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Research article

Felid-gamete-rescue within EAZA - efforts and results in biobanking felid oocytes and sperm

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Abstract

The combination of gamete banking, defined as the preservation of gametes and gonads and assisted reproductive techniques (ART) such as, artificial insemination, in vitro fertilisation and embryo transfer is an important tool to preserve the genetic diversity of endangered species. It can not only reduce the huge problem of limited space for large populations in zoos, but might facilitate conservation breeding and help to overcome barriers between in situ and ex situ populations. Within the Felid-Gamete-Rescue-Project we aim to collect and store gametes of all felid species and improve the methods of in vitro embryo production, including in vitro maturation and fertilisation and sperm storage. Gonads of 74 females and 67 males from 36 different zoological institutions were donated to the project. The majority of samples (86/141) originated from individuals younger than three years old or older than 13 years of age. Epididymal sperm was collected from 42 males (63%) and samples from 36 males (54%) were suitable for freezing. Sperm quality and quantity was sufficient for the demands of artificial insemination (>10 x 10^6 motile sperm) in 21% of males, whereas almost all cryopreserved samples can be used for intracytoplasmic sperm cell injection (ICSI). 1110 oocytes were retrieved from 62 out of 74 females (84%) and 277 (25%) oocytes matured in vitro. Forty-seven embryos were generated by in vitro maturation and fertilisation, among them nine highly valuable embryos from Asiatic golden cat and Northern Chinese leopards. Zoos are strongly encouraged to offer available gametes to the gamete bank and consider the potential benefit of artificial insemination and embryo transfer for their breeding programmes.

Introduction

Habitat fragmentation and loss are the major impacts of anthropogenic action on wild carnivore populations. Furthermore, isolation of small groups and decline of the number of individuals are intensified by human persecution and depletion of natural prey (Ripple et al. 2014; Dickman et al. 2015). Facing the rapid loss of biodiversity, storage of frozen biomaterials is an important tool to preserve the diversity of genetic information and variety of endangered species. Initiatives like the Frozen Ark and the Global Genome Initiative (Droege et al. 2014; Lermen et al. 2009) have been founded to preserve somatic cells and tissue, germplasm, and DNA, as well as germ cells and gonadal tissue, of many animal species. Most of the stored biomaterial can be used by researchers to gain more knowledge about the biology of the species and to further develop methods in several biological and veterinary disciplines (Comizzoli 2017). Within this scenario, zoos are not only predestined to contribute to biobanking by providing samples of their comprehensive species collections, they also commit themselves to sustain or re-establish captive living animal populations by professional management of breeding programmes. However, many population management programmes do not achieve the goal of sustainability (Leus et al. 2011; Long et al. 2011). Extremely small numbers of animals, particularly of successful breeders and founders, and too many individuals with undocumented ancestries result in declining populations and/or gene diversity (Lacy 2013). These current challenges to population management cannot be solved by biobanking somatic cells. Despite significant progress in stem cell and nuclear transfer technology, the conversion of these cells and DNA into living individuals has

still not been sufficiently advanced to contribute significantly to ongoing conservation programmes (Mastromonaco et al. 2014).

However, the combination of gamete banking, defined as the preservation of gametes and gonadal tissue, and assisted reproductive techniques (ARTs) like artificial insemination (AI) and in vitro fertilisation (IVF) has immediate potential to produce offspring or embryos, that may be stored by freezing or directly transferred (ET). The cryopreservation of male and female gametes, as well as embryos allows for safe long-term storage of genetic material (Wildt and Roth 1997; Jewgenow et al. 1997; Swanson 2006) which could reduce the problem of limited space for many zoo populations. Cryopreservation could also support conservation breeding and overcome barriers between in situ and ex situ populations.

Validated methodologies are a basic requirement for successful ARTs (Jewgenow et al. 2017). Development of IVF and gamete cryopreservation protocols in wild species are difficult, due to the scarcity and value of samples. In the case of felids, protocols have been developed in the domestic cat (*Felis catus*) as a model species, and were shown to be successful with wild counterparts (Jewgenow et al. 1997; Herrick et al. 2010;; Pope et al. 2012a; Fernandez-Gonzalez et al. 2015; Luther et al. 2017).

The Leibniz-Institute for Zoo and Wildlife Research runs the Felid-Gamete-Rescue-Project consisting of biobanked oocytes, sperm cells, and embryos, as well as ovarian and testicular tissue, gathered mostly from euthanized or castrated felids provided by the zoos. Some of these specimens originate from individuals that did not reproduce, but,nevertheless, carry invaluable genetic information for the population. The aim of this paper is to report and explain the efforts and results in biobanking felid gametes for the period of ten years (2007-2017).

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless stated otherwise.

