Original Report

Ex-situ preservation of Mustelidae : primer of application of genetic resource bank concept with the use of polecats as the model species

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Summary

This report presents experimental data concerning the possibility of application of a genetic resource bank concept to the Mustelidae family. Reproductive physiology and different types of preimplantation embryo development in this family are analyzed with the use of European polecat, stoat, and American mink as examples. Results of experiments on embryo transfer and embryo cryopreservation in polecats are described. The possibility of application of these technologies to endangered Mustelidae species, and especially to European mink, as well as possible obstacles are discussed.

Introduction

Reproductive technologies, most of which originated in the last 100 years, have been rapidly developed and improved recently (*Rott, 1996*). After Heap (*1891*) discovered that it was possible to obtain live offspring in rabbits after transferring preimplantation embryos to the appropriate recipient female, this technique has been successfully adopted to various domestic mammalian species (*Kraemer, 1983*). Embryo transfer (ET) technology combined with hormonally induced ovulation (superovulation) and embryo cryobanking is used widely both for laboratory (*Mobraaten, 1986*) and farm (*Calessen et al, 1998*) animals and thousands of embryos are transferred yearly (*Thibier, 1996*).

The situation with wildlife is far more complicated. More than 20 years have passed since Kraemer et al., (*1976*) reported the first successful application of ET to wild animal species. There were other, more or less effective attempts, sometimes resulting in pups developed to term (see *Rott, 1996; Loskutoff, 1998* for recent review). When applied to wildlife, the efficiency of ET and accompanying reproductive technologies was much less compared to domestic species and only limited success was achieved. The report

of Schiewe et al (*1991*) might be used as an illustration of this suggestion. This research group attempted to apply domestic cattle embryo recovery, cryopreservation, and superovulation protocol to four wild African antelope species. Findings of this investigation show that extrapolation of ET protocol from domestic to non-domestic wildlife species meets some obstacles. The main lesson of this attempt was that the basic knowledge on the reproductive physiology of each particular threatened or endangered species should be obtained before trying to adopt modern reproductive technologies to this wild species (*Wildt et al., 1992*). Sometimes, however, closely relative species might be used as experimental models, and when there are reliable data obtained with thess models, ET techniques might be applied to their threatened wild counterpart (*Wildt et al., 1992; Wildt, 1992*).

The Mustelidae is one of the most diverse and prosperous family of the Carnivora order. This family contains more than 70 extant species (*Anderson, 1989; Schreiber et al., 1989; Ternovsky and Ternovskaya, 1994*). Despite such prosperity in general, about 25 percent of Mustelidae species are nowadays endangered and even threatened to become extinct (*Ternovsky, 1975; Schreiber et al., 1979; Ternovsky and Ternovskaya, 1994; Maran, Henttonen, 1995*).

Three different reproductive patterns are inherent to mustelids. One, which is considered the most primitive (*Mead, 1989*), is found in many species of Lutrinae and Mustelinae subfamilies. The breeding season in these species occurs in the late winter or spring. The following gestation period is relatively short and constant in duration. There is no period of implantation delay in these species. The black- footed ferret (*Mustela nigripes*) which is an American endangered species; European mink (*Mustela lutreola*), which is an endangered species of the Old World, as well as polecats: European polecat (*M.putorius*), steppe polecat (*M. eversmanni*), Siberian polecat (*M. sibirica*), and domestic ferret (*M.putorius furo*) are all characterized by this reproductive pattern. The main emphasis of this study was made on this particular group of Mustelidae species, the reproductive cycles of which resemble those of ferrets. Some other Mustelidae species listed below (not included in the genus *Mustela*), which are considered endangered, also have this reproductive pattern. The pygmy spotted skunk (*Spilogale pygmala*), giant otter (*Pteronura brasiliensis*), African clawless otter (*Aonix capensis*) and oriental small-clawed otter (*Aonyx cinerca*) are considered endangered (*Schreiber et al., 1989*) and are characterized by a relatively short and constant pregnancy without any implantation delay (*Mead, 1989*).

