motility rate of 76.5% for DG and 82.3% for SW was reported. Regarding IVF, the DG group obtained a rate of 59.8% of two-cell cleavage and SW 70.0%, proving that the swim-up is the most suitable method for mice. However, there are variations for the SW method even within the murine, where some studies report longer times, as done in the Algerian mouse (*Mus spretus*), in which the cells were in a controlled environment for 60 min and showed 70% motility,²⁵ and species that required an extremely short time, such as the golden hamster (*Mesocricetus auratus*) that successfully recovered spermatozoa with 80% motility after 3 min of SW.²⁸

Although fewer studies conduct sperm selection in specimens from the Caviioidea superfamily, the methodologies that seem to stand out are simple centrifugation and DG (Table 1). These studies may suggest that spermatozoa with smaller midpiece and tail length require a higher separation force, as seen by Ni et al.,³² who combined a DG (35/85%) protocol with two centrifugation regimes of 600 × *g* and 300 × *g* for extended periods (18 min) and recovered viable sperm with 50% motility in guinea pigs. Thus, while sperm retrieval and selection constitute one of the preliminary steps in optimizing IVF due to the presumed ease of material collection, it becomes evident that this assertion holds true only for specific species. Consequently, contemporary research requires more *in vivo* studies to elucidate one efficient way to handle these vital samples, ensuring technical progress.

The critical role of sperm capacitation

In different species, the next elucidated step for sperm is capacitation. This step is often skipped in Caviioidea and Muroidea rodents, as some authors claim that the selection process alone is enough to prepare these cells for fertilization.^{7,33} Meanwhile, some authors emphasize the importance of mimicking the female's environment to ensure greater similarity with events that occur

in vivo, requiring the addition of bicarbonate (HCO_3^-) and its modulators, such as bovine serum albumin (BSA) and calcium chloride (CaCl_2).³⁴ These capacitating agents have already been used in both superfamilies, in different concentrations, and with variations in the time of exposure to the male gamete (Table 2).

Muroidea rodent *in vitro* capacitation seems to occur better in 25 mM bicarbonate. However, the addition of BSA appears to vary between species. In Algerian mice (*Mus spretus*), the authors report the need for only 0.3% BSA per 1 h to have a satisfactory capacitation rate for IVF.²⁵ Contrary to what was established by Hidalgo et al.,³⁷ when promoting capacitation in mice (*Mus musculus*), a concentration of 5 mg/mL BSA was used in association with bicarbonate for a more extended period (3 h), achieving a capacitation rate of approximately 40%. Likewise, Hirose et al.²⁸ used a high profile of BSA (15 mg/mL) in association with 3.4 mM Ca^{2+} for an even longer capacitation window (6 h), obtaining excellent results in Syrian hamsters (*Mesocricetus auratus*), reaching rates above 70%.

The data for capacitation in Caviioidea species is slightly different. Studies in guinea pigs focus on adding CaCl_2 to the capacitation medium, combined with 25 mM sodium bicarbonate (HCO_3^-). Chen et al.³⁴ and Ni et al.³² emphasized in *Cavia porcellus* epididymal sperm that adding $\text{Cl}^-/\text{Ca}^{2+}$ was essential for capacitation and acrosome reaction. In their first experiment, they maintained a constant HCO_3^- concentration but removed the chloride (Cl^-), resulting in a 30% lower capacitation rate than the control group. In their second experiment, removing calcium (Ca^{2+}) from the medium promoted a 15% reduction in the rate of acrosomal reactions, demonstrating that it is essential to assess the impact of these factors present in the *in vivo* environment and add them during *in vitro* techniques. In addition, despite the similarity between protocols in this superfamily, there is still a lot of variation between the results adjusting only one parameter, in which when using 1.71 mM CaCl_2 , different authors achieved rates of 34.2%,⁴⁴ 55%,⁴² and 80%⁴³ just increasing the sperm exposure periods in the capacitation medium of choice.

Notwithstanding recent advancements, considerable gaps persist in the comprehensive understanding of handling rodent spermatozoa. Research involving Caviioidea species is significantly underrepresented compared with the extensive work on sperm selection. Given that sperm preparation is a foundational stage of the process, the combination of deficient knowledge of sperm capacitation and inadequate selection methods can be one of the causes that prevent the increased effectiveness of IVF in these rodents.

Table 1. Sperm collection and selection methods applied in Muroidea and Caviodea rodents for IVF.

Super families	Family	Species	Sperm collection	Selection methods	Selection duration	Centrifugation parameters	Motility after selection [%]	Authors
Muroidea	Muridae	<i>Mus musculus</i>	Cauda epididymis	Density gradient (45/90%) Swim up	*15 min	300 × g – 10 min (2x) 300 × g – 5 min	76.5 82.3	Banafshi ⁷
	Muridae	<i>Mus spretus</i>	Cauda epididymis	Swim-up	60 min	Without centrifugation	70.0	Hasegawa ²⁵
	Muridae	<i>Rattus norvegicus</i>	Cauda epididymis	Density gradient (40/80%)	15 min	1500 × g – 15 min 200 × g – 20 min	ND	Safitri Purnobasuki ²⁶
	Muridae	<i>Acomys cahirinus</i>	Cauda epididymis	Swim-up	30 min	Without centrifugation	90.0	McKenna ³
	Muridae	<i>Rattus norvegicus</i>	Cauda epididymis	Swim-up	15 min	500 × g – 10 min	80.0	Agnihotri ²⁷
Caviodea	Cricetidae	<i>Mesocricetus auratus</i>	Cauda epididymis	Swim-up	3 min	Without centrifugation	80.0	Hirose ²⁸
	Cricetidae	<i>Microtus ochrogaster</i>	Epididymis	Swim-up	30 min	Without centrifugation	50.0	Horie ⁸
	Cricetidae	<i>Peromyscus maniculatus</i>	Epididymis	Swim-up	10 min	Without centrifugation	ND	Veres ²⁹
	Dasyproctidae	<i>Dasyprocta prymnolopha</i>	Cauda epididymis	Centrifugation	*	200 × g – 5 min	ND	Ferraz ⁹
	Caviidae	<i>Cavia porcellus</i>	Cauda epididymis	Density gradient (40/80%)	*	250 × g – 10 min	77.8	Cañón-Beltrán ³⁰
	Caviidae	<i>Cavia porcellus</i>	Vas deferens	Centrifugation	*	600 × g – 3 min (2x)	ND	Cañón-Beltrán ³¹
	Caviidae	<i>Cavia porcellus</i>	Cauda epididymis	Density gradient (35/85%)	*	600 × g – 18 min 300 × g – 10 min	50.0	Ni ³²

*Not informed.

ND: no data available; Min: minute.

Table 2. Sperm capacitation conditions applied in Murioidea and Caviioidea rodents for IVF.

Super families	Family	Species	Capacitation agent	Capacitation medium	Capacitation time	Capacitation evaluation	Capacitation rate (%)	Authors
Muroidea	Muridae	<i>Mus spretus</i>	0.11 mg/mL hypotaurine + 0.3% BSA	HTF medium	1 h	ND	ND	Hasegawa ²⁵
	Muridae	<i>Mus musculus</i> <i>Mus maltheyi</i> <i>Mus pahari</i>	ND	HTF medium	30 min.	ND	ND	Izquierdo-Rico ³⁵
	Muridae	<i>Mastomys coucha</i> <i>Mus musculus</i>	25 mM HCO ₃ ⁻ + 3 mg/mL BSA	TYH medium	1:30 h	Acrosome reaction: FITC	35.0	Balbach ³⁶
	Muridae	<i>Mus musculus</i>	25 mM HCO ₃ ⁻ + 5 mg/mL BSA	TYH medium	3 h	ND	40.0	Hidalgo ³⁷
	Muridae	<i>Mus musculus</i> <i>Mus spretus</i>	15 mM HCO ₃ ⁻	mT-H medium	1:30 h	ND	ND	DelBarco-Trillo ³⁸
	Muridae	<i>Mus spicilegus</i> <i>Mus musculus</i> <i>Mus spretus</i>	25 mM HCO ₃ ⁻	mT-H medium	2:30 h	ND	ND	Martin-Coello ³⁹
	Cricetidae	<i>Cricetulus griseus</i> <i>Mesocricetus auratus</i> <i>Phodopus campbelli</i> <i>Phodopus sungorus</i> <i>Phodopus roborovskii</i>	20 µM D-penicillamine + 100 µM hypotaurine + 1 µM epinephrine	mT-H medium	2 to 4 h	ND	ND	Tourmente ⁴⁰
	Cricetidae	<i>Mesocricetus auratus</i>	3.4 mM Ca ²⁺ + 15 mg/mL BSA	TALP medium	6 h	Acrosome reaction: PCM	77.0	Hirose ²⁸
	Caviidae	<i>Cavia porcellus</i>	10 mg/mL heparin + 6 mg/mL BSA	FERT-TALP	2 h	Acrosome reaction: FITC	28.4	Cañón-Beltrán ^{30,41}
	Caviidae	<i>Cavia porcellus</i>	1% BSA	HTF medium	2 h	ND	ND	Cañón-Beltrán ³¹
Caviioidea	Caviidae	<i>Cavia porcellus</i>	1.71 mM CaCl ₂	MCM medium	1 h	Acrosome reaction: CTC	55.0	Ramírez-Ramírez ⁴²
	Caviidae	<i>Cavia porcellus</i>	1.71 mM CaCl ₂	TALP medium	2 h	Acrosome reaction: CTC	80.0	Cordero-Martínez ⁴³
	Caviidae	<i>Cavia porcellus</i>	1.71 mM CaCl ₂	MCM medium	1:30 h	ND	34.2	Delgado-Buenrostro ⁴⁴
	Caviidae	<i>Cavia porcellus</i>	2.0 mM Ca ²⁺ + 25 mM HCO ₃ ⁻	MCM medium	6 h	Acrosome reaction: PCM	45.0	Chen ³⁴
	Caviidae	<i>Cavia porcellus</i>	2.0 mM Ca ²⁺ + 4 mg/mL BSA	MCM medium	6 h	Acrosome reaction: PCM	60.0	Ni ³²