Animals

Gonads from felid species were obtained from European zoos and wildlife parks, from animals that either died or underwent castration or euthanasia because of a medical condition. All animal procedures were performed in accordance with the local animal health regulations of the donating institutions. Gonads were stored and shipped preferentially at 4-6°C by using Styrofoam boxes and cool packs. Ovaries were placed in precooled physiological saline solution promptly after excision, whereas testes with epididymides were transported without any medium. The value of a donated sample was defined by the studbook keeper based on the individual's genetic representation within the population.

Oocyte collection and maturation in vitro

Immediately upon arrival in the lab, ovaries were freed of surrounding tissues, washed in Washing Medium (WM) which consisted of Medium 199 with Earle´s salts (M4530) supplemented with 3 mg/mL bovine serum albumin (BSA), 1.4 mg/mL HEPES, 0.6 mg/mL sodium lactate, 0.25 mg/mL sodium pyruvate, 0.15 mg/mL L-glutamine, 0.1 mg/mL cysteine and 0.055 mg/mL gentamicin (Hribal et al. 2013). To allow the release of the oocytes from the follicles, the outer surface of the ovaries was sliced with a scalpel in petri dishes containing 10 mL WM. Collection and quality assessment of oocytes was performed under a stereomicroscope (Hribal et al. 2013). Oocytes which had several granulosa cell layers surrounding the oocyte and a homogenous dark cytoplasm were preferred for further procedures (Figure 1A). However, oocytes with slightly granulated or a patchy cytoplasm were also taken given the value of the samples. Based on the sample origin, genetic value, amount and quality of isolated oocytes, and the availability of homologous sperm, individual decisions were made on how to process these samples. Options were in vitro maturation (IVM) for 24 or up to 34 hours, oocyte cryopreservation before or after IVM, and in vitro fertilization with or without ICSI. Different protocols were tried through the years for IVM, combining different hormones from various origins and concentrations and culture conditions (results not published), resulting in the following standard methodology: IVM of the selected oocytes are matured in WM supplemented with 0.02 IU/mL FSH and 0.05 IU/mL LH, for 24-34 hours at 38.5°C under a humidified air atmosphere with 5% CO^2 (Hribal et al. 2013).

Sperm recovery and cryopreservation

After gentle separation of the caudae epididymides and proximal ducti deferens from the testes, epididymal sperm were recovered as described for the domestic cat (Klaus et al. 2016). In brief, tissues were sliced with scissors in culture medium M199 (HEPES modification, M7528) without supplements at room temperature (20–23°C) and sperm suspension was flushed through a 30 µm filter (Sysmex Partec GmbH, Görlitz, Germany). After appropriate centrifugation at ~700 × g for 8 min, the pellet was re-suspended in a small volume of M199, and sperm concentration was determined in a counting chamber after dilution of an aliquot in distilled water.

Epididymal sperm samples were adjusted to the desired cell concentration with M199 and cryopreserved as previously described (Klaus et al. 2016; Luther et al. 2017). This concentration of sperm in M199 (preferentially aliquots of 100 µl) was diluted by two volumes TestG (buffer of 48.3 mg/mL N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonicacid, 11.6 mg/mL tris(hydroxymethyl)-aminomethane, and 2 mg/mL fructose supplemented with 15% (v/v) of the water-soluble fraction of hen's egg yolk (containing low-density lipoproteins)

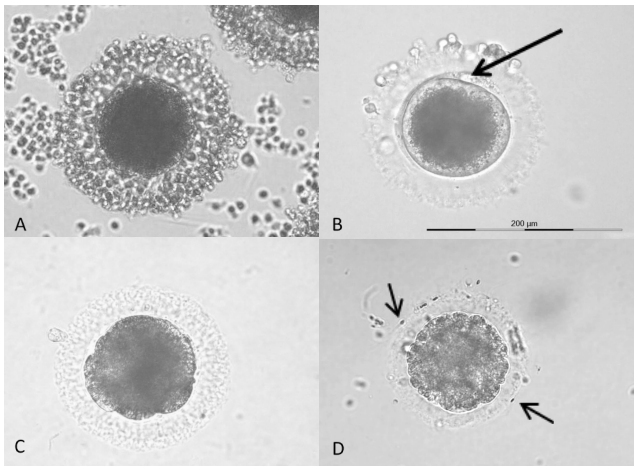

Figure 1: Oocytes and embryos obtained within the Felid-Gamete-Rescue-Project. A: Immature oocyte of a rusty-spotted cat (*Prionailurus rubiginosus*), characterized by several layers of cumulus cells and dark cytoplasm of the oocyte; B: Mature oocyte of an African lion (*Panthera leo*) the extruded polar body (arrow) is an indication of maturation; C: 4-8 cell embryo of a Northern Chinese leopard (*Panthera pardus orientalis*); D: Morula originated from an oocyte from an oncilla (*Leopardus tigrinus*) fertilized with domestic cat sperm cells. Arrows indicate on sperm cells attached to the zona pellucida. Arrow bar = 200 µm, all pictures are at the same magnification.

and with 7% (v/v) glycerol). Aliquots of 300 µL were transferred to cryovials (2 mL round bottom, Greiner, Frickenhausen, Germany) and cooled to 15°C for 40 min in a refrigerator before freezing in the vapour of liquid nitrogen for 15 min to -167°C and subsequent plunging.

