

THEMATIC SECTION: IX International Symposium on Animal Biology of Reproduction (ISABR 2022)

Biobanking and use of gonadal tissues – a promising strategy for conserving wildlife from the Caatinga biome

Alexandre Rodrigues Silva^{1*} , Aleksandra Fernandes Pereira² , Pierre Comizzoli³ 

¹Laboratório de Conservação de Germoplasma Animal, Universidade Federal Rural do Semi-Árido, Mossoró, RN, Brasil

²Laboratório de Biotecnologia Animal, Universidade Federal Rural do Semi-Árido, Mossoró, RN, Brasil

³Smithsonian National Zoo and Conservation Biology Institute, Washington, USA

How to cite: Silva AR, Pereira AF, Comizzoli P. Biobanking and use of gonadal tissues – a promising strategy for conserving wildlife from the Caatinga biome. *Anim Reprod.* 2022;19(4):e20220135. <https://doi.org/10.1590/1984-3143-AR2022-0135>

Abstract

Biological Resource Banks (BRB) or Genetic Resource Banks (GRB) are critical tools for the conservation of animal biodiversity. According to the International Union for Conservation of Nature, more than 38,500 species are threatened with extinction, out of a total of 138,300 surveyed species. These banks are repositories of biological samples and data recovered and preserved for the long term by zoos, universities, research centers and other conservation organizations. In recent years, BRB have increasingly included ovarian and testicular tissues as additional options to rescue and propagate wild species, especially those at risk of extinction. After in vitro culture or grafting, gonadal tissues are potential sources of matured gametes that can be used for Assisted Reproduction Technologies while informing about gametogenesis or mechanisms involved in infertility. It therefore is crucial to properly recover, cryopreserve, and culture these tissues using species-specific protocols. Developing BRBs is currently one of the strategies to preserve species from the Caatinga biome – an exclusively Brazilian biome with a rich wild fauna that suffers from anthropogenic activities. Among wild species from this biome, studies have been primarily conducted in collared peccaries, agoutis, cavies, and armadillos to preserve their ovarian and testicular tissues. Additionally, domestic species such as the domestic cat and donkeys have been proposed as models for wild species that are phylogenetically close. This review addresses the main technical aspects involved in obtaining BRB derived from gonadal tissues in some wild species of the Caatinga biome. It reports recent advances and perspectives to use these biological materials for wildlife conservation.

Keywords: biodiversity, wildlife conservation, genome resource banking, ovarian tissue, testicular tissue.

Introduction

Although the extinction of species is an irreversible process and is the result of natural evolution, anthropogenic activities have considerably accelerated extinctions of many species (Singina et al., 2014). According to data from the Red List of the International Union for Conservation of Nature, of a total of 138,300 species surveyed, more than 38,500 species are threatened with extinction, 26% of which are mammals (IUCN, 2022). In the current situation, zoos, universities, research centers and other conservation institutions have become important organizations for the development of conservation strategies for wildlife species (Pizzutto et al., 2021, Stadtländer, 2022). Among such important conservation strategies, the role of Biological Resource Banks (BRB) in the establishment of genetic resources and the conservation of endangered species is particularly evident in the last decade (Singina et al., 2014; Holt and Comizzoli, 2021).

The BRB or Genetic Resource Banks are considered sources of gametes, embryos, gonadal tissues, and somatic cells after recovery, processing, and cryopreservation for long term storage

*Corresponding author: alexrs@ufersa.edu.br

Received: January 2, 2023. Accepted: January 24, 2023.

Financial support: ARS, and AFP receive grants from the National Council for the Scientific Development.

Conflicts of interest: The authors have no conflict of interest to declare.



Copyright © The Author(s). This is an Open Access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

(León-Quinto et al., 2009). Though most banks store semen (Moreira et al., 2022) and embryos (Souza-Fabjan et al., 2022), the interest in BRB composed of gonadal tissues has grown over the years, especially with the rapid loss of many wild species (Campos et al., 2019a, Fernandes et al., 2023). In general, these banks can be used in conservation efforts to produce fully-grown gametes, aiming at their use in Assisted Reproduction Technologies (ARTs), such as *in vivo* and *in vitro* embryo production, as well as providing reproductive cells for *in vitro* toxicity testing. Moreover, studies related to gonadal tissues allow to understand the developmental biology of gametes, as well as understanding mechanisms involved in infertility.

Protocols to create BRB from ovarian and testicular tissues have been developed in different wild species (Campos et al., 2019b, Praxedes et al., 2020, Silva et al., 2021a,b, 2022). In general, the efficiency of BRB of preantral follicles (PAFs) enclosed in ovarian tissues or germ cells enclosed in testicular tissues depend on the technical conditions involved in the recovery, cryopreservation, *in vivo* or *in vitro* culture and characterization of these tissues, which are all species-specific (Campos et al., 2019b, Silva et al., 2020). BRBs have been developed in species from the Caatinga biome, which is characterized as an exclusively Brazilian biome with a rich wild fauna that suffers from anthropogenic activities (Silva et al., 2010). In the Caatinga biome, a total of 148 species of mammals have already been cataloged, such as collared peccaries (*Pecari tajacu*), red-rumped agoutis (*Dasyprocta leporina*), Spix's yellow-tooth cavy (*Galea spixii*), and six-banded armadillos (*Euphractus sexcinctus*). Preservation of ovarian and testicular tissues have already been attempted in those species. Additionally, domestic species such as the donkeys (Lopes et al., 2018) and domestic cat (Amelkina et al., 2022) have been proposed as models for wild species that are phylogenetically close.

The objective of this review is to address the main technical aspects involved in obtaining BRB derived from gonadal tissues in some wild species of the Caatinga biome. It also reports advances and perspectives to use these biological materials for wildlife conservation.