ND: no data available.

BSA: bovine serum albumin; HCO₃⁻: sodium bicarbonate; HTF: human tubal fluid medium; TYH: Toyoda Yokoyama, Hoshi medium; mT-H: HEPES-buffered modified Tyrode's medium; TALP: Tyrode, albumin, lactate, and pyruvate medium; MCM: minimum capacitation medium; FITC: fluorescein isothiocyanate; PCM: phase-contrast microscopy; CTC: chlortetracycline staining.

Addressing obstacles in female gamete retrieval for IVF

The rodents of these two superfamilies show slight differences in their *cumulus*-oocyte complexes (COCs) morphology. Regarding the size, smaller species representing the superfamily Muroidea, such as mice (*Mus musculus*), have a COC size of around 65 μm ,⁴⁵ while larger species of the superfamily Caviioidea, such as the black-rumped agouti (*D. prymnolopha*) and red-rumped agouti (*D. leporina*), have COCs of approximately 100 and 90 μm , respectively.^{9,46} Additionally, the definition of a viable COC may vary according to its characteristics. In contrast, in black-rumped agouti (*D. prymnolopha*), a compact *cumulus* structure containing more than three layers of cells and ooplasm with fine and homogeneous granulations filling the interior of the zona pellucida is preferable.⁴⁷ Still, for guinea pigs (*C. porcellus*), an ideal oocyte has granular cytoplasm and four or more layers of *cumulus* cells.⁴⁸ These specificities surrounding the female gamete can dictate how these structures will interact with the environment in which they are inserted, requiring different supplementations to reach maturation and how they will be recovered from the animals (Table 3).

Most of the research done for the COC recovery from the Muroidea superfamily is with the laboratory mouse (*Mus musculus*). In this species, the oocytes are recovered already matured, with well established

protocols that promote superovulation with intraperitoneal injection of gonadotrophin to secure more samples from these small rodents.⁵⁵ However, this is not a reality for wild rodents or even less studied laboratory species, such as hamsters (*Mesocricetus*), as few studies have shed light on understanding the hormonal cycles of these females and how to increase this sample gain efficiently. Significant progress in this field has only begun recently, as demonstrated by the research of Yao et al.,⁵² who, using a protocol based on mice with human menopausal gonadotrophin subcutaneously injected into the back of the guinea pigs' necks, obtained an average of six oocytes per female, when the non-super stimulated group produced an average of only two oocytes per guinea pig. Therefore, in light of these challenges, concerted efforts appear to have been directed within both superfamilies to secure COCs, even in their immature states, to enhance the efficacy of their *in vitro* maturation (IVM).

Why does IVM optimization fall short of enhancing IVF efficiency?

The primary stage in developing an optimal IVM methodology for diverse rodent species involves acquiring viable immature oocytes, which can quickly become the first major difficulty. Collection can be performed post-mortem when working with a large number of laboratory animals, such as mice (*Mus musculus*), with a

Table 3. Oocyte collection and *in vitro* maturation in Muroidea and Caviioidea rodents.

Super families	Family	Species	Collection method	Females (n)	No. of oocytes/ females recovered	IVM duration	MII (%)	Authors
Muroidea	Muridae	<i>Rattus norvegicus</i>	Slicing	57	8 oocytes	30 h	ND	Oliveira ⁴⁹
	Muridae	<i>Rattus norvegicus</i>	Slicing	74	7 oocytes	30 h	55.7	Paim ⁵⁰
	Muridae	<i>Rattus norvegicus</i>	Slicing	72	5 oocytes	24 h	36.6	Alcoba ⁵¹
	Muridae	<i>Mus musculus</i>	Follicle	3	25 oocytes	17 h	93.0	Martín-Coello ³⁹
		<i>Mus spretus</i>	aspiration	3	20 oocytes		86.0	
		<i>Mus spicilegus</i>		6	11 oocytes		91.0	
Caviioidea	Caviidae	<i>Cavia porcellus</i>	Follicle aspiration	110	15 oocytes	17 h	78.3	Cañón-Beltrán ⁴¹
	Caviidae	<i>Cavia porcellus</i>	Slicing	48	14 oocytes	24 h	52.5	Yao ⁵²
	Caviidae	<i>Cavia porcellus</i>	Follicle aspiration	ND	564 oocytes*	17 h	75.9	Cañón-Beltrán ⁵³
	Cuniculidae	<i>Cuniculus paca</i>	LOPU	8	45 oocytes	24 h	54.1	Barros ⁵⁴
	Dasyproctidae	<i>Dasyprocta prymnolopha</i>	Slicing	17	17 oocytes	ND	ND	Ferraz ⁴⁷
	Dasyproctidae	<i>Dasyprocta prymnolopha</i>	Slicing	17	17 oocytes	24 h	27.4	Ferraz ⁹
	Dasyproctidae	<i>Dasyprocta leporina</i>	Slicing	21	18 oocytes	24 h	52.1	Praxedes ⁴⁶

ND: no data available.

LOPU: laparoscopic ovum-pick up.

*Total oocyte recovery without the number of females available for proportion.

standard process after cervical dislocation, when the ovaries can be excised, and immature oocytes for the IVM can be obtained by gently teasing the cortical region of the ovaries.⁵⁶ On the other hand, the optimal approach would entail *in vivo* recovery in cases in which the animal population is limited, whether wild-strained murines or wild caviids. This *in vivo* methodology ensures the survival of the egg donors, allowing multiple collections over the lifespan of a single animal.⁵⁷

Only one has successfully implemented laparoscopic ovum pick-up among those who have optimized IVM steps to establish IVF in their rodent species. Barros et al.⁵⁷ described a successful oocyte recovery in the lowland paca (*Cuniculus paca*), in which eight adult females were anesthetized (25 mg/kg ketamine and 0.5 mg/kg midazolam) and had their follicles (1 mm) aspirated with an 18-gauge needle attached to a vacuum system with pressure not exceeding 65 mmHg. They were successful in 27 procedures, in which 445 follicles were observed (14 ± 2.23 per animal) and 366 were aspirated (11.44 ± 1.82 per animal). One hundred and forty oocytes were recovered, with a mean of 4.38 ± 0.86 oocytes per paca, with an overall oocyte recovery rate of 38.25%. Meanwhile, the recovery of postmortem immature oocytes is still the method of choice (Table 3) for other wild rodents such as the black-rumped agouti (*D. prymnolopha*), red-rumped agouti (*D. leporina*), Algerian mice (*Mus spretus*) and spiny mouse (*Acomys cahirinus*), significantly limiting the experimental samples of these authors, which may delay the progress in optimizing their IVF protocols.^{3,9,39,46}

Between Caviodea and Muroidea rodents, the former, despite being advanced in all other stages of IVF, do not present more significant updates for IVM optimization. This can be explained by the practicality of obtaining matured oocytes through broad functional protocols, as mentioned previously.⁵⁸ Nevertheless, we can observe varied results, whether very successful, as seen by Martín-Coello et al.,³⁹ when promoting IVM for 24 h in three different species of murines (*Mus musculus*, *Mus spretus* and *Mus spicilegus*), obtained metaphase II (MII) rates ranging from 86% to 93% (Table 3). As well as gradual increases in the percentage of success, as was seen with rats (*Rattus norvegicus*), which initially used a protocol with only 24 h of IVM, the authors achieved MII rates of only 36.6%.⁵¹ But in 2015, Paim et al.⁵⁰ increased the IVM time of rats to 30 h while maintaining the same supplements in the medium, observing a mature oocyte rate of 55.7%, an increase of almost 20% in a few years (Figure 3).