The stoat, whose reproduction is different, is used here as a reference. This Mustelidae species was included into another group of species according to the reproductive classification of Ternovsky (*1977*) and Mead (*1989*). The species of this group are characterized by long gestation periods accompanied by prolonged obligatory embryonic diapause and implantation delay. The possibility of application of a genetic resource bank concept to these diapausing species is a special problem, which is beyond the main goals of our present study. Nonetheless, it should be noticed that stoat and other Mustelidae species exhibiting prolonged gestation accompanied by delayed implantation are very promising for embryological experiments, due to the availability of embryos during most of the year.

Moreover, the possibility of conservation of the diapausing embryo is of special concern. Some other Mustelidae species listed below, which are considered endangered, have this (stoat-like) reproductive pattern. The European marbled polecat (*Vormela peregusna*), wolverine (*Gulo gulo*), Taiwan yellow-throated marten (*Martes flavigula*) and sea otter (*Enhydra lutris*) are endangered (*Schreiber et al., 1989*) and have a long period of delayed implantation in their embryo development (*Ternovsky,1977; Mead, 1989*).

Additionally to these two main reproductive patterns mentioned above, another one has been described in Mustelidae (*Ternovsky, 1977; Mead, 1989*). This pattern is characterised by relatively short, but variable gestation periods. This variability is due to occurrence of a short period of delayed implantation, the duration of which is determined by the day of mating. Mead (*1989*) listed only American mink (M. vison) and striped skunk (*Mephitis mephitis*) as known Mustelidae species, which have this pattern. There is no known endangered Mustelidae species in this group, so we did not emphase on this pattern of reproduction in the present research.

Ovulation in the majority of Mustelidae species is induced by copulation (*Mead, 1989, Murphy, 1989; Ternovsky and Ternovskaya, 1994*). Nevertheless, some exceptions exist. In spotted skunk (*Spilogale putorius latifrons*) ovulation occurs spontaneously (*Greensides and Mead, 1973*).

Reproductive physiology in mustelides has been studied mainly in ferrets , American mink and sable, which have a long history of captive breeding (see *Manteifel, 1947; Murphy, 1989; Sundkvist et al., 1989; Ternovsky and Ternovskaya, 1994* for review). Some other wildlife Mustelidae species were also thoroughly investigated: the western spotted skunk (*Spilogale putorius*) (*Enders and Mead, 1996*); European badger (*Canivenc, Bonnin, 1981*) and stoat (*Mustela erminea*) (*Deanesly, 1943; Ternovsky, 1977; Amstislavsky et al., 1993 a; 1997; Ternovsky, Ternovskaya, 1994*). Some data, not so comprehensive, but nevertheless very interesting, have been obtained in some other Mustelidae species, such as otter, marten, wolverine, sea otter and others (see *Mead , 1989; Ternovsky and Ternovskaya, 1994* for review).

The ferret was the first species of carnivorous animal that embryo transfer technology was successfully applied to (*Chang, 1968*). There have also been reports about pups born after embryo transfer in the American mink (*Zhelezova, Golubitsa, 1978; Adams, 1982*). Nevertheless, until recently, embryo transfer has not been investigated in Mustelidae species on a large scale. Moreover, in mustelids only occasional attempts of embryo cryopreservation (*Amstislavsky et al., 1993b*) and accompanying procedures such as *in vitro* culture of the embryos (*Whittingham, 1975*) have been performed earlier. The aim of this study is to fill this gap. Results of our experiments on application of the cryobanking technology (i.e. embryo transfer, and embryo cryopreservation) to the Mustelidae are summarized here. Details of the unpublished experiments will be published elsewhere (*Lindeberg et al., 1999*).

Synchronization of donor and recipient animals

Synchronization of donor and recipient females in domestic animals is accomplished through precisely timed injections of prostaglandins.. Moreover, hormonally induced superovulation of donors is a common practice in monoovulatory species. In mustelids, synchronization and superovulation is complicated by the fact that most mustelids are seasonal breeders and induced ovulators. To induce estrus in seasonal breeders out of breeding season you need melatonin or special lighting regimens. Meanwhile, exact timing of estrus has not been accomplished yet. In mustelids, embryo transfer has to be done during their breeding season and ovulation can be induced by mating or gonadotrophine injection.