Preservation and use of ovarian tissues

The cryopreservation associated with the *in vitro* or *in vivo* culture of PAFs represents a promising alternative for the preservation of female germplasm. Almost all (>90%) of the oocyte reserve of the ovary is found in these follicles, making it possible to preserve genetic material from a single female, (i) before it goes to atresia, (ii) from individuals at any stage of development (fetus, pre-pubescent, and adult) and (iii) individual status (alive animals or post-mortem) (Silva et al., 2015). This technology allows the creation of BRB with biomaterials from females of high genetic value that suddenly die, or females that, for medical reasons, have to undergo ovariectomy (Campos et al., 2019a). In addition to allowing the storage of a homogeneous population of oocytes from the same individual, it allows the study of factors related to follicular development, contributing to the elucidation of the role of toxic substances, for instance.

Given their importance and prospects for use, several protocols for cryopreservation and culture of PAFs have been developed in different species, with promising results. In general, studies on PAFs enclosed in ovarian tissues involve studying the ovarian features, conditions for transportation, cryopreservation, *in vitro* and *in vivo* culture of tissues. In collared peccaries, red-rumped agoutis, Spix's yellow-tooth cavy, armadillos, and Northeastern donkeys, significant advances have been achieved (Table 1).

Table 1. Advances and main references in ovarian tissue preservation protocols in species from the Caatinga biome.

Species	Follicle population characterization	Cryopreservation	<i>In vitro</i> culture	Ovulation post xenografting
Six-banded armadillo	Brasil et al. (2021)	--	--	--
Northeastern donkey	Lopes et al. (2017)	Lopes et al. (2018)	--	--
Spix's yellow-tooth cavy	Praxedes et al. (2017)	Praxedes et al. (2017)	--	--
Collared peccary	Lima et al. (2012)	Campos et al. (2019b) Lima et al. (2019)	Lima et al. (2018) Gomes et al. (2020) Campos et al. (2021)	--
Red-rumped agouti	Santos et al. (2018)	Wanderley et al. (2012) Praxedes et al. (2020) Praxedes et al. (2021)	Praxedes et al. (2021)	Praxedes et al. (2018b)

Studying the ovarian features

The first step in the elaboration of a preservation protocol for PAFs included in ovarian tissues consists in understanding the characteristics of these tissues in the species of interest. Studies in different species have reported differences in the population of PAFs as well as in the percentages of follicular categories (Table 2). These characteristics probably play a key role in the success of the cryopreservation and culture protocols. Furthermore, the quantity and quality parameters of the PAF population define reproductive potential (Doležel et al., 2004). In collared peccaries (Lima et al., 2012), the PAF population found (33 273 PAFs per ovary) is very low compared to the domestic swine (the domestic species most closely related to them), in which an ovarian population of 420 000 primordial follicles per pair of ovaries was reported (Gosden and Telfer, 1987). On the other hand, values found for ovarian population in collared peccaries are like those reported for goats, in which 32 204 PAFs were found per ovary (Lucci et al., 1999), indicating that these numbers could be related to the different ovulation rates among species. In addition, studies conducted under transmission electron microscopy revealed an abundance of lipids present in the ooplasm of oocytes included in peccary PAFs. Since the presence of large amounts of lipids could make cryopreservation difficult, the adoption of vitrification protocols for the preservation of PAFs in this species has been suggested (Lima et al., 2012).

Differences related to PAFs population have also been observed in species of the same infraorder. In two species of South American hystricomorph rodents, the PAF population found for red-rumped agouti was greater (Santos et al., 2018) than that reported for Spix's yellow-tooth cavy (Praxedes et al., 2017) (Table 2); however, these cavyes present a larger amount of primary than primordial follicles, different from what is commonly observed in other species.

Table 2. Characterization of preantral ovarian follicle populations in wild species from the Caatinga biome.

	Collared peccary (Lima et al., 2012)	Red-rumped agouti (Santos et al., 2018)	Spix's yellow-tooth cavy (Praxedes et al., 2017)	Six-banded armadillo (Brasil et al., 2021)	Northeastern donkey (Lopes et al., 2017)	
Follicle numbers	Right ovary	--	4419 ± 532	220 ± 175	--	11614 ± 3039
	Left ovary	--	5397 ± 574	195 ± 182	--	9520 ± 2523
	Total	33273 ± 579	--	416 ± 342	6175 ± 1923	21135 ± 10646
Category (in %)	Primordial	91.6	86.6	32.2	59.4	91.3
	Primary	6.3	13.0	63.7	46.3	8.2
	Secondary	2.2	0.4	4.1	2.4	0.4

In six-banded armadillos (Table 2), it was observed that the follicular population is significantly influenced by infection by *Mycobacterium leprae*, since the species is a natural reservoir for this microorganism that transmits leprosy (Brasil et al., 2021). In Northeastern donkeys (Table 2), it was observed that the follicular population is influenced by both age and body condition of the females (Lopes et al., 2017). Thus, these studies show that there are some particularities regarding the population and follicular category between species. This information is important in defining ovarian tissue preservation protocols.

Short-term storage for transportation

Since many wild species are located far from specialized laboratories for cryopreservation and culture, short-term preservation of ovarian tissue during transport has been proposed, especially in collared peccaries (Lima et al., 2014). Such protocols need to maintain the viability of PAFs between the collection of biological material and its processing for cryopreservation and/or culture.

In collared peccaries, two media based on phosphate buffered saline solution (PBS) and media based on powdered coconut water (ACP®, ACP Biotechnology®, Fortaleza, Brazil) were evaluated for short-term storage of PAFs for morphology, ultrastructure, and follicular viability (Lima et al., 2014). Under refrigeration, ovarian tissues were evaluated at different times (from 6 to 36 h). In this study, ACP® provided a more adequate medium for short-term preservation of collared peccary PAFs at low temperatures compared to PBS based medium.

Cryopreservation of ovarian tissues

The cryopreservation of ovarian tissue has been developed to increase the number of female genetic materials in BRB (Praxedes et al., 2018c). One of the main strategies for the preservation of ovarian tissue is the development of field-friendly protocols. Two methods of cryopreservation of ovarian tissue can be chosen, slow freezing and vitrification, the latter being widely applied in the field for the preservation of ovarian tissues from wild species (Table 3). Among vitrification methods, solid-surface vitrification (SSV) has been prominent for the preservation of ovarian tissues. SSV and other vitrification methods have the main advantage of reducing the formation of ice crystals and their use in the field, as they allow manipulation with low-cost materials (Carvalho et al., 2011).