This gradual efficiency increase phenomenon can be observed in Caviodea species (Figure 3). In guinea pigs

(*Cavia porcellus*), the authors consolidated the methodology by applying a 17-h IVM, in which 75.9%⁵³ turned into 78.3% of matured COCs,⁴¹ emphasizing the success of the protocol. In the genus *Dasyprocta*, the efficiency of *in vitro* maturation increased by almost 30%, as the first results in *D. prymnolopha* were only 27.4% MII,⁹ and in *D. leporina* was improved to 52.1% of oocytes on the MII nuclear stage.⁴⁶ However, these outcomes can be explained by the variances in protocol applications, such as incorporating different supplements in the media for each species and the different IVM durations.

The first example can be seen in the IVM outcomes of the guinea pig, in which despite Cañon-Beltrán et al.⁴¹ having obtained a rate above 78% MII, Yao et al.⁵² with the same species managed to get only 52.5% of the oocytes to mature *in vitro*, but with a more extended IVM protocol, reaching 24 h. Thus, knowing the ideal time the gamete would take *in vivo* to complete its maturation can enhance the reproductive biotechnologies of interest. Accounting for temporal aspects, some authors design their experiments with this aim. Three different times of oocyte maturation were tested (16 h vs. 20 h vs. 24 h), as in the IVM of black-rumped agouti (*D. prymnolopha*), defining the best time for the species as 24 h.⁹ Due to this previous work in the genus *Dasyprocta*, Praxedes et al.⁴⁶ optimized different parameters, such as the influence of various concentrations of follicle-stimulating hormone (10, 50, or 75 mIU/mL, FSH) and the synergistic effects of adding an epidermal growth factor (10, 50, or 100 ng/mL, EGF) in the maturation medium, concluding that the combination of 10 mIU/mL FSH and 10 ng/mL EGF for 24 h is ideal for red-rumped agouti oocytes.

Therefore, although *in vitro* oocyte maturation represents a crucial step in understanding the behavior of female gametes *in vitro* and serves as an alternative to using fully mature and fertilization-ready cells, this technique still exhibits incredibly variable efficiency among these rodents, with rates far from the ideal, unlike the consistent success seen in *Mus musculus*. These factors and the challenges in obtaining sufficient samples to increase the number of studies hinder the technique from reaching its full potential in optimizing IVF protocols for rodents within the Caviodea and Muroidea superfamilies.

The true state of rodent IVF: diverse efficiency and limited success

Initially, the sperm concentration to be added to the IVF droplet is one of the most critical factors, as excess cells can induce oxidative stress and polyspermy.^{59,60}

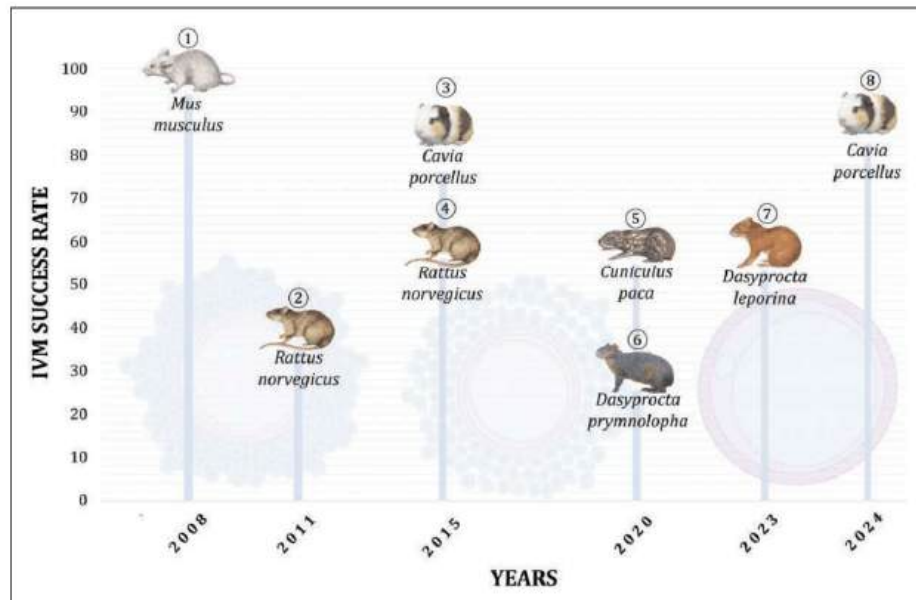


Figure 3. Representative graph demonstrating the efficiency of the oocyte in vitro maturation in different species of Muroidea and Caviioidea rodents over the years. [1] Martín-Coello et al.;³⁹ [2] Alcoba et al.;⁵¹ [3] Cañón-Beltrán et al.;⁵³ [4] Paim et al.;⁵⁰ [5] Barros et al.;⁵⁴ [6] Ferraz et al.;⁹ [7] Praxedes et al.;⁴⁶ [8] Cañón-Beltrán et al.⁴¹

There is no established standard for Muroidea species (Table 4), leading to a wide variation in the concentrations used. For instance, for Algerian mice (*Mus spretus*), authors used a low concentration of 0.032×10^6 (or 32,000 spz/mL) during co-incubation with mature oocytes, achieving approximately a 15% cleavage rate and a 10% efficiency in birth rate post-embryo transfer.²⁵ Conversely, a higher concentration of 1.0×10^6 (or 1,000,000 spz/mL) was used in the IVF of the Japanese grass vole (*Microtus montebelli*), resulting in a 2.7% blastocyst formation rate and a birth efficiency rate of 36.7% following embryo transfer.⁶⁵

A significant disparity in sperm concentrations is evident among the three studies that successfully established IVF protocols in Caviioidea rodents. Ferraz et al.⁹ adapted a bovine protocol for *D. prymnolopha*, employing a 25×10^6 spz/mL concentration with IVM oocytes, yielding a low cleavage rate of 2.9%. In contrast, Cañón-Beltrán et al.^{31,41} adapted mouse protocols for application in guinea pigs (*C. porcellus*), opting for a much lower concentration of 1.0×10^6 spz/mL in the IVF droplet with mature oocytes. This approach resulted in cleavage rates exceeding 80% for two-cell embryos and, subsequently, a 29.5% rate of morula formation.

Another critical factor to consider is co-incubation duration during fertilization, as extended exposure can

induce oxidative stress in the in vitro environment.⁶⁶ The Muroidea superfamily exhibits one wide range of appropriate co-incubation times, ranging from 3 h to 8 h. The optimal duration for these rodents appears to be around 6 h, as evidenced by Meguro et al.¹⁴ in their study on the large Japanese field mouse (*Apodemus speciosus*). They used a standard concentration of 1.0×10^6 spz/mL with previously matured oocytes for a 6-h co-incubation period, yielding a 29.4% rate of four-cell stage embryos after in vitro development. Longer durations are observed in earlier studies, such as in the southern African mastomys (*Mastomys coucha*) in 1998, in which a lower sperm concentration of 0.1×10^6 spz/mL was used over an 8-h window, resulting in a cleavage rate exceeding 43%.⁶² A similar disparity in co-incubation times is evident for Caviioidea species, as seen in sperm concentration choices. Using a cattle-adapted protocol, Ferraz et al.⁹ opted for a longer IVF duration of 15 h, whereas Cañón-Beltrán et al.^{31,41} chose a shorter co-incubation period of 5 h.

A third parameter that influences the success of the gamete's co-incubation in vitro is the choice of the appropriate medium. Due to their varied compositions, they can perform different functions in different species, as they differ in their energy, protein and ionic sources.⁶⁷ For Caviioidea rodents, the medium used

Table 4. Different conditions of *in vitro* fertilization and embryo production in Murioidea and Caviodea rodents.