So far, we have worked for two breeding seasons at Juankoski Fur Animal Research Station of the Institute of Applied Biotechnology, University of Kuopio in Finland and at the Experimental Research Station Institute of Zootaxy and Ecology of Animals, Russian Academy of Sciences in Novosibirsk .

Detection of estrus and determining the proper mating time were done on the basis of observation of vulva swelling degree and checking the vaginal smears. To induce

ovulation the females were mated with males. Mating was done once a day on two consecutive days in Finland, but only one day in Novosibirsk. Donor females were mated with fertile males, and recipient females to sterile males. At Juankoski the sterile males were prepared surgically by cutting the spermatic cords. In Novosibirsk hybrid genetically sterile males were used. One such sterile hybrid male originated from a cross between a European mink (*Mustela lutreola)* female to a European polecat (*Mustela putorius*) male. Another one originated from a steppe polecat (*Mustela eversmanni*) female and a domestic ferret *(Mustela putorius furo*) male. Mating of donor-recipient pairs were organized on the same day or so that the recipient was mated one day after the donor. Fertility of mating was secured by taking a sample from the vagina. If spermatozoa were found, the mating of a donor was considered successful. Females mated with genetically sterile or surgically sterilized males were also checked after mating and no spermatozoa were found.

Stoat females bred in captivity manifest their estrus at age 20 days (*Ternovsky, Ternovskaya 1994*). Young females chosen as embryo donors (26-92 days old) were mated to adult males of proven fertility and vaginal smears were checked for presence of spermatozoa after mating as described earlier (*Amstislavsky et al. 1993 a*).

Embryo transfer technique

For embryo recovery the donor females were sacrificed. The advantage of sacrificing a donor is that you are able to collect embryos not only from the uterine horns, but also from the oviducts. The uterine horns were flushed with phosphate buffered saline (PBS) supplemented with bovine serum albumin (BSA). Flushing solution was collected to Petri dishes, which were studied under a stereo microscope for evaluation of the developmental stage of the recovered embryos. The embryos were washed in the flushing solution before they were transferred, *in vitro* cultured or frozen. If embryos were chosen for transfer, they were stored in a CO_2 -incubator until the moment of transfer. Embryos were transferred surgically into the uterus of synchronized recipients under general anaesthesia. The recipient animals were anaesthetized with a combination of medetomidine hydrocloride and ketamine. The transfer was made with a fine glass capillary, which was sharpened in advance and connected to a plastic tube. The distal end of this plastic tube was connected to a holder, which made it possible to guide the instrument by mouth and to insert the embryos into the uterine horn by gently increasing the pressure in the system. The number of transferred embryos was in the range of 2-15. The embryos were transferred with a minimal amount of flushing solution. All embryos were transferred into the same uterine horn. All recipients were given post-operative antibiotics.

Preimplantation embryo development and embryo transfer in mustelids.

Ovulation, fertilization and early embryo development in ferret species has been studied earlier (*Robinson, 1918; Hammond, Marshall, 1930; Hamilton ,1934; Hammond, Walton, 1934; Chang and Yanagimachi,1963; Chang, 1965; Enders and Schlafke , 1972; Carroll et al., 1985*). Excellent reviews are available for more details (*Murphy, Douglas 1992; Lagerkvist, 1992*). It is well known that ferrets are induced ovulators (*Robinson, 1918*) and have been shown to ovulate approximately 30 hours after initial intromission (*Hammond and Walton, 1934*). Chang (*1965*), in his elegant experiments, has demonstrated that ferret spermatozoa retain their fertilizing capacity in the reproductive tract of females for 120 hours after being placed there. We also observed that stoat spermatozoa survived in female reproductive tract and were actively moving for up to four days. This relatively long period of sperm viability is one of the specific features of Mustelidae species which might be concerned with induced ovulation.