Table 3. Studies on cryopreservation of ovarian tissues in wild species from the Caatinga biome.

Species	Aim	Cryopreservation			References
		Technique	Cryoprotectants ^a	Main results	
Collared peccary	(1) To establish a vitrification protocol using different cryoprotectants	Solid-surface vitrification (SSV)	3 M EG vs. 3 M DMSO vs. 3 M DMF vs. 6 M EG vs. 6 M DMSO vs. 6 M DMF	3 M EG was more efficient for the vitrification of ovarian tissues	Lima et al. (2019)
	(2) To evaluate different vitrification methods using distinct cryoprotectants	Solid surface vitrification (SSV) method or the ovarian tissue cryosystem (OTC)	3 M EG vs. 3 M DMSO vs. 1.5 M DMSO + 1.5 M EG	Utilization of a closed system, the OTC, with 3 M EG for the vitrification of ovarian tissue	Campos et al. (2019b)
Red-rumped agouti	(1) To develop an efficient protocol for cryopreservation	Slow freezing	1.5 M EG vs. 1.5 M DMSO vs. 1.5 M PROH	1.5 M PROH was more efficient for the vitrification of ovarian tissues	Wanderley et al. (2012)
	(2) To establish a vitrification protocol using different cryoprotectants	Solid-surface vitrification (SSV)	3 M EG vs. 3 M DMSO vs. 6 M EG vs. 6 M DMSO vs. 3 M DMSO + 3 M EG	3 M EG was more efficient for the vitrification of ovarian tissues	Praxedes et al. (2020)
	(3) To evaluate the effect of open and closed systems used for ovarian tissue vitrification on the microbiological load and preservation of follicles	Solid surface vitrification (SSV) method or the ovarian tissue cryosystem (OTC)	3 M EG	Both open and closed systems were equally efficient in preserving ovarian tissues; however, the OTC seems to provide a less adequate environment for bacterial proliferation.	Praxedes et al. (2021)
Spix's yellow-tooth cavy	To establish vitrification using DMSO	Solid-surface vitrification (SSV)	3 M DMSO	PAFs cryopreservation using an SSV process and 3 M DMSO	Praxedes et al. (2017)
Northeastern donkey	To compare different cryoprotectants and concentrations	Solid-surface vitrification (SSV)	3 M EG vs. 3 M DMSO vs. 6 M EG vs. 6 M DMSO vs. 3 M DMSO + 3 M EG	3 M DMSO + 3 M EG was more efficient for the vitrification of ovarian tissues	Lopes et al. (2018)

Abbreviations: DMSO: dimethyl sulfoxide; EG: ethylene glycol; DMF: dimethyl formamide; PROH: propanediol. ^aAll cryopreservation solutions contained sucrose and fetal bovine serum.

In collared peccary (Table 3), two studies focused on the development of vitrification as a standard method for ovarian tissue preservation. Lima et al. (2019) evaluated different combinations of cryoprotectants (3 M ethylene glycol – EG vs. 3 M dimethyl sulfoxide – DMSO vs. 3 M dimethyl formamide – DMF vs. 6 M EG vs. 6 M DMSO vs. 6 M DMF). In this study, the authors observed that SSV is an effective method to preserve collared peccary female germplasm, regardless of the cryoprotectants. The use of 3 M EG appears to be the most effective cryoprotectant concentration and compound for preservation from both a viability and morphological perspective of peccary PAFs. Also, DMF appears to be a viable alternative as a cryoprotectant for mammalian ovarian tissue.

Despite being used for the preservation of the female germplasm of domestic (Carvalho et al., 2011) and wild species (Lima et al., 2019), SSV is considered as an open system that allows contact between the sample and liquid nitrogen, which represent a potential exposure to cryo-resistant pathogens. Thus, the ovarian tissue cryosystem (OTC) is an alternative closed system that has been proven to efficiently preserve ovarian tissue derived collared peccaries (Campos et al., 2019b).

For red-rumped agouti (Table 3), preservation of up to 64% PAFs was achieved using a slow freezing with different 1.5 M DMSO or EG or propanediol – PROH. However, transmission electron microscopy revealed that PROH provided the most efficient preservation of the ovarian tissue ultrastructure (Wanderley et al., 2012). Since SSV has been a promising technique in the field, it was developed in red-rumped agouti and Spix's yellow-tooth cavy, proving to be an efficient technique in the preservation of ovarian tissues from these rodent species. For red-rumped agoutis, the cryoprotective solution composed of 3 M EG was the most efficient for the vitrification of ovarian tissues. Recently, Praxedes et al (2021) evaluated the effect of open and closed systems (SSV vs. OTC) used for ovarian tissue vitrification on the microbiological load and preservation of PAFs in the red-rumped agoutis. Authors concluded that both open and closed systems were equally efficient in preserving agouti ovarian tissues, based on PAFs morphology and DNA integrity. However, the OTC provides a less adequate environment for pathogen contamination.

For Spix's yellow-tooth cavy (Table 3), a pioneering study on the species using SSV demonstrated that 3.0 M DMSO was efficient in preserving follicular morphology and DNA integrity (Praxedes et al., 2017). As for northeastern donkeys, the best results regarding ovarian tissue cryopreservation were achieved when using the combination of EG and DMSO in the SSV (Lopes et al., 2018).

In vitro and in vivo culture of ovarian tissues

There are two main applications of the culture (*in vivo* and *in vitro*) of PAFs from wild species, (i) understanding the events involved in the activation, growth, and maturation of PAFs, and (ii) obtaining fully-grown gametes for use in ARTs. Several factors can influence the efficiency of *in vitro* culture, from those related to the culture system itself, to the reproductive stage of the ovary donor and categories of cultured follicles (Figueiredo et al., 2020). One key factor consists of evaluating the composition of the media, which has a direct relationship with the success of PAFs cultured *in vitro*.