Super families	Family	Species	Sperm concentration (sptz/mL)	Co-incubation medium	IVF duration	IVC medium	Efficiency rate [%]	Live birth [% efficiency]	Authors
Muroidea	Muridae	<i>Mus musculus</i>	0.67×10^6	TALP	5h	TALP	67.3 (2 cells)	No	Suzuki; Fujinoki ⁶¹
	Muridae	<i>Mus musculus</i>	*	HTF	5h	KSOM-AA	70.0 (2 cells)	Yes – [20]	Banafshi ⁷
	Muridae	<i>Mus spretus</i>	0.032×10^6	HTF	4h	CZB	15.2 (2 cells)	Yes – [10]	Hasegawa ²⁵
	Muridae	<i>Mus musculus</i>	1.0×10^6	HTF	5h	KSOM-AA	81.2 (2 cells)	No	Izquierdo-Rico ³⁵
		<i>Mus mattheyi</i>					0.0 (2 cells)		
		<i>Mus pahari</i>					3.5 (2 cells)		
		<i>Mastomys coucha</i>					0.8 (2 cells)		
	Muridae	<i>Rattus norvegicus</i>	*	M16	5h	M16	86.0 (BI)	No	Safitri; Purnobasuki ²⁶
	Muridae	<i>Acomys cahirinus</i>	0.25×10^6	MT6	3h	MT6	*	Yes – [*]	McKenna ³
	Muridae	<i>Apodemus speciosus</i>	1.0×10^6	HTF	6h	KSOM	29.4 (4 cells)	No	Meguro ¹⁴
	Muridae	<i>Mus musculus molossinus</i>	0.5×10^6	HTF	5h	CZB	97.4 (2 cells)	Yes – [100]	Mochida ¹
		<i>Mus musculus musculus</i>					97.4 (2 cells)	Yes – [83]	
		<i>Mus musculus domesticus</i>					96.3 (2 cells)	Yes – [83]	
		<i>Mus musculus castaneus</i>					84.6 (2 cells)	Yes – [75]	
	Muridae	<i>Mus spicilegus</i>	1.0×10^6	HTF	4h	HTF	87.0 (2 cells)	No	Martin-Coello ³⁹
	Muridae	<i>Mastomys coucha</i>	0.1×10^6	HTF	8h	*	43.7 (2 cells)	No	Nohara ⁶²
	Cricetidae	<i>Mesocricetus auratus</i>	0.022×10^6	TALP	6h	*	*	No	Hirose ²⁸
	Cricetidae	<i>Microtus ochrogaster</i>	*	HTF	5h	G-1 PLUS	32.6 (BI)	Yes – [50]	Horie ⁸
	Cricetidae	<i>Microtus fortis</i>	0.8×10^6	HTF	6h	HTF	93.0 (IB)	No	Liu ⁶³
	Cricetidae	<i>Calomys musculus</i>	1.0×10^6	M2	4h	*	65.3 (2 cells)	No	Lasserre ⁶⁴
Caviodea		<i>Calomys laucha</i>					74.6 (2 cells)		
	Cricetidae	<i>Microtus montebell</i>	1.0×10^6	M2	6h	*	2.7 (BI [#])	Yes – [36.7]	Wakayama ⁶⁵
	Caviidae	<i>Cavia porcellus</i>	1.0×10^6	TM	18–22 h	SOF	29.5 (morula)	No	Canón-Beltrán ⁴¹
	Caviidae	<i>Cavia porcellus</i>	1.0×10^6	HTF	5h	KSOM	81.4 (2 cells)	No	Canón-Beltrán ³¹
	Dasyproctidae	<i>Dasyprocta prymnolopha</i>	25×10^6	*	15h	SOF	2.9 (morula)	No	Ferraz ⁹

*No data available.

[#]In vivo development.

IVC: in vitro culture of embryos; HTF: human tubal fluid medium; TM: Tyrode's medium; M16: modified Tyrode's medium; MT6: modified Tyrode's medium with potassium; KSOM-AA: optimized simplex medium with potassium and pyruvate medium; M2: modified Krebs-Ringer bicarbonate solution; KSOM: optimized simplex medium with potassium; KSOM-AA: optimized simplex medium with potassium and amino acids; CZB: glucose-free and serum-free medium; G-1 PLUS: commercial medium for the culture of human embryos; SOF: synthetic oviductal fluid; BI: blastocyst; IB: initial blastocyst.

was the human tubal fluid (HTF) in guinea pigs by Cañón-Beltrán et al.,³¹ in which the authors reported an 81.4% cleavage success rate after IVF. Likewise, for Muroidea rodents, most studies use HTF in different rodents, such as *Mus musculus*,⁷ *Apodemus speciosus*,¹⁴ and *Microtus ochrogaster*,⁸ obtaining efficiency rates ranging from 29.4% to 70.0%.

From these observations, we can conclude that the IVF in these two rodents' superfamilies is far from being considered efficient for everyone. In murines, of the 15 works presented during this review, six species (*Calomys laucha*, *Microtus fortis*, *Microtus ochrogaster*, *Mus musculus*, *Mus spicilegus* and *Rattus norvegicus*) showed cleavage rates above 70% after embryonic development. Only four species (*Microtus fortis*, *Microtus montebell*, *Microtus ochrogaster* and *Rattus norvegicus*) showed blastocyst formation. Even within the genus *Mus*, under identical IVF conditions, Izquierdo-Rico et al.³⁵ noted disparities in embryonic development rates, spanning from 81.2% for two-cell cleavage in *Mus musculus* to 3.5% and 0% in *Mus pahari* and *Mus matthevi*, respectively. Additionally, among the protocols used, only five of 15 species (*Acomys cahirinus*, *Mus musculus*, *Mus spretus*, *Microtus ochrogaster* and *Microtus montebell*) had offspring after embryo transfer. However, within Caviodea rodents, the generation of blastocysts has not been documented to date, exhibiting a halt in development after morula formation, and no offspring have been produced thus far.

In summary, establishing a successful IVF technique in rodents requires significant modifications to optimize several species-specific initial steps. It is imperative not to rely on exact replicas of protocols designed for other mammals, even those within the same families or genus as the study species.

Conclusions

Despite recent advances in increasing the efficiency of the initial stages of IVF, as the best method for sperm collection, selection and capacitation, followed by oocyte collection and in vitro maturation, and the best conditions for gamete co-incubation and embryo culture, there is still a long way to go to guarantee high efficiency for Muroidea and Caviodea rodents. One of the primary reasons for the limited advancement in these methodologies is the lack of studies dedicated to elucidating the protocols involved. This is primarily due to the challenges encountered during the initial stages of collecting viable male and female gametes. Additionally, the low interest in investigating species that do not offer significant commercial returns, such as wild rodents, further impedes the progress of these reproductive biotechniques.

Moreover, this review highlights the discrepancy in the number of species studied between the two superfamilies. In Muroidea, more species (20) are explored for reproductive studies compared with Caviodea (four). However, even within the Muroidea superfamily, research is concentrated on only two families (Muridae and Cricetidae). Thus, the notion that fertilization is a common and optimized technique for rodents holds true only for the laboratory mouse (*Mus musculus*).

In conclusion, for IVF to be regarded as a viable and promising option for these rodents, it is imperative to prioritize morphophysiological studies and advance research aimed at understanding their in vivo environment. This understanding is essential for effectively translating in vivo conditions to the in vitro environment, thereby ensuring species-specific success for each animal of interest, whether the goal is conservation or commercial reproduction.

Consent to participate

Not applicable.

Consent to publication

Not applicable.

Data availability

The data that supports this study are available in the article. **[AQ1]**

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Réflexions et implications d'une analyse comparative du succès de la fécondation in vitro chez les rongeurs Cavoidea et Muroidea

Résumé

La fécondation in vitro (FIV) est une technique de procréation assistée largement utilisée en raison de sa capacité à générer une progéniture plus importante dans un court laps de temps tout en fournissant simultanément des données physiologiques fondamentales par le biais des interactions gamètes. Son développement sous-optimal chez la plupart des espèces s'avère toutefois un problème lorsque la FIV est appliquée aux rongeurs. Malgré de nombreuses études portant sur les superfamilles Cavoidea et Muroidea, certaines espèces ont du mal à atteindre des taux de clivage satisfaisants, tandis que d'autres obtiennent plus de 90% d'efficacité avec une progéniture vivante. Les efforts pour améliorer ces taux comprennent l'adoption de méthodologies couramment utilisées chez d'autres mammifères ou l'élaboration de protocoles entièrement nouveaux. Les différences morphophysiologiques distinctes entre ces animaux nécessitent cependant un examen attentif pour éviter la surgénéralisation lors de l'optimisation. Nous avons donc cherché à examiner ce qui a été établi pour les deux superfamilles de rongeurs les plus recherchées, Cavoidea et Muroidea, en ce qui concerne les étapes de la FIV, telles que la manipulation des gamètes et le développement embryonnaire, afin de mieux comprendre la diversification du protocole dans l'ordre des rongeurs et la manière dont elle affecte l'efficacité de la FIV. Après une analyse approfondie des données accumulées au fil des ans, il s'avère évident que la superfamille Muroidea est utilisée plus largement dans les études sur la reproduction que la famille Cavoidea. Les procédures de FIV atteignent en outre une efficacité totale chez une seule espèce de rongeur, à savoir la souris de laboratoire (*Mus musculus*). Le développement d'une technique de FIV réussie pour toutes les espèces de rongeurs nécessite par conséquent des modifications substantielles pour optimiser les étapes précoces spécifiques à l'espèce - un défi compliqué par le fait qu'il est difficile d'obtenir des gamètes viables pendant les étapes initiales et par l'intérêt limité de la recherche pour les espèces sans valeur commerciale significative, comme les rongeurs sauvages.