Nevertheless, due to changes in ovulated oocytes, fertilization of ova rarely occurs more than 30 hours after ovulation (*Chang, Yanagimachi, 1963*). The current opinion is that under natural conditions fertilization occurs in ferrets within 24 hours after ovulation (*Chang, Yanagimachi, 1963*). This species is polyovulatory and on the average about 12 ova ovulate as a result of mating (*Lagerkvist, 1992*). For this reason, there is no need to induce superovulation in ferrets hormonally. Results of our large scale experiment with flushing and transfer of embryos in European polecat are described in details (*Lindeberg et al. 1999- in press*). Briefly some of these data are presented below. There seems to be a pronounced variation in time of implantation and duration of pregnancy among Mustelidae species, which is much more prominent than the variation in the canine species we described earlier (*Valtonen, Jalkanen, 1993*). Table 1 illustrates three different types of pregnancy and embryo development, which are found in the Mustelidae family.

The European polecat represents the simplest reproductive pattern, as there is no embryonic diapause, the pregnancy is relatively short and of constant duration. Alternatively, implantation in the stoat is delayed and, due to this long period of obligatory embryonic diapause, the pregnancy encompasses a long period of time. The American mink represents a relatively rarely observed type of embryonic development, where pregnancy is short, but extremely variable in duration, due to the occurrence of a short period of delayed implantation, whose duration is determined by the date of mating.

Table 1. Timing of entering the uterus and implantation in some Mustelidae species.

Events	MUSTELIDAE SPECIES		
	European polecat (Mustela putorius)	American mink (Mustela vison)	Stoat (Mustela erminea)
Entrance into the uterus	Day 6-7	Day 7-8	Day 11-12
Expansion of embryos	Day 6-11	*Day 7-8 (49)	* Day 12- 240
Implantation	Day 11-14	Day 8-49	Day 240- 272

Day 0 = first day of mating
* In these cases expansion coincides with embryonic diapause
Referenced from Hamilton, 1934; Enders, 1952, Enders and Schlafke 1972; Kolpovsky, 1978; Mead, 1989; Enders and Schlafke 1972; Murphy, Douglas, 1992; Amstislavsky, 1993 (a) and our unpublished experiments in Finland and in Novosibirsk.

Figure 1 represents different stages of embryonic development in the European polecat. Based on our early observation on the stoat (*Amstislavsky et al., 1993 a; Maksimovsky et al., 1994*) and recent experiments with European polecats, we can conclude that the embryos in Mustelidae move from the oviduct to the uterus mainly in the stages from compact morulae to blastocysts. We have transferred embryos of different stages collected both from the oviduct and uterus. The pregnancy rate was high (totally 20 transfers in Finland and in Novosibirsk with an overall success rate of 45%, unpublished). Cleavage stages, morulae, early blastocysts, blastocysts, and expanded blastocysts were successfully transferred (Figure 1, c-h).

In summary, we conclude that the most suitable embryonic developmental stages for transfer are blastocysts and expanded blastocysts. We used this stage of embryonic development also for transfer between ferret subspecies. Seven offspring were produced as a result of embryo transfer of expanded blastocysts between European polecat and domestic ferret in Novosibirsk (Table 2).

Table 2. Embryo transfer between European polecat (*Mustela putorius*) and domestic ferret (*Mustela putorius furo*)

Direction of embryo transfer	Date of mating	Number of transferred blastocysts	Recipient number	Date of delivery	Number of pups born	Success rate (%)
From *M. putorius* to *M. putorius* *	14.05	6	262	23.06	6	100
From *M. putorius* to *M. putorius furo*	11.06	6	328	22.07	2	33
the same	16.04	10	326	26.05	5	50
the same	17.04	2	332	-	0	0
the same	24.04	2	336	-	0	0