In wild species from the Caatinga biome, the first studies have been carried out to identify the culture conditions involved in the development of PAFs. In collared peccaries, three studies exemplify this. Initially, Lima et al. (2018) verify the effect of follicle-stimulating hormone (FSH) supplementation to alpha minimum essential medium (α -MEM⁺) or tissue culture medium-199 (TCM199⁺) media on the *in vitro* development of PAFs by 7 days derived from collared peccaries. The FSH promotes the maintenance of the ovarian extra-cellular matrix, contributing to the follicle development. Therefore, authors suggested the use of TCM199⁺ medium supplemented of FSH for the *in vitro* development of collared peccaries PAFs under 7-day culturing conditions.

Gomes et al. (2020) confirmed that the addition of 25 ng/mL of BMP-15 to the culture medium of collared peccary PAFs maintained a high number of morphologically healthy follicles and stimulated the activation of primordial follicles after 6 days in culture. In 2021, Campos et al. observed that GDF-9, especially at a 200 ng/mL inclusion in the culture medium, was actively involved in the *in vitro* development of collared peccary PAFs. Therefore, BMP-15 and GDF-9 contribute for follicle activation and development during *in vitro* culture. These collective results improve the understanding of initial follicle development in collared peccaries and can be used to improve the protocols for *in vitro* culturing of PAFs.

For red-rumped agoutis, initial evaluations have been carried out from cultured non-cryopreserved and cryopreserved ovarian fragments. In this study, authors evaluated the cryopreservation conditions using a culture system of ovarian fragments in TCM-199 plus ITS medium (insulin 10 μ g/mL, transferrin 5.5 μ g/mL, and selenium 5.0 ng/mL), 0.23 mM sodium pyruvate, 2 mM glutamine, 2 mM hypoxanthine, and 1.25 mg/mL bovine serum albumin –

Praxedes et al., 2021). In this species, studies aimed at identifying the most suitable components of the culture medium for PAFs are still necessary, to evaluate follicular activation and development.

In addition to *in vitro* culture, ovarian tissue grafting has been used in the evaluation of cryopreservation protocols for PAFs, allowing it to be a tool for assessing the recovery of ovarian function. In red-rumped agoutis, Praxedes et al. (2018a) evaluated the development of fresh and vitrified ovarian tissue after xenografting in female C57Bl/6 mice with severe combined immunodeficiency (SCID). The authors reported that according to the characteristics of the external genitalia and the predominance of cornified cells on vaginal cytology, ovarian activity returned in 80% of the xenografting using non-cryopreserved tissues and 17% in the xenografting using vitrified tissues. At 42 days after xenografting, follicle development and occurrence of ovulation was confirmed by the recovery and histological evaluation of the xenografted fragments. Therefore, red-rumped agouti PAFs are likely to be vitrified and subsequently resume their development through xenografting in SCID mice.

Perspectives of preserving ovarian tissues

Among the many challenges in establishing strategies for the conservation of wild species, it is worth highlighting the fact that the reproductive physiology of many of these species remains unknown. Despite this, numerous studies have shown the efforts of researchers to fill this gap. Thus, the continuity of studies focused on the understanding of ovarian physiology and its preservation is justifiable. This perspective is evident, mainly because despite the need for improvements in the protocols for the preservation and culture of ovarian tissue, the birth of mice offspring from frozen material has already been obtained (dela Peña et al., 2002), encouraging the continuity of studies.

Preservation and use of testicular tissues

Previously, preservation of male germplasm had been limited to well-established protocols for cryopreservation of spermatozoa obtained by ejaculation or retrieval from the epididymis. In recent years, the possibility of safeguarding testicular tissue has emerged as a promising tool for ARTs. According to this technology, the undifferentiated spermatogonia preserved into the testicular tissue could be cryopreserved and cultured, guaranteeing the unlimited production of viable spermatozoa to be used in ARTs. Therefore, the cryopreservation of testicular tissues represents an important technology for the preservation of fertility in wild mammals, enabling essential advances for the knowledge and maintenance of the species (Silva et al., 2020).

In general, protocols for germ cells enclosed in testicular tissues involves the study the testicular features, conditions for cryopreservation, *in vitro* and *in vivo* culture of tissues. Significant advances have been achieved and the establishment of protocols has allowed important results in collared peccaries, red-rumped agoutis, and Spix's yellow-tooth caviés (Table 4).

Table 4. Advances and main references in testicular tissue preservation protocols in species from the Caatinga biome

Species	Study of testicular features	Cryopreservation	<i>In vitro</i> culture	Xenografting
Spix's yellow-tooth cavy	Santos et al. (2012), Santos et al. (2014)	Silva et al. (2019b)	--	--
Collared peccary	Costa et al. (2010)	Silva et al. (2019a), Silva et al. (2021a)	Silva et al. (2021b)	Campos-Junior et al. (2014)
Red-rumped agouti	Assis-Neto et al. (2003), Arroyo et al. (2017)	Silva et al. (2022)	--	--

Understating the morphology and physiology of testicular tissue

Similar to what is observed in ovarian tissue, for testicular tissue technology variations in the characteristics of spermatogenesis and testicular histological parameters may result in variations in the establishment of protocols for cryopreservation and culture of testicular tissue. In general, different species have varied testicular characteristics, not only in terms of spermatogenesis, but

also in terms of histological architecture and cellular characteristics. In collared peccary, for example, the daily sperm production per gram of testis in collared peccaries is approximately $23.4 \pm 2 \times 10^6$, which is similar to domestic and wild pigs (Costa et al., 2010). It is even necessary to point out that collared peccaries have a very peculiar testicular histological architecture, with isolated niches of Leydig cells being found in the middle of the interstitial tissue (Costa et al., 2010).

Cryopreservation of testicular tissues

In collared peccary (Table 5), slow freezing and vitrification in cryotubes were the most suitable methods in the cryopreservation of testicular tissues derived from adult animals and using the combination of DMSO and EG as cryoprotectants in the cryopreservation solution (Silva et al., 2019a, 2021a). In these studies, glycerol-based cryoprotectants were not suitable for any of the vitrification methods employed [slow freezing, vitrification in cryotubes and SSV]. Furthermore, when other combinations of cryoprotectants [DMSO and EG alone] were evaluated, they were efficient in SSV. In these studies, all procedures were performed on adult wild animals, which are generally difficult to preserve due to their cellular diversity and vulnerability to *ex vivo* conditions. Although tissues from adult animals have already been cryopreserved in different species, no protocol is effective enough to obtain spermatozoa from cryopreserved testicular tissues. This fact highlights the need to continue studies to produce viable sperm and, consequently, live offspring.