Erkenntnisse und Schlussfolgerungen aus einer vergleichenden Analyse des in vitro Fertilisation-Erfolgs bei Cavoidea und Muroidea-Nagetieren

Abstract

Die In vitro Fertilisation (IVF) ist eine weit verbreitete Technik der assistierten Reproduktion, da mit ihr innerhalb eines kurzen Zeitraums mehr Nachkommen erzeugt und gleichzeitig durch die Interaktion der Gameten grundlegende physiologische Daten geliefert werden können. Die suboptimale Entwicklung der meisten Arten stellt jedoch im Falle der Anwendung der IVF bei Nagetieren eine Herausforderung dar. Trotz zahlreicher Studien, die sich auf die Überfamilien der Cavoidea und Muroidea konzentrieren, haben einige Arten Schwierigkeiten, zufriedenstellende Teilungsraten zu erreichen, während andere einen Erfolg von über 90% mit lebenden Nachkommen erzielen. Zu den Bemühungen, diese Raten zu verbessern, gehören die Übernahme von Methoden, die bei anderen Säugetieren üblich sind, oder die Entwicklung völlig neuer Protokolle. Die deutlichen morphophysiologischen Unterschiede zwischen diesen Tieren müssen jedoch sorgfältig berücksichtigt werden, um eine Übergeneralisierung bei der Optimierung zu vermeiden. Daher haben wir uns zum Ziel gesetzt, die für die beiden am meisten erforschten Nager-Überfamilien, Cavoidea und Muroidea, ermittelten Schritte der IVF, wie z. B. die Gametenmanipulation und die Embryonalentwicklung, zu überprüfen, um die Diversifizierung der Protokolle in der Nagerordnung und deren Auswirkungen auf die Effizienz der IVF besser zu ergründen. Nach einer umfassenden Analyse der im Laufe der Jahre gesammelten Daten wird deutlich, dass die Überfamilie der Muroidea in Reproduktionsstudien umfassender genutzt wird als die Cavoidea. Außerdem erreichen IVF-Verfahren nur bei einer Nagetierart, der Labormaus (*Mus musculus*), vollen Erfolg. Folglich erfordert die Entwicklung einer erfolgreichen IVF-Technik für alle Nagetierarten erhebliche Modifikationen, um die artspezifischen frühen Schritte zu optimieren - eine Herausforderung, die durch die Schwierigkeiten bei der Gewinnung lebensfähiger Gameten in der Anfangsphase und das begrenzte Forschungsinteresse an Arten ohne bedeutenden kommerziellen Wert, wie z. B. Wildnager, erschwert wird.

Conocimientos e implicaciones de un análisis comparativo del éxito de la fecundación in vitro en roedores Caviioidea y Muroidea

Resumen

La fecundación in vitro (FIV) es una técnica de reproducción asistida muy utilizada por su capacidad para generar más descendencia en un breve plazo de tiempo, al tiempo que proporciona datos fisiológicos fundamentales a través de las interacciones de los gametos. No obstante, su desarrollo subóptimo en la mayoría de las especies supone un reto cuando la FIV se aplica a roedores. A pesar de los numerosos estudios centrados en las superfamilias Caviioidea y Muroidea, algunas especies luchan por alcanzar tasas de escisión satisfactorias, mientras que otras obtienen una eficacia superior al 90% con crías vivas. Los esfuerzos para mejorar estos índices incluyen el uso de metodologías utilizadas habitualmente en otros mamíferos o el desarrollo de protocolos totalmente nuevos. No obstante, las marcadas diferencias morfofisiológicas entre estos animales requieren una atenta consideración para evitar una generalización excesiva durante la optimización. Por tanto, nos propusimos revisar lo que se ha establecido para las dos superfamilias de roedores más investigadas, Caviioidea y Muroidea, respecto a los pasos de la FIV, como la manipulación de gametos y el desarrollo embrionario, para comprender mejor la diversificación del protocolo en el orden de los roedores y cómo afecta a la eficacia de la FIV. Tras un extenso análisis de los datos acumulados a lo largo de los años, resulta evidente que la superfamilia Muroidea se utiliza más en los estudios sobre reproducción que la Caviioidea. Asimismo, los procedimientos de FIV únicamente alcanzan una eficacia total en una especie de roedor, el ratón de laboratorio (*Mus musculus*). En consecuencia, el desarrollo de una técnica de FIV satisfactoria en todas las especies de roedores requiere modificaciones significativas para optimizar los primeros pasos específicos de cada especie, un reto complicado por las dificultades para obtener gametos viables durante las fases iniciales y el escaso interés de la investigación en especies sin un valor comercial significativo, como los roedores salvajes.

ANEXO D – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 2

CAPÍTULO 2 - INSIGHTS AND IMPLICATIONS FROM A COMPARATIVE ANALYSIS OF *IN VITRO* FERTILIZATION SUCCESS IN CAVIOIDEA AND MUROIDEA RODENTS

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4701 ANEXO E – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 3
 4702
 4703 CAPÍTULO 3 - COMPARATIVE ANALYSIS OF SPERM SELECTION METHODS
 4704 AND THEIR IMPACT ON *IN VITRO* FERTILITY IN THE RED-RUMPED AGOUTI
 4705 (*Dasyprocta leporina*)

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- 4707 ANEXO F – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 4
- 4708
- 4709 CAPÍTULO 4 - TIME AND MEDIA-DEPENDENT MODULATION OF SPERM
- 4710 CAPACITATION IN RED-RUMPED AGOUTI (*Dasyprocta leporina* LINNAEUS, 1758)

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- 4712 ANEXO G – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 5
- 4713
- 4714 CAPÍTULO 5 - EFFECTS OF FERTILIZATION MEDIA ON *IN VITRO* EMBRYONIC
- 4715 DEVELOPMENT IN RED-RUMPED AGOUTI (*DASYPROCTA LEPORINA*) USING A
- 4716 HETEROLOGOUS SYSTEM WITH GUINEA PIG OOCYTES

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- 4718 ANEXO H – CERTIFICADO DE REVISÃO DO INGLÊS DO CAPÍTULO 6
- 4719
- 4720 CAPÍTULO 6 - EFFECTS OF EMBRYO PRODUCTION METHOD AND CULTURE
- 4721 MEDIUM ON EMBRYONIC DEVELOPMENT AND QUALITY IN RED-RUMPED
- 4722 AGOUTI (*Dasyprocta leporina* LINNAEUS, 1758)

REVIEW

CERTIFICATE

To whom it may concern:

We hereby declare that the manuscript described below

Title of the Paper: Effects of embryo production method and culture medium on embryonic development and quality in red-rumped agouti (*Dasyprocta leporina* Linnaeus, 1758)

Authors: Lhara Ricarlany Medeiros de Oliveira, Leonardo Vitorino Costa de Aquino, Luana Grasielle Pereira Bezerra, Moacir Franco de Oliveira, Alexandre Rodrigues Silva, Alexsandra Fernandes Pereira

Was reviewed into the English language and edited for being clearer, according to the scientific writing, by the professional scientific translation team from Cia das Traduções (MS Traduções Ltda.- National Registry of Legal Entities – CNPJ n. 08.966.620/0001-91).

We testify that the manuscript was carefully reviewed for being clearer and with the proper scientific language in English, including grammar, punctuation, spelling and general style. However, the study content and the authors' purposes, as understood from the original text, were not changed anyhow.

The respective author read the manuscript reviewed into English by us before the final review, and the same had the opportunity of selecting the terms and expressions suggested by the reviewer in case of doubts, so that the final version was adjusted according to his intentions and prepared for publishing.

A copy of both reviews of the original manuscript sent by the client and the review made by Cia das Traduções will be made available under request, so that it is verified, within minimum time period of one year.