Sperm evaluation and preparation for fertilisation in vitro

Samples were thawed by slewing a single vial for 90 s in a 5 L circulating water bath (LAUDA, Lauda-Koenigshofen, Germany) at 38°C. The sperm suspension was transferred to a pre-warmed, 2 mL tube and slowly diluted with an equal volume of pre-warmed M199. After a 10 min incubation at 38°C, an aliquot was used to determine sperm motility by subjective estimation (two competent observers) or a computer assisted sperm analysis system (AndroVision, Minitüb, Tiefenbach, Germany) as described recently (Luther et al. 2017). Subsequently, sperm suspension was centrifuged at ~500 × g for 5 min and re-suspended in WM according to the intended fertilisation procedure.

In some cases, a test aliquot was thawed to assess the post-thaw motility and suitability of sperm for AI, IVF, or ICSI. All samples with motile sperm were considered at least suitable for ICSI. If the total number of motile sperm after thawing all cryopreserved aliquots of a male exceeded 1.5×10^6 (extrapolated from post-thaw motility and frozen sperm quantity) samples were regarded as useable for IVF and if more than 10×10^6 motile sperm were present the sample was regarded as useable for AI (Swanson 2007). Since valuable samples were only thawed when requested for fertilisation, post-thaw motility had not yet been evaluated in 20 applicable samples. In these 20 samples post-thaw motility was approximated as 50% of the motility prior to cryopreservation (Medeiros et al. 2002) and thus considered to have potential application for AI, IVF or ICSI.

Fertilisation in vitro

For ICSI, sperm which had been stripped of cumulus cells by gently pipetting with a micropipette (The Stripper®, BioTipp, Waterford, Ireland) were placed in a 6 cm petri dish that had been prepared with 10 µl droplets of a ready-to-use polyvinylpyrrolidone solution (Origio, Berlin, Germany) and diluted 1:2 (v:v) with 5 µL droplets of WM supplemented with 0.5 mg/mL HEPES. Oocytes with a visible polar body assessed under 200 x magnification on an inverted microscope (Axiovert 100, Carl Zeiss Jena, Germany) were judged as mature (Figure 1B). Motile and morphologically normal spermatozoa were immobilised and injected into mature oocytes from the 3 o'clock position after placing the polar body at the 6 or 12 o'clock position.

For IVF, 50 µL of sperm was added to the fertilisation drop (350 µL of WM supplemented with 10 µg/mL heparin in 4 well dishes covered with mineral oil) to reach a final concentration of 1×10^6 motile sperm/mL or 2×10^5 motile sperm/mL, for frozen-thawed (African lion, *Panthera leo*, and Northern Chinese leopard, *Panthera pardus japonensis*) or fresh samples (*domestic cats*), respectively. Co-incubation of sperm and oocytes was performed for 18 hours at 38.5°C in humidified air atmosphere.

In a few occasions, where no homologous sperm was accessible, heterologous IVF was performed using fresh sperm to assess the fertilization potential of the oocytes under our in vitro conditions: these embryos were never intended for transfer to a female. Lion oocytes were fertilised with fresh tiger and domestic cat sperm, leopard oocytes were fertilised with lion sperm and oncilla oocytes were fertilised with domestic cat sperm. Domestic cat sperm cells were obtained on the same day that the animals were being neutered and the sperm prepared for IVF as described before (Ringleb et al. 2011). For oocytes from genetically valuable individuals, the sperm sample was always chosen according to prior agreement with the respective studbook keeper.

Embryo culture

Directly after microinjection by ICSI, or after 18 hours of gamete co-incubation in the cases of IVF, presumptive zygotes were transferred to 400 µL embryo culture medium (EC), which was Ham´s F-10 medium supplemented with 5% FCS, 100 IU/mL penicillin, 0.11 mg/mL sodium pyruvate, 0.1 mg/mL streptomycin, and 0.075 mg/mL L-glutamine and cultured at 38.5°C under a humidified air atmosphere with 5% CO^2. Evaluation of embryo development was performed every 24 hours.

Vitrification of oocytes and embryos was performed with a three-step method developed for the domestic cat (Mikolajewska et al. 2012) or slow freezing according to Gomez et al. (2003). Genetically important and timely developed embryos were cryopreserved at day 3 to avoid exposure of prolonged in vitro conditions or even risk embryo loss in an attempt to reach blastocyst stage (Maheshwari et al. 2016).