* Control

In vitro culture of polecat embryos

Polecat embryos can be cultured *in vitro* from the 1-2 cell stage to the expanded blastocyst stage. After a culture period of 2-4 days, depending on the developmental stage at the beginning of the culture, the great majority of embryos are successfully developed to blastocyst stage (*Whittingham, 1975; Lindeberg, 1998*). Whittingham (*1975*) was the first who reported about successful *in vitro* culture of ferret embryos from the 1-cell to the blastocyst stage, but provided little other information. We reached even more prominent results compared to the study of Whittingham (*1975*), when after a 144 hour *in vitro* culture, some polecat embryos hatched (*Lindeberg et al., 1998*). It has been shown earlier that it is possible to culture embryos of American mink (*Mustela vison*) from blastocyst stage to hatching (*Moreau et al., 1996*). Domestic cat embryos develop *in vitro* to the blastocyst stage, but hatching has not been demonstrated (*Johnston et al., 1991*). It has been also shown that fox (*Vulpes vulpes*) embryos develop *in vitro* to expanded blastocyst, but only those embryos which were beyond the 8 cell stage (*Lindeberg et al., 1993*). Until recently, only American mink (*M. vison*) embryos were demonstrated to hatch *in vitro* among carnivorous animals by Mureau et al. (*1996*), but unfortunately only blastocysts were cultured in their study. It can be concluded that the European polecat is another Mustelidae species which is possible to culture *in vitro* to the stage of hatched blastocyst. *In vitro* culture might also be used to verify the viability of the embryos after cryopreservation .

Embryo cryopreservation in polecats

The embryos of the Mustelidae species contain more lipid droplets as compared to most laboratory and farm animals (*Kizilova et al., 1998*) and are relatively large: expanded blastocysts achieve a size of about 1 mm in diameter (*Amstislavsky et al., 1993 a; Maksimovsky et al., 1994*). These two peculiarities are the main obstacles to successful freezing of Mustelidae embryos.

Glycerol and DMSO are traditionally used for embryo cryopreservation in different animal species (*Niemann, 1991, Rott, 1996*). It has been shown recently, that ethylene glycol (EG), when used as a cryoprotectant, gives better

results as compared to glycerol in some circumstances (*Pollard, Leibo, 1994; Martinez, Matkovic, 1998*).

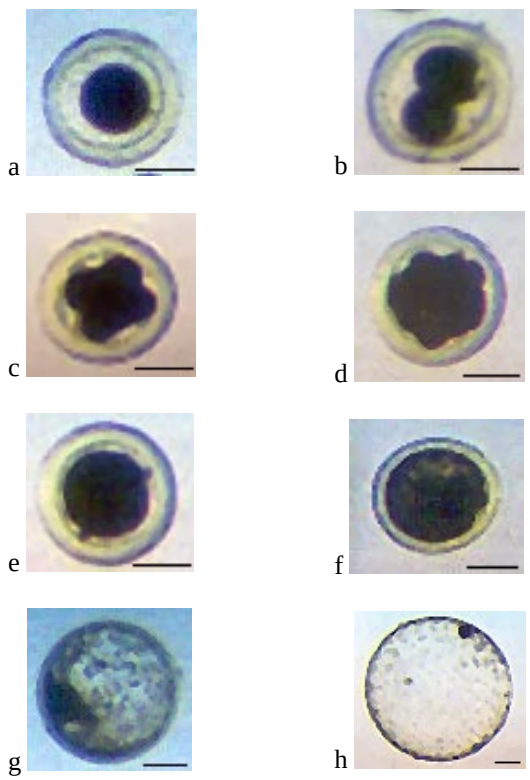


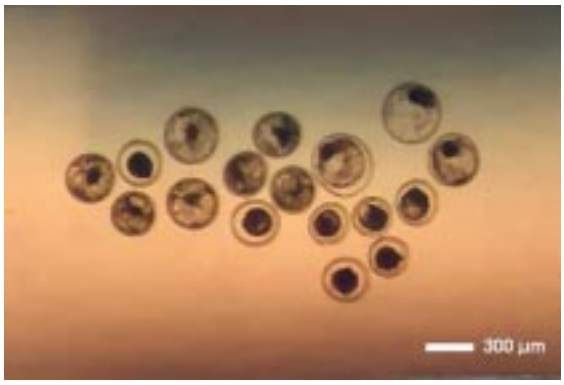
Fig. 1. Stages of preimplantation embryo development in European polecat (*M. putorius*) Bar indicates 100 µm; the day of mating was considered as Day 0 of pregnancy.