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4724 ANEXO I – RESUMO I PUBLICADO NO X INTERNATIONAL SYMPOSIUM ON
4725 ANIMAL BIOLOGY OF REPRODUCTION (ISABR) – 2024



X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)
MALE REPRODUCTIVE BIOLOGY

Influence of swim-up on the DNA damage rates in red-rumped agouti (*Dasyprocta leporina* Linnaeus, 1758) sperm

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Financial support: CNPq and CAPES

The red-rumped agouti is a medium size wild rodent included in the Caviioidea superfamily. Despite these animals have an important role in their ecosystems as seed scatterers, their populations are decreasing considerably due to hunting and habitat fragmentation (1). Consequently, a collective effort has been made with the purpose of studying their germplasm and apply it in reproductive biotechniques (2). However, the manipulation of these samples can directly influence their viability, as a previous study has observed that rodent spermatozoa are very susceptible to mechanical stresses (3). Therefore, we aimed to evaluate the effect of swim-up as a classical in vitro sperm selection method on the DNA damage rates in epididymal spermatozoa of red-rumped agoutis. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 20/2021) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 76655-1). Six red-rumped agoutis were anesthetized (50 mg/kg sodium thiopental) and euthanized (1 mL/kg potassium chloride), the testes-epididymis complexes were recovered, the cauda region was excised and the epididymal spermatozoa were obtained by retrograde flushing. The samples were divided into two groups: without selection (WS), and swim-up (SP). For SP, spermatozoa were added at the bottom of a tilted tube (45°) for 15 min and then centrifuged 300×g for 10 min. Thus, for the DNA damage evaluation, spermatozoa samples were smeared and left to dry in the air. The slides were fixed in Carnoy's solution for 3 h, incubated for 25 min in a buffer solution composed of 15 mM Na₂HPO₄ and 80 mM citric acid (pH 2.5) at 75 °C. Smears were stained using 0.2 mg/mL acridine orange for 10 s. A total of 100 cells per group were evaluated using fluorescence microscopy (40×). According to the sperm head color, they were classified as: intact, low, medium, or high DNA damage. (4). All data were expressed as mean ± standard error (one male/one repetition) and analyzed by ANOVA followed by Tukey test (P < 0.05). The percentage of intact DNA spermatozoa was maintained SP (68.8% ± 0.9) similar to the WS samples (86.2% ± 7.2, P > 0.05). Similar results (P > 0.05) were observed for low DNA damage [WS (9.0% ± 5.7), SP (8.4% ± 3.0)] and high DNA damage [WS (2.5% ± 1.1), SP (10.5% ± 5.7)]. However, we were able to observe a significant increase in the percentage of sperm with medium DNA damage in the SP group (12.1% ± 5.1), when compared to WS (2.1% ± 0.9, P < 0.05). These results may be correlated to the swim-up protocols, where the combination of centrifugation and longer periods in the incubator with non-viable sperm could lead to an increase in reactive oxygen species levels, thus, promoting the denaturation of the DNA strand and increasing the risk of cell apoptosis (5). In conclusion, swim-up increased the DNA damage rate in red-rumped agouti epididymal spermatozoa. These results represent an important step towards understanding the male germplasm of these animals, and how they will behave during future applications of in vitro reproduction techniques.

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4727 ANEXO J – RESUMO II PUBLICADO NO X INTERNATIONAL SYMPOSIUM ON
4728 ANIMAL BIOLOGY OF REPRODUCTION (ISABR) – 2024



X INTERNATIONAL SYMPOSIUM ON ANIMAL BIOLOGY OF REPRODUCTION (ISABR)

MALE REPRODUCTIVE BIOLOGY

Effects of centrifugation on oxidative stress status and membrane functionality of red-rumped agouti (*Dasyprocta leporina* Linnaeus, 1758) epididymal sperm

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The epididymal sperm collection procedure includes various physical manipulations such as mixing, pipetting and centrifugations (1). These preparation protocols are crucial to eliminate contaminants such as epithelial and red blood cells, bacteria, and cell debris, which can affect the spermatozoa functionality by increasing the oxidative stress status. This stress condition has been observed in laboratory rodents (2), thus becoming a concern during the sperm cell manipulation for reproductive biotechniques in wild rodents. Therefore, our aim was to analyze the effects of centrifugation on oxidative stress status and membrane functionality of red-rumped agouti spermatozoa. All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (no. 20/2021) and Chico Mendes Institute for Biodiversity Conservation (ICMBio, no. 76655-1). Six males were anesthetized (15 mg/kg ketamine hydrochloride and 1 mg/kg xylazine hydrochloride) and euthanized (1 mL/kg potassium chloride). Subsequently, sperm recovery was performed from the epididymal tail by retrograde flushing. The samples were divided into two groups: fresh sperm (FS) and centrifuged (CT). The centrifugation protocol consisted of 300×g for 3 min twice, the supernatant was discarded, and the pellet was resuspended for evaluation. To determine oxidative stress, reactive oxygen species (ROS) and intracellular glutathione (GSH) levels were quantified using 10 µM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) and 10 µM 7-amino-4-chloromethylcoumarin (CellTracker Blue), respectively. Spermatozoa were incubated in the dark with the different probes for 30 min at 37 °C in a dry bath. The cells were analyzed by fluorescence microscopy (40×), and the arbitrary fluorescence units (AFU) were quantified using ImageJ software. For the membrane functionality evaluation, a hypo-osmotic swelling test (HOST) was conducted with a solution composed of distilled water with sodium citrate and fructose solution (50 mOsm/L). Spermatozoa were evaluated in a phase-contrast light microscope (400×, 200 cells/group). Sperms that had a swollen coiled tail were classified with a functional membrane. All data were expressed as mean ± standard error (one male/one repetition) and analyzed by ANOVA followed by Tukey test ($P < 0.05$). Centrifugation ($85.9\% \pm 3.25$) was able to preserve the functionality of the sperm membrane, with values similar to FS ($90.7\% \pm 1.69$, $P > 0.05$). Moreover, sperm from the CT group demonstrated a lower production of ROS ($0.57 \text{ AFU} \pm 0.15$) when compared with FS ($1.00 \text{ AFU} \pm 0.42$, $P < 0.05$). A GSH increase was observed on CT group ($1.82 \text{ AFU} \pm 0.94$) when compared with FS ($1.00 \text{ AFU} \pm 0.42$, $P < 0.05$). These results demonstrate that centrifugation did not cause a difference in the membrane functionality of these cells, while was capable of select better quality cells, thus increasing the levels of GSH, an important intracellular antioxidant, and decreasing the levels ROS, parameters crucial for maintaining the stability and viability of spermatozoa for subsequent reproductive techniques, such as in vitro fertilization (3). In conclusion, the centrifugation method proved effective in processing red-rumped agouti spermatozoa without causing osmotic instability and preventing the imbalance of oxidative status. These findings represent a notable stride forward of assisted reproduction biotechnologies, offering a more nuanced understanding of the appropriate handling of wild rodent's sperm cells.

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4730 ANEXO K – RESUMO PUBLICADO NO XXX SEMINÁRIO DE INICIAÇÃO
 4731 CIENTÍFICA DA UFRSA (XXX SEMIC) – 2024



Núcleo de Avaliação: Núcleo I
 Área temática: Ciências Agrárias
 Área do Conhecimento: Medicina Veterinária

Avaliação de diferentes concentrações de albumina sérica bovina durante a capacitação espermática em *Dasyprocta leporina* (Linnaeus, 1758)

Ana Livia Rocha Rodrigues, Lhara Ricarlany Medeiros de Oliveira, Antônia Beatriz Mendonça Pereira, Alexandre Rodrigues Silva, Alexsandra Fernandes Pereira

A fertilização *in vitro* (FIV) possui grande potencial para aumentar a capacidade reprodutiva de diversas espécies, incluindo a *Dasyprocta leporina*. Contudo, são escassos os estudos que exploram a otimização das etapas da FIV para essa espécie, como a capacitação espermática, resultando em uma limitada disponibilidade de informações. Portanto, o objetivo foi avaliar diferentes concentrações de albumina sérica bovina (BSA) como agente capacitante de espermatozoides epididimários de *D. leporina*. Todos os procedimentos foram aprovados pelo CEUA/UFRSA (no. 20/2022) e ICMBio (no. 71837-1). Para tanto, seis animais foram anestesiados e eutanasiados para o experimento. A recuperação dos espermatozoides foi realizada por lavagem retrógrada da cauda do epidídimo, seguida da capacitação espermática em meio mínimo capacitante (MMC) composto de 105 mM cloreto de sódio, 26 mM bicarbonato de sódio, 10 µg/mL vermelho de fenol, 5,56 mM glicose, 25 mM HEPES, 21,6 mM lactato de sódio, 2 mM cloreto de cálcio, 0,25 mM piruvato de sódio, e 1% solução de antibiótico-antimicótico. Três grupos foram considerados para comparação: meio MMC adicionado de baixa concentração de BSA (↓BSA, 4 mg/mL), alta concentração de BSA (↑BSA, 15 mg/mL) ou sem BSA (GC, controle). Após 6 h de incubação em ambiente controlado (38,5 °C em 6,5% de CO₂), os espermatozoides foram avaliados quanto à motilidade, morfologia, integridade funcional da membrana plasmática, capacitação propriamente dita e reação acrossômica. Todos os dados foram analisados para normalidade pelo teste de Shapiro-Wilk e a homogeneidade da variância pelo teste de Bartlett, posteriormente transformados e analisados usando o teste de Tukey ($P < 0,05$). Quanto à obtenção das amostras, essas apresentaram coloração esbranquiçada, com pH 7,0 e índice de vigor de 4,5 (escala de 0 a 5). A concentração média foi de $252,3 \times 10^6$ espermatozoides/mL, variando de 179 a 290×10^6 espermatozoides/mL. Nenhuma diferença foi observada entre os grupos para a motilidade e integridade da membrana plasmática ($P > 0,05$). Todos os tratamentos (GC: $63,5\% \pm 1,0$; ↑BSA: $68,5\% \pm 1,2$; ↓BSA: $72,7\% \pm 3,3$) foram capazes de promover taxas de capacitação acima de 60%, e não diferiram entre si ($P > 0,05$). Resultados similares foram observados para a reação acrossômica [controle ($54,7\% \pm 1,5$), ↑BSA ($56,2\% \pm 3,2$) e ↓BSA ($60,2\% \pm 2,0$, $P > 0,05$)]. Contudo, na análise de morfologia espermática, o grupo ↓BSA ($17,3\% \pm 3,4$) apresentou significativamente ($P < 0,05$) mais defeitos na cauda que o GC ($6,5\% \pm 1,1$) e ↑BSA ($9,5\% \pm 2,0$), podendo associar a uma maior viabilidade espermática. Ainda, ambos os grupos com ↑BSA ($9,3\% \pm 3,0$) e ↓BSA ($12,8\% \pm 5,1$) foram superiores ao grupo controle ($5,0\% \pm 1,3$) no número de espermatozoides de velocidade média ($P < 0,05$). Portanto, a partir desses dados recomenda-se a adição de diferentes concentrações de BSA (15 mg/mL e 4 mg/mL) no MMC durante a capacitação, para manter as características viáveis dos espermatozoides de *D. leporina*. Essa otimização representa um importante passo para entender a fisiologia