Statistical analysis

Data are presented as medians and range (min - max). For comparison of IVF and ICSI fertilisation outcome two-tailed contingency tables were analysed with Fisher's Exact Test. P levels < 0.05 were considered as significant. Statistical analysis were performed by using the statistical program InStat3 (GraphPad Software, Inc., California, USA).

Results

The Felid-Gamete-Rescue-Project has so far received gonads from 74 females and 67 males from 36 different zoological institutions. The age distribution of donated individuals is shown in Figure 2. The majority of all samples (86/141) came from males or females younger than three years (mainly prepubertal) or older than 13 years of age ("post-reproductive" defined by zoos as animals that no longer reproduce naturally on a regular basis). Males from the Panthera genus younger than 1.5 years were not regarded for this

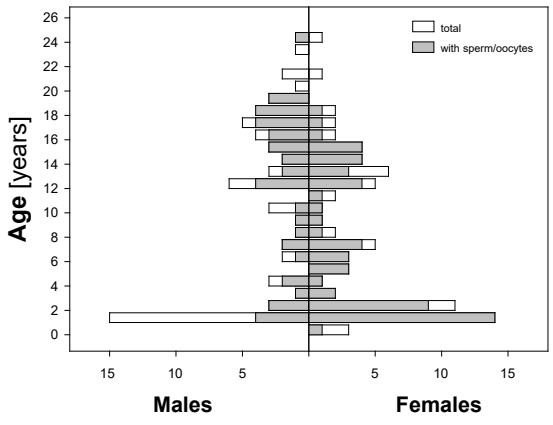

Figure 2: Age structure of individuals from which testes/epididymides or ovaries were recruited within the Felid-Gamete-Rescue-Project (2007-2017): Total numbers of males and females are given as well as the numbers of individuals where gametes could be collected. Males younger than 1.5 years were not considered for the project.

Table 1: Oocyte in vitro maturation, fertilization and cleavage rates of female felids, donated to the Felid-Gamete-Rescue-Project. Subspecies as declared by the zoos.

Species	Subspecies	N females	N retrieved oocytes	N mature oocytes (%)	Fertilisation procedure	N embryos/ mature oocytes
Lion	*Panthera leo*	37	538	171 (31.8)		22 (12.9)
	Panthera leo leo	3[p]	66	24/34 (70.6)	IVF	3/23 (13.0)
				23/32 (71.9)	ICSI	3/23 (13.0)
		1	14	6/14 (42.9)	IVF	2/6 (33.3)
		8	149	41/149 (27.5)	ICSI	14/41 (34.1)
		4	59	24/59 (40.7)	IVF	0
		11	164	36/164 (21.9)	ICSI	0
		2	62	17/62* (27.4)		
		2	7	0		
		4	0			
	Panthera leo persica	1	17	0		
		1	0			
Tiger	*Panthera tigris*	13	165	30 (18.2)		1 (3.3)
	Panthera tigris tigris	1	12	3/12 (25.0)	ICSI	1/3 (33.3)
		1	10	5/10 (50.0)	ICSI	0
	Panthera tigris altaica	2	47	12/47 (25.5)	ICSI	0
		1	16	0		
		1	40	1/40* (2.5)		
		1	4[c]			
		3	0			
	Panthera tigris sumatrae	1	19	0		
	Panthera tigris jacksoni	1	5	2/5* (40.0)		
	Panthera tigris corbetti	1	12	7/12 (58.3)	IVF	0
Leopard	*Panthera pardus*	9	286	61 (32.8)		22 (36.1)
	Panthera pardus japonensis	2[p]	136	25/68 (36.8)	ICSI	8/25 (32.0)
				23/68 (33.8)	IVF	6/23 (26.1)
		1	28	7/28 (25.0)	ICSI	6/7 (85.7)
	Panthera pardus orientalis	1	10	6/10 (60.0)	ICSI	2/6 (33.3)
		3	112	0		
		2	0			
Jaguar	*Panthera onca*	1	3[c]			
Asiatic golden cat	*Catopuma temminckii*	1	3	1/3 (33.3)	ICSI	1/1 (100)
		2	13	6/13 (46.2)	ICSI	0
Black-footed cat	*Felis nigripes*	1	4	0		
		1	10	0		

*no fertilization trial due to poor oocyte quality; [c]oocyte cryopreservation; [p]oocytes of animals were pooled; [h]heterologous IVF with domestic cat sperm in the absence of species specific sperm.

Table 1: Oocyte in vitro maturation, fertilization and cleavage rates of female felids, donated to the Felid-Gamete-Rescue-Project. Subspecies as declared by the zoos (continued).