Table 5. Cryopreservation of testicular tissues in some wild species from the Caatinga biome.

Species	Aim	Cryopreservation			Authors
		Technique	Cryoprotectors ^a	Main results	
Collared peccary	(1) To compare different cryoprotectants and concentrations	Solid-surface vitrification (SSV)	3 M EG vs. 3 M DMSO vs. 1.5 EG + 1.5 M DMSO	DMSO/EG in combination is better than DMSO or EG alone for SSV of testicular tissue from peccaries	Silva et al. (2019a)
	(2) To evaluate the effects of different cryopreservation techniques including glycerol-based cryoprotectant combinations	Solid-surface vitrification (SSV) and slow freezing (SF), conventional vitrification (CV)	1.5 M or 3.0 M [DMSO + EG vs. DMSO + Glycerol vs. EG + Glycerol]	SF and CV appeared to be the most suitable methods for the cryopreservation of adult testicular tissues DMSO + EG was more efficient for the vitrification of testicular tissues	Silva et al. (2021a)
Red-rumped agouti	To evaluate the effects of different cryopreservation techniques and cryoprotectant combinations	Solid-surface vitrification (SSV) and slow freezing (SF), conventional vitrification (CV)	1.5 or 3 M [EG vs. DMSO vs. DMSO + EG]	The use of SSV with DMSO + EG was the best protocol for the preservation of testicular tissues	Silva et al. (2022)
Spix's yellow-tooth cavy	To compare different cryoprotectants and concentrations	Solid-surface vitrification (SSV)	3 M EG vs. 3 M DMSO vs. 6 M EG vs. 6 M DMSO	3 M EG was more efficient for the vitrification of testicular tissues	Silva et al. (2019b)

Abbreviations: DMSO: dimethyl sulfoxide; EG: ethylene glycol; DMF: dimethyl formamide; PROH: propanediol. ^aAll cryopreservation solutions contained sucrose and fetal bovine serum.

For this reason, steps to optimize SSV have been developed in collared peccaries and in red-rumped agoutis, with the goal of developing SSV as a safe bioprocess in the conservation of biodiversity. In red-rumped agoutis (Table 5), the use of SSV with DMSO plus EG was the best protocol for the preservation of testicular tissues from adult individuals (Silva et al., 2021b). In another hystricognath rodent - the Spix' yellow-tooth cavy (Table 5), however, the isolated use of 3 M EG promoted the best results in the vitrification of testicular tissues (Silva et al., 2019a).

Testicular tissue culture

The culture of testicular tissue in wild species is still a recent tool. In general, all systems have advantages and disadvantages in relation to the development of spermatogenesis, with the *in*

in vitro culture of testicular samples being carried out mainly in systems of organotypic culture of tissue fragments, and 2D culture of cell suspensions. Currently, *in vitro* culture systems involve base media and supplements with variations in protein source and growth factors, and the temperature in a CO₂ incubator can vary from 31 to 37 °C, depending on the species.

A pioneering study conducted by our collared peccaries research group has established means and supplements for the development of spermatogenesis. In collared peccary, after 28-days *in vitro* culture, the StemPro-34 SFM seemed to be the most adequate medium for *in vitro* culture of prepubertal peccary testicular tissue. Moreover, the supplementation with glial-derived neurotrophic factor (GDNF, 10.0 ng/mL) seems to be essential for the maintenance of cell survival and proliferation (Silva et al., 2021b).

Perspectives of preserving testicular tissues

Technologies for testicular tissue preservation are relatively recent compared to other ARTs. Despite this, numerous advances have been described around the world, to either develop cryopreservation protocols or to establish adequate culture systems. Birth of viable offspring from frozen testicular tissue and subsequently submitted to grafting protocols has already been described not only in mice (Shinohara et al., 2002), but also in domestic pigs (Kaneko et al., 2013) and primates such as the Rhesus monkey (Fayomi et al., 2019). These impressive results are encouraging examples for wildlife conservation.

Final considerations

Over the past decades, biodiversity loss has accelerated at a global scale. To maintain and sustain biodiversity, ARTs have been developed and implemented by many researchers around the world. Cryopreservation of ovarian and testicular tissue, when properly performed, can be useful approaches for understanding the biology of germ cells in different species. It allows the evaluation of the mechanisms of contraceptive agents responsible for infertility, the correction of the causes of infertility by editing the genome of these cells, and the obtaining of viable sperm to be used in the embryo production.

In some species from the Caatinga biome, important progress has been made. Regarding the preservation of PAFs, it still has limitations when it comes to being a source of oocytes for ARTs in wild animals. On the other hand, the use of cryopreservation and *in vitro* culture of PAFs have been important tools for the knowledge of folliculogenesis in different species. Several protocols for cryopreservation and *in vitro* culture of PAFs have already been developed, and their results vary according to the conditions established for the preservation protocols, *in vitro* culture systems, media and supplements used, and, mainly, according to the investigated species.

Although testicular tissues from adult individuals have already been cryopreserved in different species, no protocol is effective enough to produce mature spermatozoa from cryopreserved testicular tissues. This fact highlights the need to continue studies to produce viable sperm and, consequently, live offspring. For this reason, steps to optimize SSV have been developed in collared peccaries and agoutis, wild mammals of the national fauna, with the aim of developing SSV as a safe bioprocess in the conservation of biodiversity.

Acknowledgements

This study was financed in part by the National Counsel of Technological and Scientific Development (CNPq).

References

- Amelkina O, Silva AM, Silva AR, Comizzoli P. Feline microRNAome in ovary and testis: exploration of in-silico miRNA-mRNA networks involved in gonadal function and cellular stress response. *Front Genet.* 2022;13:1009220. <http://dx.doi.org/10.3389/fgene.2022.1009220>. PMID:36226169.
- Arroyo MAM, Silva FFS, Santos PRS, Silva AR, Oliveira MF, Assis-Neto AC. Ultrastructure of spermatogenesis and spermatozoa in agoutis during sexual development. *Reprod Fertil Dev.* 2017;29(2):383-93. <http://dx.doi.org/10.1071/RD14442>. PMID:26336816.