espermática da espécie, além de auxiliar nas tecnologias de reprodução assistida, como a FIV, para a conservação da *D. leporina*.

Palavras-chave: Roedor silvestre, Fertilização *in vitro*, Manipulação espermática.

Agência financiadora: PIBIC/CNPq.

Campus: Mossoró.

4735 ANEXO L – RESUMO I PUBLICADO NO 32º CONGRESSO BRASILEIRO DE
4736 ZOOTECNIA (ZOOTEC) – 2023

**QUANTIFICAÇÃO DOS NÍVEIS DE EROs E GSH PARA AVALIAÇÃO DO ESTRESSE
OXIDATIVO EM ESPERMATOZOIDES EPIDIDIMÁRIOS DE *Dasyprocta leporina***

Antonia Beatriz Mendonça Pereira ¹; Lhara Ricarlany Medeiros de Oliveira ¹; Leonardo Vitorino Costa de Aquino ¹; Luana Grasielle Pereira Bezerra ²; Luanna Lorena Vieira Rodrigues ¹; Ana Livia Rocha Rodrigues ¹; Alexandre Rodrigues Silva ⁴; Alessandra Fernandes Pereira ³

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Resumo:

O estresse oxidativo é uma condição de desequilíbrio provocada pela alta produção de espécies reativas de oxigênio (EROs) durante o cultivo *in vitro*. Esse fenômeno pode afetar diretamente a viabilidade das células, podendo causar danos na morfologia e função celular. Como a fertilização *in vitro* (FIV) envolve etapas de cultivo é essencial averiguar o status do estresse oxidativo dos espermatozoides antes de sua incubação nas condições ambientais *in vitro*. O objetivo foi avaliar o estresse oxidativo em espermatozoides de *D. leporina* de acordo com os níveis de EROs e glutatona intracelular (GSH). Para tanto, sete *D. leporina* foram anestesiadas e eutanasiadas para a recuperação de espermatozoides epididimários usando a lavagem retrógrada. Espermatozoides recuperados foram avaliados quanto aos níveis de EROs e GSH. Para os níveis de EROs, espermatozoides foram incubados com a sonda diacetato de 2',7'-diclorofluoresceína (H₂DCFDA) por 30 min a 37°C. Para os níveis de GSH, espermatozoides foram incubados com a sonda CellTracker Blue por 30 min a 37°C. Após a incubação, células foram centrifugadas (500×g, 10 min), o sobrenadante descartado e espermatozoides avaliados sob microscopia de fluorescência. Para cada animal e para cada análise, 100 células foram contadas para a quantificação da intensidade de fluorescência usando o software ImageJ. Todos os dados foram expressos como média ± erro padrão. Os dados foram analisados por ANOVA seguida de teste de Tukey (P < 0,05). Após a lavagem retrógrada, uma concentração de 567×10^6 espermatozoides/mL foi observada com uma taxa de motilidade de $85,2\% \pm 2,2$. Ambos os níveis de EROs e GSH apresentaram uma média de $1,0 \pm 0,42$ unidades de fluorescência arbitrárias, sem diferença estatística (P > 0,05). Em conclusão, não houve alterações nos índices de EROs e GSH em espermatozoides de *D. leporina* recuperados após a coleta, e a utilização desses parâmetros é uma importante ferramenta para análise do estresse oxidativo na espécie.

Palavras-chave: Rodentia; FIV; Glutatona; Análise fluorescência

Apoio

CAPES e CNPq.

4738 ANEXO M – RESUMO II PUBLICADO NO 32º CONGRESSO BRASILEIRO DE
4739 ZOOTECNIA (ZOOTEC) – 2023

ENSAIO DE APOPTOSE COMO MÉTODO DE AVALIAÇÃO DA QUALIDADE DE ESPERMATOZOÍDES EPIDIDIMÁRIOS DE *Dasyprocta leporina*

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Resumo:

A fertilização *in vitro* (FIV) é uma técnica de reprodução assistida voltada para aumentar o potencial reprodutivo de uma espécie, bem como contribuir para sua conservação. O estabelecimento da FIV em *D. leporina*, roedor silvestre de significativo papel ecológico, econômico e científico, representa um passo importante na geração de conhecimento da espécie. Neste contexto, uma etapa para o sucesso da técnica consiste no desenvolvimento de metodologias que permitam avaliar de maneira mais precisa os espermatozoides. Portanto, o objetivo foi avaliar a integridade do DNA de espermatozoides epididimários de *D. leporina* usando o ensaio de apoptose com laranja de acridina. Para tanto, sete *D. leporina* foram anestesiadas (15 mg/kg cloridrato de cetamina e 1 mg/kg cloridrato de xilazina) e eutanasiadas (1 mL/kg cloreto de potássio). Em seguida, a recuperação dos espermatozoides foi realizada a partir da cauda do epidídimo por lavagem retrógrada. Espermatozoides foram avaliados para a motilidade usando análise computadorizada, e integridade do DNA usando laranja de acridina. Para este ensaio, 15 µL de espermatozoides foram colocados em lâminas de vidro, secados ao ar e fixados em solução de Carnoy por 2 h. Posteriormente, as lâminas foram incubadas em solução tampão (80 mM de ácido cítrico e 15 mM de Na₂HPO₄, pH 2,5) a 75°C por 20 min. Em seguida, as lâminas foram marcadas com laranja de acridina, lavadas com água destilada e visualizadas sob microscopia de fluorescência. Finalmente, 100 espermatozoides foram avaliados por repetição (um animal/uma repetição), sendo as células classificadas como viáveis (núcleo verde), início apoptose (núcleo amarelado), apoptose tardia (núcleo alaranjado) e necrose (núcleo vermelho). Todos os dados foram expressos como média ± erro padrão. Os dados foram analisados por ANOVA seguida de teste de Tukey (P < 0,05). Após sete repetições, a média de recuperação espermática foi de 567 × 10⁶ espermatozoides/mL, com uma taxa de motilidade de 85,2% ± 2,2, comprovando a eficiência do método de recuperação. Quanto à taxa de apoptose, observou-se uma maior porcentagem de células viáveis (86,2% ± 7,3), e menores percentuais de células em início de apoptose (9,0% ± 5,7), apoptose tardia (2,1% ± 0,9) e necrose (2,5% ± 1,2). Em conclusão, esse estudo foi o primeiro a demonstrar que é possível analisar a qualidade dos espermatozoides epididimários por meio de um ensaio de apoptose, contribuindo para o aprimoramento das pesquisas em *D. leporina*.