Species	Subspecies	N females	N retrieved oocytes	N mature oocytes (%)	Fertilisation procedure	N embryos/ mature oocytes
Caracal	*Caracal caracal*	1	0			
Cheetah	*Acinonyx jubatus*	1	20	0		
		1	13	1/13 (7.7)	ICSI	0
Geoffroys's cat	*Leopardus geoffroy*	1	9	1/9* (11.1)		
Oncilla	*Leopardus tigrinus*	1	4	3/4 (75.0)	IVF[h]	1/3 (33.3)
Pallas cat	*Otocolobus manul*	1	0			
Rusty-spotted cat	*Prionailurus rubiginosus*	1	27	0		
Sand cat	*Felis margarita*	1	8	3/8 (37.5)[c]	-	
Snow leopard	*Panthera uncia*	1	7	0		
Sum		74	1110	277		47

*no fertilization trial due to poor oocyte quality; [c]oocyte cryopreservation; [p]oocytes of animals were pooled; [h]heterologous IVF with domestic cat sperm in the absence of species specific sperm.

Table 2. Success of oocyte recovery, maturation and fertilization rate in relation to different age/ reproductive classes of felids. "Prepubertal" are females before onset of puberty (e.g. for Panthera species ≤ 2 years old); "Adult" are animals in the prime reproductive age, and "Post-reproductive" was defined as females ≥14 years, that do not reproduce naturally on a regular base in zoos anymore.

Age class (N animals)	Animals with gametes	N of oocytes	Range of oocytes/animal	Matured oocytes (in %)	Embryos (n animals)	Embryo/ animal
all (73[a])	61	994[b]	3 - 68	253 (21.4%)	47 (19)	2.5
Prepubertal (26)	24	491	3 - 68	157 (32.0%)	33 (10)	3.3
Adult (31)	26	408	4 - 40	81 (19.9%)	8 (4)	2
Post-reproductive (16)	11	95	3 - 31	15 (15.8%)	5 (5)	1

[a]One animal of unknown age excluded
[b]Seven animals of different age classes with together 94 oocytes were processed in groups and had therefore been excluded

analysis since sperm cells could never be found in these animals. Depending on the location of the donor institution, transportation time to the lab varied between 4 and 24 hours (females) or even 48 hours (males).

Females

In total, 1110 oocytes of acceptable quality were retrieved from 62 individual felids (Table 1). Ovaries were collected from an additional 12 females, but no viable oocytes were obtained due to (i) damage of the ovarian tissue during transportation (elevated temperature or freezing to the ice packs; n = 5), or (ii) the age of the females (senescent ovaries; n = 7). In another 14 animals, none of the retrieved oocytes were able to mature in vitro. The number of oocytes per animal ranged from 3 – 68 and was lowest in the "post-reproductive" group (3 – 31; Table 2). Overall, 277 oocytes matured in vitro, evaluated by the visual presence of polar body (ICSI) and/or DNA staining of uncleaved oocytes after IVF.

Of the matured oocytes, 166 had been fertilized using ICSI, 87 oocytes were fertilised by IVF and 47 felid embryos were produced (Table 1). Due to the lack of suitable sperm cells, 12 oocytes were cryopreserved by either slow-freezing (sand cat *Felis margarita*) or vitrification (Amur tiger *Panthera tigris altaica,* jaguar *Panthera onca*). No fertilisation was performed in 21 Metaphase II oocytes as they were of low quality.

Table 3 presents the data from 19 animals from which embryos were obtained. These females ranged from 1 to 18 years old

Table 3. Data about female felids in the Felid-Gamete-Rescue-Project, where embryos were produced.

Species	N females	Age (y)	Shipping time (h)	N retrieved oocytes	Oocytes per group	N mature oocytes (%)	Fertilisation procedure	Species of sperm source#	N embryos/ matured oocytes (%)
African lion	4*	2	10	68	33[a]	13 (39.4)	ICSI	Lion/cryo	4/13 (30.8)
					35[a]	12 (34.3)	ICSI	Lion/cryo	7/12 (58.3)
					34[a]	23 (67.7)	IVF	Domestic cat/fresh	2/23 (8.7)
	3*	2	10	66	32[a]	23 (81.3)	ICSI	Lion/cryo	3/23 (13.1)
						3	IVF	Lion/cryo	1/3 (33.3)
	1	5	24	26	-	5 (19.2)	ICSI	Tiger/fresh	2/5 (40)
	2*	6	10	55	-	11 (20)	ICSI	Lion/cryo	1/11 (9.1)
	1*	14							
	1	14	10	14	-	6 (42.9)	IVF	Lion/cryo	2/6 (33.3)
Bengal tiger (hybrid)	1	15	8	12	-	3 (25)	ICSI	Tiger/cryo	1/3 (33.3)
Amur leopard	1	1,5	10	10	-	6 (60)	ICSI	Leopard/fresh	2/6 (33.3)
Northern Chinese leopard	2*	1	10	136	68[a]	25 (36.8)	ICSI	Leopard/fresh	8/25 (32)
					68[a]	23 (33.8)	IVF	Leopard/fresh	6/23 (26)
	1	3,5	12	28	-	7 (25)	ICSI	Lion/fresh	6/7 (85.7)
Oncilla	1	14	22	4	-	3 (75)	IVF	Domestic cat/fresh	1/3 (33.3)
Asiatic golden cat	1	18	10	3	-	1 (33.3)	ICSI	Golden Cat/cryo	1/1 (100)
Sum total N	19			422		161			47
Sum median		6	10			25%			26%

#fresh = freshly isolated; cryo = frozen-thawed sperm from the gamete bank; *refers to animals who were processed together; [a] represents distribution on several groups based on different fertilisation procedures.