- Assis-Neto ACD, Melo MIVD, Carvalho MAMD, Miglino MA, Oliveira MFD, Menezes DJAD, Papa PC, Kfoury JR Jr. Qualitative analysis of the established spermatogenesis in agoutis (*Dasyprocta aguti*) raised in captivity. *Braz J Vet Res Anim Sci.* 2003;40:180-4. <http://dx.doi.org/10.1590/S1413-95962003000900004>.
- Brasil AV, Silva AM, Campos LB, Praxedes ECG, Antunes JMAP, Silva AR. Estimation of the preantral follicles ovarian population in six-banded armadillos (*Euphractus sexcinctus*) infected or not by the *Mycobacterium leprae*. *Anim Reprod.* 2021;29:42.
- Campos LB, Praxedes ECG, Saraiva MVA, Comizzoli P, Silva AR. Advances and challenges of using ovarian preantral follicles to develop biobanks of wild mammals. *Biopreserv Biobank.* 2019a;17(4):334-41. <http://dx.doi.org/10.1089/bio.2018.0130>. PMID:30896983.
- Campos LB, Silva AM, Praxedes ECG, Bezerra LGP, Lins TLBG, Menezes VG, Matos MHT, Lima GL, Rodrigues APR, Silva AR. Vitrification of collared peccary ovarian tissue using open or closed systems and different intracellular cryoprotectants. *Cryobiology.* 2019b;91:77-83. <http://dx.doi.org/10.1016/j.cryobiol.2019.10.193>. PMID:31639331.
- Campos LB, Silva AM, Praxedes ECG, Freitas J, Bezerra LGP, Melo LM, Pereira AF, Figueiredo JR, Silva AR. Effect of growth differentiation factor 9 (GDF-9) on *in vitro* development of collared peccary preantral follicles enclosed in ovarian tissues. *Anim Reprod Sci.* 2021;226:106717. <http://dx.doi.org/10.1016/j.anireprosci.2021.106717>. PMID:33579546.
- Campos-Júnior PHA, Costa GM, Avelar GF, Lacerda SM, da Costa NN, Ohashi OM, Miranda MS, Barcelos LS, Jorge EC, Guimarães DA, de França LR. Derivation of sperm from xenografted testis cells and tissues of the peccary (*Tayassu tajacu*). *Reproduction.* 2014;147(3):291-9. <http://dx.doi.org/10.1530/REP-13-0581>. PMID:24324205.
- Carvalho AA, Faustino LR, Figueiredo JR, Rodrigues APR, Costa APR. Vitrification: an alternative for preserving embryos and genetic material mammalian females in cryobanking. *Acta Vet Bras.* 2011;5(3):236-48.
- Costa GMJ, Leal MC, Silva JV, Ferreira ACS, Guimarães DA, França LR. Spermatogenic cycle length and sperm production in a feral pig species (Collared Peccary, *Tayassu tajacu*). *J Androl.* 2010;31(2):221-30. <http://dx.doi.org/10.2164/jandrol.109.008524>. PMID:19745218.
- dela Peña EC, Takahashi Y, Katagiri S, Atabay EC, Nagano M. Birth of pups after transfer of mouse embryos derived from vitrified preantral follicles. *Reproduction.* 2002;123(4):593-600. <http://dx.doi.org/10.1530/rep.0.1230593>. PMID:11914121.
- Doležel R, Kyliánková R, Kummer V, Mašková J, Stará P, Vitásek R. Follicular population and oestrogen receptor alpha in ovary of the bitch. *Acta Vet Brno.* 2004;73(1):37-43. <http://dx.doi.org/10.2754/avb200473010037>.
- Fayomi AP, Peters K, Sukhwani M, Valli-Pulaski H, Shetty G, Meistrich ML, Houser L, Robertson N, Roberts V, Ramsey C, Hanna C, Hennebold JD, Dobrinski I, Orwig KE. Autologous grafting of cryopreserved prepubertal rhesus testis produces sperm and offspring. *Science.* 2019;363(6433):1314-9. <http://dx.doi.org/10.1126/science.aav2914>. PMID:30898927.
- Fernandes DP, Praxedes EA, Freitas CIA, Pereira AF. *Ex situ* approaches for the conservation of genetic resources in the superorder Xenarthra. *Biopreserv Biobank.* 2023. In press. <http://dx.doi.org/10.1089/bio.2021.0182>. PMID:36409545.
- Figueiredo JR, Cadenas J, Lima LF, Santos RR. Advances in *in vitro* folliculogenesis in domestic ruminants. *Anim Reprod.* 2020;16(1):52-65. <http://dx.doi.org/10.21451/1984-3143-AR2018-0123>. PMID:33936289.
- Gomes HAN, Campos LB, Praxedes ECG, Oliveira MF, Pereira AF, Silva AR, Saraiva MVA. BMP-15 activity on *in vitro* development of collared peccary (*Pecari tajacu* Linnaeus, 1758) preantral follicles. *Reprod Domest Anim.* 2020;55(8):958-64. <http://dx.doi.org/10.1111/rda.13735>. PMID:32473042.
- Gosden RG, Telfer E. Numbers of follicles and oocytes in mammalian ovaries and their allometric relationships. *J Zool.* 1987;211(1):169-75. <http://dx.doi.org/10.1111/j.1469-7998.1987.tb07460.x>.
- Holt WV, Comizzoli P. Genome resource banking for wildlife conservation: promises and caveats. *Cryo Lett.* 2021;42(6):309-20. PMID:35366296.
- International Union for Conservation of Nature – IUCN. The International Union for Conservation of Nature's (IUCN) red list of threatened species. Version 2021-2 [Internet]. 2022 [cited 2023 Jan 2]. Available from: <https://www.iucnredlist.org>
- Kaneko H, Kikuchi K, Nakai M, Somfai T, Noguchi J, Tanihara F, Ito J, Kashiwazaki N. Generation of live piglets for the first time using sperm retrieved from immature testicular tissue cryopreserved and grafted into nude mice. *PLoS One.* 2013;8(7):1-8. <http://dx.doi.org/10.1371/journal.pone.0070989>. PMID:23923039.