a. Ovulated oocyte – Day 2-3
b. Two-cell embryo – Day 2-3
c. Four-cell embryo – Day 4-5
d. Early morula – Day 5-6
e. Compact morula – Day 6-7
f. Early blastocyst – Day 6-7
g. Blastocyst – Day 7-8
h. Expanded blastocyst – Day 8-11

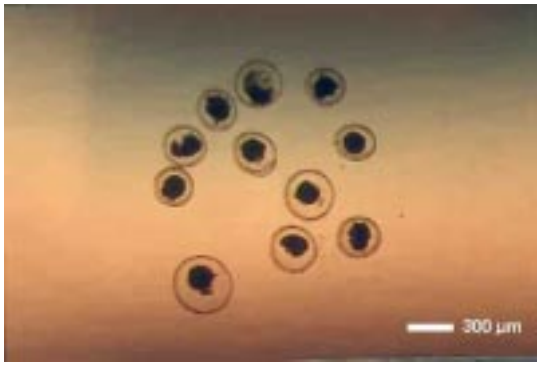
Earlier we investigated the ultrastructural changes in steppe polecat embryos after cryopreservation (*Kizilova et al., 1998*). We froze these embryos by conventional programmed freezing method with the use of either 1,4 M glycerol or 1,4 M DMSO as a cryoprotectant and analyzed thawed embryos by electronic microscopy. When DMSO was used as a cryoprotectant, the ultrastructure of most of the cells of the trophectoderm (TE) was not damaged, and the only change observed was a lower osmiofility of the lipid droplets as compared to unfrozen controls. Some of the TE cells and the large portion of the cells of ICM (inner cell mass) were more damaged. In all the cases, when glycerol was used as a cryoprotectant, damages of embryos were heavier as compared to cases when DMSO was used. In embryos cryopreserved with glycerol, blastomeres appeared shrunken, some of the cisternae of the endoplasmic reticulum were abnormally dilated, and destruction of cell contacts was observed in many cases. About 30% of trophoblastic cells demonstrated numerous interruptions of cell membranes. The observed changes of size and of electron density in the majority of blastomeres led us to the conclusion that glycerol was toxic to polecat embryos. Bruyas et al. (*1993*) found an evidence that freeze-thawing with glycerol induced considerable cellular damage also in horse embryos.

In cryopreservation experiments with European polecat embryos, EG was chosen as the cryoprotectant. Totally 17 blastocysts flushed from two donor females at Day 7-8 p.c. were frozen-thawed by a conventional method borrowed from bovine studies. Equilibration with EG was achieved by transferring embryos into Petri dish, which contained 4 % EG. After 5 minutes equilibration, embryos were transferred into the medium with a final concentration of 8 % EG and equilibrated

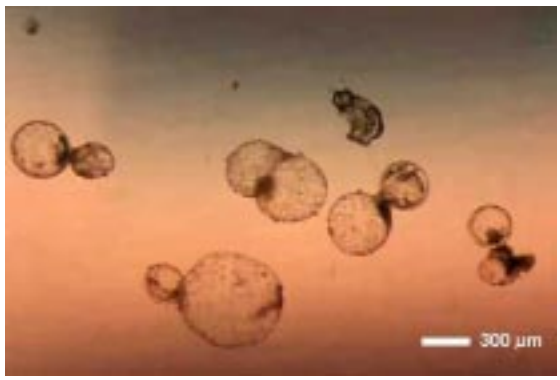
additionally for 20 minutes. After equilibration with cryoprotectant, the embryos were aspired into 0.25 ml plastic straws (L'Aigle, IVM, France) with 8% EG and put in the freezer. The controlled freezing was performed using a programmable freezer (Freeze Control). According to this technique, the straws were cooled at 3 °C/ min to -6°C after which ice formation was immediately induced by touching the top of the liquid column containing the embryos with forceps cooled in liquid nitrogen (seeding). After 10 minutes, cooling continued at 0,3°C/ min to -35°C. The straws were then plunged into liquid nitrogen and in this experiment were stored one hour at -196°C before thawing.