Table 4. Success of sperm retrieval within the Felid-Gamete-Rescue-Project: Number of males from which testes/epididymides were recruited (2007-2017) are given as well as the number of males where sperm could be collected and cryopreserved. A male's sample with a total of >10×10^6 or >1.5×10^6 motile sperm (extrapolated after thawing a test aliquot) were classified as suitable for AI or IVF, respectively.

Species	Subspecies	Males total	with Sperm		Sample suitability		
			collected	frozen	AI	IVF	ICSI
Lion	*Panthera leo*	17	8	7	4	6	7
	Panthera leo persica	4	0				
Tiger	*Panthera tigris altacica*	5	4	4	1	3	4
	Panthera tigris tigris	1	1	1	0	1	1
	Panthera tigris sumatrae	3	1	0			
Leopard	*Panthera pardus orientalis*	6	5	5	2	3	5
	Panthera pardus japonensis	3	3	3	3	3	3
	Panthera pardus melas	2	1	1	0	1	1
	Panthera pardus tulliana	3	1	1	1	1	1
Jaguar	*Panthera onca*	1	0				
Asiastic golden cat	*Catopuma temminckii*	3	3	3	0	1	1
Ocelot	*Leopardis pardalis*	2	2	2	2	2	2
Margay	*Leopardis wiedii*	1	1	1	1	1	1
Geoffroy's cat	*Leapardis geoffroyi*	2	2	2	0	2	2
Puma	*Puma concolor*	1	1	0			
Cheetah	*Acinonyx jubatas jubatas*	2	2	1	0	1	1
	Acinonyx jubatas soemmergingii	1	1	0			
Leopard cat	*Prionailurus bengalensis*	1	1	1	0	1	1
Rusty-spotted cat	*Prionailurus rubiginosus*	1	1	1	0	1	1
Fishing cat	*Prionailurus viverrinus*	2	1	1	0	0	0
Pallas cat	*Otocolobus manul*	3	1	0			
Black-footed cat	*Felis nigipes*	3	2	2	0	1	2
Sum		67	42	36	14	28	35
Percentage (%)		100	63	54	21	42	52

(median 6), and sample shipping time was no longer than 24 h (median 10 h). From these animals 422 oocytes were retrieved, 25% matured in vitro (median) and fertilisation rate determined by the cleavage of zygotes was 26% (median). There was no difference in the fertilisation rate between ICSI (35/106) and IVF (12/61) produced embryos (P = 0.0752, Fisher's Exact Test). It was still possible to produce embryos from "post-reproductive" animals, though to a lesser extent than in the adult or the prepubertal group (Table 2).

Of particular importance was the timely development of a 4-cell Asiatic golden cat embryo (*Catopuma temminckii)* and eight day 3 embryos at 4-8 cell stage from two Northern Chinese leopards (Figure 1C). These cells were cryopreserved and are available for embryo transfer. All other embryos were of less genetic importance because they originated from overrepresented or hybrid animals, or from individuals with uncertain ancestries: 22 embryos were from African lions; two from one Amur leopard (*Panthera pardus orientalis*); six from Northern Chinese leopard; one from an oncilla (*Leopardus tigrinus*; Figure 1D); and one from Bengal tiger *Panthera tigris tigris*. In the case of two African lions, one Northern Chinese leopard and oncilla, fertilisation was performed with heterologous sperm for evaluating the developmental competence of the in vitro matured oocytes; as no compatible sperm were available at the time of the trials.

Males

Table 4 presents an overview of male felids which were recruited from 15 species and 20 European zoos. Sperm was recovered from 42 males resulting in 36 samples which could be dedicated to cryopreservation. The sperm of six males was calculated to be less than 1000 so these samples were not cryopreserved. The number of recovered spermatozoa ranged from ~100 to 1.9×10^9 (median 21×10^6). The motility before cryopreservation was 0 to 82% (median 50%). For 16 samples, the post-thaw motility ranged from 0 (only in the fishing cat *Prionailurus viverrinus*) to 59% (median 28%). The resulting total number of motile sperm after thawing was estimated to range from 0 to 193×10^6 (median 5×10^6) per male. Reasons for missing sperm and/or low sperm quality can be related not only to age, season, and health issues, but also transportation time (up to 48 h, median 20 h) and temperature (raising to ambient temperature upon long transportation). As expected, in very young (1.5-2 years) and very old (>19 years) males sperm recovery was less successful.