- León-Quinto T, Simon MA, Cadenas R, Jones J, Martínez-Hernández FJ, Moreno JM, Vargas A, Martínez F, Soria B. Developing biological resource banks as a supporting tool for wildlife reproduction and conservation The Iberian lynx bank as a model for other endangered species. *Anim Reprod Sci.* 2009;112(3-4):347-61. <http://dx.doi.org/10.1016/j.anireprosci.2008.05.070>. PMID:18585877.
- Lima GL, Luz VB, Lima LF, Rocha RMP, Castro SV, Castelo TS, Rodrigues A, Figueiredo JR, Silva AR. Interactions between different media and follicle-stimulating hormone supplementation on *in vitro* culture of preantral follicles enclosed in ovarian tissue derived from collared peccaries (*Pecari tajacu* Linnaeus, 1758). *Reprod Domest Anim.* 2018;53(4):880-8. <http://dx.doi.org/10.1111/rda.13179>. PMID:29604127.
- Lima GL, Luz VB, Lunardi FO, Souza AL, Peixoto GC, Rodrigues APR, Oliveira MF, Santos RR, Silva AR. Effect of cryoprotectant type and concentration on the vitrification of collared Peccary (*Pecari tajacu*) ovarian tissue. *Anim Reprod Sci.* 2019;205:126-33. <http://dx.doi.org/10.1016/j.anireprosci.2019.04.012>. PMID:31047761.
- Lima GL, Santos EAA, Lima LF, Luz VB, Rodrigues APR, Silva AR. Short-term preservation of *Pecari tajacu* ovarian preantral follicles using phosphate buffered saline (PBS) or powdered coconut water (ACP) media. *Arq Bras Med Vet Zootec.* 2014;66(6):1623-30. <http://dx.doi.org/10.1590/1678-7297>.
- Lima GL, Santos EAA, Luz VB, Rodrigues APR, Silva AR. Morphological characterization of the ovarian preantral follicle population of collared peccaries (*Tayassu tajacu* Linnaeus, 1758). *Anat Histol Embryol.* 2012;42(4):304-11. <http://dx.doi.org/10.1111/ahe.12021>. PMID:23278244.
- Lopes KRF, Lima GL, Bezerra LGP, Barreto-Junior RA, Oliveira MF, Silva AR. Characterization of the ovarian preantral follicle populations and its correlation with age and nutritional status in Brazilian Northeastern donkeys (*Equus assinus*). *Anim Reprod Sci.* 2017;187:193-202. <http://dx.doi.org/10.1016/j.anireprosci.2017.11.007>. PMID:29126831.
- Lopes KRF, Praxedes EC, Campos LB, Bezerra MB, Lima GL, Saraiva MVA, Silva AR. Vitrification of ovarian tissue of Brazilian North-eastern donkeys (*Equus asinus*) using different cryoprotectants. *Reprod Domest Anim.* 2018;53(5):1060-7. <http://dx.doi.org/10.1111/rda.13203>. PMID:29809292.
- Lucci CM, Amorim CA, Rodrigues APR, Figueiredo JR, Bão SN, Silva JRV, Gonçalves PB. Study of preantral follicle population *in situ* and after mechanical isolation from caprine ovaries at different reproductive stages. *Anim Reprod Sci.* 1999;56(3-4):223-36. [http://dx.doi.org/10.1016/S0378-4320\(99\)00045-7](http://dx.doi.org/10.1016/S0378-4320(99)00045-7). PMID:10497918.
- Moreira SSJ, Lago AEA, Moura AAA, Silva AR. Impact of seminal plasma composition on sperm freezability in wild mammals: a review. *Biopreserv Biobank.* 2022;20(1):90-6. <http://dx.doi.org/10.1089/bio.2021.0026>. PMID:34726507.
- Pizzutto CS, Colbachini H, Jorge-Neto PN. One Conservation: the integrated view of biodiversity conservation. *Anim Reprod.* 2021;18(2):e20210024. <http://dx.doi.org/10.1590/1984-3143-ar2021-0024>. PMID:34122656.
- Praxedes EA, Borges AA, Santos MVO, Pereira AF. Use of somatic cell banks in the conservation of wild felids. *Zoo Biol.* 2018a;37(4):258-63. <http://dx.doi.org/10.1002/zoo.21416>. PMID:29722907.
- Praxedes ÉCG, Lima GL, Bezerra LGP, Santos FA, Bezerra MB, Guerreiro DD, Rodrigues APR, Domingues SFS, Silva AR. Development of fresh and vitrified agouti ovarian tissue after xenografting to ovariectomised severe combined immunodeficiency (SCID) mice. *Reprod Fertil Dev.* 2018b;30(3):459-68. <http://dx.doi.org/10.1071/RD17051>. PMID:28784201.
- Praxedes ECG, Peixoto GCX, Silva AM, Silva AR. Reproduction in agouti (*Dasyprocta* spp.): a review of reproductive physiology for developing assisted reproductive techniques. *Anim Reprod.* 2018c;15(4):1181-92. <http://dx.doi.org/10.21451/1984-3143-AR2018-0058>. PMID:34221130.
- Praxedes ÉCG, Bezerra LCG, Moreira SSJ, Santos CS, Brasil AV, Silva AMD, Guerreiro DD, Pereira AF, Rodrigues APR, Silva AR. Microbiological load and preantral follicle preservation using different systems for ovarian tissue vitrification in the red-rumped agouti. *Cryobiology.* 2021;103:123-8. <http://dx.doi.org/10.1016/j.cryobiol.2021.08.003>. PMID:34454959.
- Praxedes ECG, Lima GL, Silva AM, Apolinário CA, Bezerra JA, Souza AL, Oliveira MF, Rodrigues APR, Silva AR. Characterisation and cryopreservation of the ovarian preantral follicle population from Spix. *Reprod Fertil Dev.* 2017;29(3):594-602. <http://dx.doi.org/10.1071/RD15249>. PMID:28442066.
- Praxedes ECG, Lima GL, Silva AM, Campos LB, Souza CM, Moreira SSJ, Oliveira MF, Silva AR. Comparison of different intracellular cryoprotectants on the solid surface vitrification of red-rumped agouti (Lichtenstein, 1823) ovarian tissue. *Reprod Domest Anim.* 2020;55(2):154-61. <http://dx.doi.org/10.1111/rda.13600>. PMID:31804747.
- Santos EA, Lima GL, Praxedes ECG, Silva AM, Maia KM, Oliveira MF, Rodrigues APR, Silva AR. Estimation, morphometry and ultrastructure of ovarian preantral follicle population in agouti (*Dasyprocta leporina*). *Pesq Vet Bras.* 2018;38(1):175-82. <http://dx.doi.org/10.1590/1678-5150-pvb-4946>.