Palavras-chave: fertilização *in vitro*; roedor silvestre; reprodução; integridade do DNA; análise de fluorescência

Apoio

CAPES e CNPq

4741 ANEXO N – RESUMO III PUBLICADO NO 32º CONGRESSO BRASILEIRO DE
4742 ZOOTECNIA (ZOOTEC) – 2023

EFEITO DO GRADIENTE DE PERCOLL DURANTE A PREPARAÇÃO ESPERMÁTICA SOBRE A VIABILIDADE E ATIVIDADE MITOCONDRIAL EM *Dasyprocta leporina*

Ana Livia Rocha Rodrigues* ¹; Lhara Ricarlany Medeiros de Oliveira ¹; Leonardo Vitorino Costa de Aquino ¹; Luana Grasielle Pereira Bezerra ²; Luanna Lorena Vieira Rodrigues ¹; Antonia Beatriz Mendonça Pereira ¹; Alexandre Rodrigues Silva ⁴; Alessandra Fernandes Pereira ³

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Resumo:

Em mamíferos silvestres, a fertilização *in vitro* (FIV) vem sendo desenvolvida como estratégia de multiplicação e conservação. Contudo, ainda apresenta uma eficiência reduzida e variável. Um dos fatores que pode influenciar esta eficiência é a presença de espermatozoides não viáveis durante a FIV. Portanto, o objetivo foi avaliar o gradiente de Percoll como método de preparação de espermatozoides de *D. leporina* para FIV, um importante roedor silvestre para a biodiversidade. Para tanto, espermatozoides epididimários foram recuperados por lavagem retrógrada a partir de sete *D. leporina*. Após a recuperação, 250 µL dos espermatozoides foram depositados em um microtubo contendo gradiente de Percoll (85/35%) e centrifugados (300×g por 15 min). Em seguida, o sobrenadante foi removido e adicionou-se 500 µL de meio nutritivo para uma segunda centrifugação (300×g por 10 min). Posteriormente, o sobrenadante foi descartado, a amostra foi homogeneizada e submetidas às análises de viabilidade e atividade mitocondrial. Brevemente, espermatozoides foram marcados com Hoechst 33342, iodeto de propídio e CMXRos por 10 min, e, em seguida, analisados em microscópio de fluorescência, considerando cabeça azul (membrana plasmática intacta, MPI), cabeça vermelha (membrana plasmática rompida, MPR), peça intermediária vermelha (função mitocondrial normal, FMN) e peça intermediária escura (função mitocondrial anormal, FMA). Espermatozoides não submetidos ao gradiente de Percoll foram considerados como grupo controle. Todos os dados foram expressos como média ± erro padrão e analisados por ANOVA seguida de teste de Tukey (P < 0,05). Após sete repetições, um menor percentual de espermatozoides viáveis foi observado no grupo Percoll (41,5% ± 7,4) quando comparado aos espermatozoides do grupo controle (73,5% ± 6,6) de acordo com os valores de MPI/FMN (P < 0,05). Além disso, um maior percentual de espermatozoides não viáveis de acordo as taxas de MPR/FMA (32,5% ± 11,5) foi observado no grupo Percoll quando comparado ao grupo controle (4,7% ± 4,0). Ainda, o índice de espermatozoides totais com FMN foi maior no grupo controle (74,1% ± 6,6) quando comparado aos espermatozoides do grupo Percoll (41,7% ± 7,4). Portanto, as condições avaliadas do gradiente de Percoll no presente estudo não foram adequadas para a preparação de espermatozoides epididimários de *D. leporina*. Mais estudos são necessários para a otimização destes protocolos de preparação espermática para a FIV na espécie.

Palavras-chave: roedor silvestre; FIV; gradiente de densidade; epididimo; seleção espermática

Apoio

CAPES e CNPq

4744 ANEXO O – RESUMO IV PUBLICADO NO 32º CONGRESSO BRASILEIRO DE
4745 ZOOTECNIA (ZOOTEC) – 2023

AVALIAÇÃO DA LAVAGEM POR CENTRIFUGAÇÃO SOBRE A MORFOLOGIA ESPERMÁTICA EM *Dasyprocta leporina*

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Resumo:

A *Dasyprocta leporina* é um roedor silvestre do continente americano de importante papel para a manutenção dos ecossistemas. Associada a sua boa adaptabilidade em cativeiro, estudos voltados para o emprego de técnicas de reprodução assistida vêm sendo desenvolvimentos na espécie. Nesse sentido, uma das técnicas a ser estabelecida em *D. leporina* seria a fertilização *in vitro* (FIV). Para tanto, o preparo dos espermatozoides representa uma etapa importante para o sucesso da FIV. Considerando a possível sensibilidade de espermatozoides à centrifugação, o objetivo foi avaliar a lavagem por centrifugação sobre a morfologia espermática em *D. leporina*. Espermatozoides epididimários foram recuperados por lavagem retrógrada de sete indivíduos adultos mantidos em criatório científico. Após a coleta, espermatozoides foram centrifugados duas vezes (300×g por 3 min) em meio nutritivo. Em seguida, o sobrenadante foi descartado e o pellet ressuspenso em 200 µL de meio. A análise morfológica foi realizada adicionando 5 µL de amostra espermática em 45 µL de corante rosa de bengala por 30 min. Em seguida, um esfregaço desta mistura foi realizado em lâmina de vidro e visualizado em microscopia de luz em um aumento de 100×. Espermatozoides não submetidos à nenhuma centrifugação foram considerados como grupo controle. Para cada grupo e animal, 100 espermatozoides foram classificados morfológicamente de acordo com morfologia normal (MN) ou anormal (MA) quanto às alterações da região espermática (cabeça, peça intermediária ou cauda). Todos os dados foram expressos como média ± erro padrão e os dados foram analisados por ANOVA seguida de teste de Tukey (P < 0,05). Após sete repetições, nenhuma diferença foi observada nos valores de MN (81,4% ± 3,4 e 82,6% ± 2,2) e MA (18,6% ± 3,4 e 17,4% ± 2,2) para espermatozoides não centrifugados e centrifugados, respectivamente. Além disso, nenhuma diferença foi observada quanto às alterações da cabeça (5,7% ± 2,5 e 1,8% ± 0,3), peça intermediária (2,5% ± 0,9 e 2,7% ± 0,8) e cauda (10,4% ± 2,0 e 11,8% ± 1,5) dos espermatozoides não centrifugados e centrifugados, respectivamente. Em conclusão, a lavagem por centrifugação não altera a morfologia de espermatozoides epididimários de *D. leporina*, podendo ser aplicado na espécie para estudos de FIV.

Palavras-chave: Rosa de bengala; Preparação espermática; Epidídimo; FIV

Apoio

CAPES e CNPq.

- 4747 ANEXO P – RESUMO PUBLICADO NO I SIMPÓSIO POTIGUAR EM CIÊNCIA
4748 ANIMAL (SIMPA) – 2023



ANÁLISE DO MÉTODO DE CENTRIFUGAÇÃO SOBRE A MOTILIDADE E VIABILIDADE DE ESPERMATOZOÍDES DE *Dasyprocta leporina*

*CENTRIFUGATION METHOD ANALYSIS ON THE MOTILITY AND VIABILITY OF *Dasyprocta leporina* SPERMATOZOA*

Morfofisiologia e Biotecnologia Animal

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Objetivos: Analisar o efeito da centrifugação como método de processamento espermático nas taxas de motilidade e viabilidade em *Dasyprocta leporina*, visando desenvolver a fertilização *in vitro* (FIV) na espécie. **Material e Métodos:** Espermatozoides epididimários de sete machos foram recuperados e centrifugados, onde foi adicionado 250 µL dos espermatozoides e a mesma quantidade de meio mínimo capacitante (MMC). As amostras foram centrifugadas duas vezes a 300×g por 3 min. Espermatozoides não centrifugados foram usados como controle (grupo NC). Para motilidade, as amostras foram avaliadas por análise computadorizada (CASA, IVOS 12.0). Já para análise de viabilidade, espermatozoides foram incubados com 40 µg/mL de Hoechst 33342 por 10 min, seguido de 0,5 mg/mL de iodeto de propídio e 500 µM de Mito Tracker Red por 8 min. Após marcação, espermatozoides foram avaliados por microscopia de fluorescência. Todos os dados foram expressos como média ± erro padrão e os dados foram analisados por ANOVA seguida de teste de Tukey ($p < 0,05$). **Resultados:** No parâmetro de motilidade, não houve diferença entre o grupo NC e o centrifugado, onde ambos foram superiores a 85% ($p > 0,05$). Já quanto à viabilidade, o grupo centrifugado apresentou uma maior taxa de espermatozoides com membrana plasmática intacta ($94,7\% \pm 1,3$) em relação ao NC ($78,2\% \pm 4,3$), e respectivamente, também uma menor taxa de espermatozoides com membrana rompida e perda de atividade mitocondrial ($4,7\% \pm 1,2$ vs. $20,1\% \pm 4,1$) ($p < 0,05$). **Conclusões:** O método de centrifugação não alterou a taxa de motilidade espermática e foi capaz de selecionar espermatozoides com maior viabilidade, tornando-se uma opção adequada para otimizar as etapas da FIV em *D. leporina*.

Palavras-chave: roedor silvestre, fertilização *in vitro*, processamento espermático, espermatozoides epididimários.

Keywords: wild rodent, *in vitro* fertilization, sperm processing, epididymal sperm.

Protocolo CEUA: no. 20/2021. **Protocolo ICMBio:** no. 76655-1.

Fonte de Fomento: CAPES, bolsa de doutorado, processo no. 001; CNPq, auxílio à pesquisa, processo no. 309078/2021-0.