Discussion

Saving the genetic material of threatened species by the establishment of biobanks is scientifically feasible and essential (Comizzoli 2015). As a repository of rare species, zoos may provide biobanks with essential samples. Whereas biobanking of somatic cells and tissues is undoubtedly of general importance, gamete and embryo banking is of particular interest for zoos because this material can be directly beneficial for population management strategies. By gamete transfer between in situ and ex situ populations gene flow could be recreated without the need to exchange living animals (Swanson 2006). However, the success of a gamete bank is dependent on availability of gametes and on reliable ART.

Recovery of gametes from living animals (oocyte pick-up, electro-ejaculation, urethral catheterization) is highly desirable but associated with costs (specialized staff and equipment, hormonal treatment of females) and risks (anaesthesia and surgery) and is, therefore, only rarely performed in zoos. Alternatively, gametes are more easily available from gonads recovered after castration or euthanasia. Nearly all of the samples collected (99%) by the Felid-Gamete-Rescue-Project have been gathered post-euthanasia, but this option is also associated with two major disadvantages: i) Oocytes extracted from nonstimulated ovaries are of much lower developmental potential than in vivo-matured oocytes gathered by ovum pick-up after hormonal treatment (Bogliolo et al. 2004). Even the well established in vitro maturation procedure in the domestic cat already leads to a loss of 20-50 % of the oocytes prior to the fertilisation procedure (Johnston et al. 1991; Comizzoli et al. 2003; Sowinska et al. 2017). The average maturation rate of 25% achieved in this study with several wild felid species is markedly lower than maturation rates of domestic cat oocytes (Ringleb et al. 2004). Similarly, it is recognised that felid males produce low quantity and quality sperm cells (Wildt et al. 1988). This deficit is further increased by the fact that epididymal sperm has lower motility and viability compared to ejaculated or catheterized specimens (Hermansson and Axner 2007); ii) Euthanasia of animals associated with health and/or age-related factors can influence gamete quantity and quality (Johnston et al. 1991; Rao et al. 2015). In the case of the females, 16/74 samples were obtained from animals of 14 years or older. Although oocytes could still be recovered, the mean number of oocytes per animal and the maturation rate of these oocytes were markedly reduced. The probability of sperm recovery seems to decline only in very old males (> 19 years); iii) Recovery, assessment, and processing of oocytes and sperm cells requires expertise, but only a few labs are qualified for the species-specific demands for felid species, which are different from the human and livestock IVF systems. Consequently, samples have to be sent to specialised labs, but prolonged and inappropriate transportation can cause a loss in gamete quality. Based on data from domestic cats (Wolfe and Wildt 1996) and data in the current study, it is recommended that ovaries be properly cooled and processed within 24 h, whereas epididymal sperm cells may survive in the epididymis for at least 48h, or even 72 h, if samples are kept cool (Ringleb et al. 2011; Angrimani et al. 2018).

The expertise on oocyte maturation/fertilisation, embryo culture or gamete cryopreservation in felid species can be acquired by working with domestic cats, but differences between felid species might occur. For example, oocytes from the bigger cats of the Panthera genus, seem to require more time for maturation (Wiedemann et al. 2012) and embryo development (Fernandez-Gonzalez et al. 2015). To continuously improve expertise in wild felid species, however, samples from genetically unimportant (overrepresented or hybrid) individuals are highly needed. The Felid-Gamete-Rescue-Project has extensive experience with IVM/IVF of African lions. Former reports of in vitro fertilisation in lions showed that IVM and IVF techniques can be performed for embryo production, with cleavage rates of fertilized oocytes from 11.4% to 31.6% (Johnston et al. 1991; Jewgenow et al. 1997). Our fertilisation rate of 23% for lion oocytes is in this range. However, recent improvements of culture media in one trial with oocytes from four lions, led to a cleavage rate of 58.3% (7/12 oocytes; Table 3) with two embryos developing to the blastocyst stage (28.6%) (Fernandez-Gonzalez et al. 2015). Depending on the availability of sperm, IVF as well as ICSI were successfully applied in this study. The use of ICSI within the Felid-Gamete-Recue-Project offers the option to overcome poor sperm cell quality and quantity (Fernandez-Gonzalez et al. 2015).

There are only a few reports on IVM/IVF in other cat species. Successful IVF in the tiger has been described with fresh and frozen-thawed ejaculated sperm (Donoghue et al. 1990; Johnston et al. 1991; Donoghue et al. 1992) and the cleavage rate was up to 70.2% when in vivo matured oocytes had been used. When oocytes were obtained directly from ovaries, maturation rates ranged from 23.5 to 48.9% and fertilisation rates were less than 10% (Johnston et al. 1991). Johnston et al. (1991) also performed IVM/IVF on leopard oocytes (subspecies not specified) with a cleavage rate of up to

28.6%. Similarly, one third of in vitro matured and fertilized (ICSI) oocytes from Northern-Chinese leopards cleaved in this study.