- Santos PRS, Oliveira MF, Arroyo MAM, Silva AR, Rici REG, Miglino MA, Assis Neto AC. Ultrastructure of spermatogenesis in Spix's yellow-toothed cavy (*Galea spixii*). *Reproduction*. 2014;147(1):13-9. <http://dx.doi.org/10.1530/REP-13-0452>. PMID:24101585.
- Santos PRS, Oliveira MF, Silva AR, Assis AC No. Development of spermatogenesis in captive-bred Spix's yellow-toothed cavy (*Galea spixii*). *Reprod Fertil Dev*. 2012;24(6):877-85. <http://dx.doi.org/10.1071/RD12015>. PMID:22781939.
- Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K, Kurome M, Nagashima H, Toyokuni S, Kogishi K, Honjo T, Ogura A. Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination. *Hum Reprod*. 2002;17(12):3039-45. <http://dx.doi.org/10.1093/humrep/17.12.3039>. PMID:12456600.
- Silva AM, Bezerra LGP, Praxedes ECG, Moreira SSJ, Souza CMP, Oliveira MF, Pereira AF, Comizzoli P, Silva AR. Combination of intracellular cryoprotectants preserves the structure and the cells proliferative capacity potential of adult collared peccary testicular tissue subjected to solid surface vitrification. *Cryobiology*. 2019a;91:53-60. <http://dx.doi.org/10.1016/j.cryobiol.2019.10.199>. PMID:31678072.
- Silva AM, Pereira AG, Praxedes ECG, Moreira SSJ, Oliveira MF, Comizzoli P, Silva AR. Vitrification of testicular tissue from adult Spix's yellow-toothed cavy (*Galea spixii*) using different cryoprotectants. In: *Proceedings of the Society for the Study of Reproduction 2019 Annual Meeting*; 2019; San José. San José: Society for the Study of Reproduction; 2019b.
- Silva AM, Pereira AF, Comizzoli P, Silva AR. Cryopreservation and culture of testicular tissues: an essential tool for biodiversity preservation. *Biopreserv Biobank*. 2020;18(3):235-43. <http://dx.doi.org/10.1089/bio.2020.0010>. PMID:32282240.
- Silva AM, Bezerra LGP, Pereira AG, Pereira AF, Oliveira MF, Comizzoli P, Silva AR. *In vitro* culture of testicular tissue from prepubertal collared peccaries using different culture media supplemented with various GDNF concentrations. *Anim Reprod*. 2021a;19:65.
- Silva AM, Pereira AG, Brasil AV, Souza-Junior JBF, Moura CEB, Pereira AF, Oliveira MF, Comizzoli P, Silva AR. Influence of freezing techniques and glycerol-based cryoprotectant combinations on the survival of testicular tissues from adult collared peccaries. *Theriogenology*. 2021b;167:111-9. <http://dx.doi.org/10.1016/j.theriogenology.2021.03.013>. PMID:33813051.
- Silva AM, Pereira AG, Bezerra LGP, Moreira SSJ, Pereira AF, Oliveira MF, Comizzoli P, Silva AR. Cryopreservation of testicular tissue from adult red-rumped agoutis (*Dasyprocta leporina* Linnaeus, 1758). *Animals*. 2022;12(6):738. <http://dx.doi.org/10.3390/ani12060738>. PMID:35327135.
- Silva AR, Castelo TS, Lima GL, Peixoto GCX. Conservation of germplasm from wild animals of the Caatinga biome. *Acta Sci Vet*. 2010;38:373-89.
- Silva AR, Lima GL, Peixoto GCX, Souza AL. Cryopreservation in mammalian conservation biology: current applications and potential utility. *RRBS*. 2015;4:1-8. <http://dx.doi.org/10.2147/RRBS.S54294>.
- Singina GN, Volkova NA, Bagirov VA, Zinovieva NA. Cryobanking of somatic cells in conservation of animal genetic resources: prospects and successes. *Sh Biol*. 2014;6(6):3-14. <http://dx.doi.org/10.15389/agrobiology.2014.6.3eng>.
- Souza-Fabjan JMG, Batista RITP, Melo LM, Oliveira MAL, Chaves MS, Fonseca JF, Freitas VJF. Transcervical versus laparotomy embryo recovery: what strategy is best for embryo bank formation in the Canindé goat conservation Program? *Biopreserv Biobank*. 2022;20(2):204-7. <http://dx.doi.org/10.1089/bio.2021.0041>. PMID:34491078.
- Stadtländer CTH. The role of zoological institutions in a changing world: a review of the ark and beyond: the evolution of zoo and aquarium conservation, 2022. *Zoo Biol*. 2022;20:21698. In press. <http://dx.doi.org/10.1002/zoo.21698>.
- Wanderley LS, Luz HK, Faustino LR, Lima IM, Lopes CA, Silva AR, Bão SN, Campello CC, Rodrigues AP, Figueiredo JR. Ultrastructural features of agouti (*Dasyprocta aguti*) preantral follicles cryopreserved using dimethyl sulfoxide, ethylene glycol and propanediol. *Theriogenology*. 2012;77(2):260-7. <http://dx.doi.org/10.1016/j.theriogenology.2011.07.038>. PMID:21924476.

Author contributions

ARS: Conceptualization, Supervision, Writing – original draft, Writing – review & editing; AFP: Conceptualization, Writing – original draft, Writing – review & editing; PC: Conceptualization, Writing – original draft, Writing – review & editing.