Single IVM/IVF trials have been reported for several small felid species, for example cheetah *Acinonyx jubatus*, clouded leopard *Neofelis nebulosa*, bobcat *Lynx rufus*, Geoffroys`s cat *Leopardus goeffroyi* or leopard cat *Proinailurus begalensis* (Johnston et al. 1991). In the case of the sand cat, IVF was so far only performed after hormonal stimulation of the females (Herrick et al. 2010). Our trial to mature sand cat oocytes in vitro was successful. Three of eight isolated oocytes were assessed after IVM and all three were mature. Based on this promising result it was decided to freeze the other five oocytes without assessing maturation state. Similarly, of four oocytes with good morphological characteristics that could be retrieved from a 14-year-old oncilla, three oocytes matured in vitro. Moreover, one oocyte cleaved after heterologous fertilisation with sperm from domestic cat.

Cryopreservation of gametes and embryos is a central issue in gamete banking. Beside the rare events when fresh semen can be applied for artificial insemination, sperm cells, mature or immature oocytes, and embryos have to be cryopreserved. Cryopreservation of felid sperm cells was established a long time ago (Platz et al. 1978; Lengwinat and Blottner 1994; Zambelli et al. 2010) and may work sufficiently well not only in the domestic cat, but also in wild felid species (Donoghue et al. 1992; Jewgenow et al. 1997; Swanson 2007; Herrick et al. 2010). The most important problem, is that very old or diseased animals, as used in this study, can have impaired sperm quality. Cryopreservation usually induces a drop in viability and if the original sample is of very low quality and/or quantity it comes to a point where not only AI (<1.5×106 motile sperm), but also IVF ihas nearly no chance of success. After thawing, only 21% and 42% of all received testis samples (39% and 78% of all frozen samples) revealed suitable sperm samples for AI or IVF, respectively. Thus in many cases, ICSI is the only promising and validated alternative fertilisation method (Bogliolo et al. 2001; Fernandez-Gonzalez et al. 2015). For ICSI, only a few viable sperm cells are required (Ringleb et al. 2011), which can be collected and cryopreserved even after transportation and cold storage of testes/epididymides for up to 72 h.

Immature and mature felid oocytes have previously been frozen with limited success with both slow-freeze and vitrification protocols (Murakami et al. 2004; Braun et al. 2006; Luciano et al. 2009; Mikolajewska et al. 2012; Arayatham et al. 2017; Fernandez-Gonzalez and Jewgenow 2017). Live offspring have been reported after the transfer of vitrified in vitro matured domestic cat oocytes fertilized by ICSI (Pope et al. 2012b). Freezing oocytes is of particular importance if adequate sperm cells for fertilisation are not available. Adequate sperm means not only homologous sperm, but also sperm from genetically valuable males recommended for use with the oocyte in question. Although little data is available for felid species, cryopreservation of embryos is supposed to be more successful than oocyte freezing (Saragusty and Arav 2011). In the domestic cat, embryos at different developmental stages have been frozen or vitrified with similar protocols as for oocytes. Pope et al. (2012a) reported the birth of domestic cat kittens and one black-footed cat kitten after transfer of frozen-thawed embryos into domestic cat recipients. In this study, embryos at the 4-8 cell stage were frozen and are awaiting an ET trial.

Summary

Gamete banking has the significant potential to save genetic diversity of zoo populations, support gene flow within breeding programmes, and might be the last chance to transfer genes of new founders into zoo populations. However, ART is a collection of several complicated methods from hormonal cycle induction, to culture and fertilisation techniques, cryopreservation, and finally ET that are still far from being fully established. Therefore, gonads of overrepresented or hybrid animals or individuals of unknown ancestry provide valuable material for the continuous refinement of ART, and are as important to the progress of conservation as genetically important individuals. Based on our experiences gonads should be shipped cooled but not frozen and transportation should be terminated within 24h. During the 10 years reviewed of this gamete rescue project, embryo production systems have been greatly improved though a direct comparison between them is not possible due to the uniqueness of every single case. Of particular importance is the availability of a cryopreserved 4-cell Asiatic golden cat embryo and eight 4-8 cell Northern Chinese leopard embryos for embryo transfer. In the future, cryopreservation of oocytes (in case of the lack of sperm cells) and embryos will be focussed upon.

Everything starts with the availability of samples. A gamete bank needs gametes! Zoos with their profound species collections are strongly encouraged to exploit all options by offering available gametes to gamete banks. Effective management decisions could include: the early castration of animals which are no longer designated for breeding; use routine health checks for semen collection from animals at the most fertile age; or apply ART to the generation of offspring which are genetically important, but in non-breeding situations. All these measures can help to save the genetic diversity of zoo populations.

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