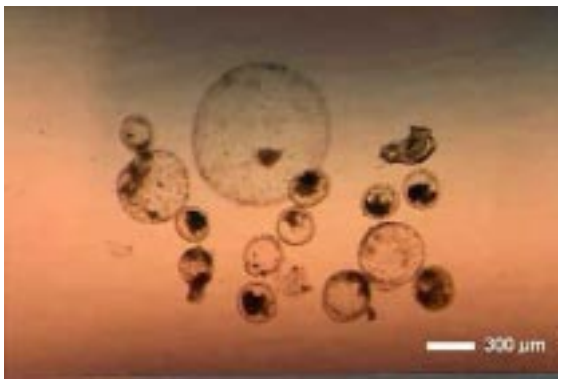
a) 17 embryos from two donors. Both donor females were flushed on Day 7-8 from mating and sixteen blastocysts and one early blastocyst were recovered. 11 of the embryos were chosen for freezing.



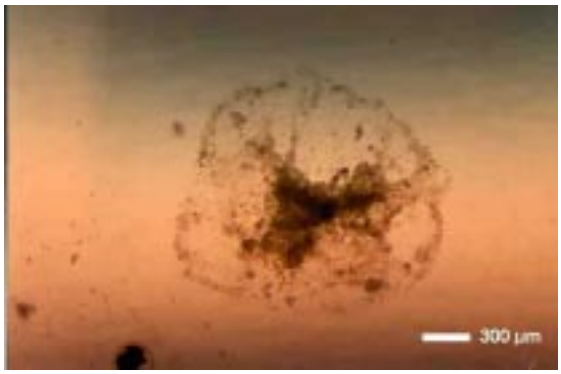
b) The 11 embryos were frozen with 1,5 M ethylene glycol as cryoprotectant using a programmable freezer (Freeze Control). The photograph was taken immediately after thawing the embryos.



c) The embryos have been cultured in modified TCM199 for two days. Four of them are hatching.



d) The embryos after four days in culture. Hatched embryos are more expanded than the others.



e) A hatched embryo attached to the bottom of a Petri dish after eight days in culture.

Fig. 2. In vitro culture of frozen-thawed European polecat (*M. putorius*) embryos

The straws were thawed quickly with body warm expired air and pushed from the straws to a small Petri dish. They were equilibrated 6-7 minutes in 4% sucrose and two times for 5 minutes in the flushing solution after which the embryos were ready to be transferred into the culture system or into recipients.

From the 17 frozen-thawed polecat embryos 11 were cultured *in vitro* in order to verify their viability. Three of the 11 cultured embryos hatched and increased in size markedly after being cultured for 4 days. One of them even "implanted" (attached to the bottom of the Petri dish) on day 8 of *in vitro* culture (Figure 2). In this experiment no detrimental effects on the subsequent development during in vitro culture were observed after 20 minutes exposure to 8% EG and cryopreservation for one hour. This experiment suggests that conventional freezing of polecat embryos with EG results in viable embryos.

On the one hand, we have already mentioned that severe ultrastructural changes have been observed in some cells of polecat embryos after freeze-thawing with glycerol or DMSO (*Kizilova et al., 1998*). On the other hand, the present study confirms that EG is non-toxic for Mustelidae embryos and can be used to cryopreserve polecat embryos. In summary, these results favor ethylene glycol for subsequent experiments on cryopreservation of Mustelidae embryos.

Current status and prospects of *ex situ* preservation of endangered Mustelidae species

There are a number of Mustelidae species which are endangered or even threatened with extinction (*Ternovsky, 1975; Schreiber et al., 1989; Ternovsky, Ternovskaya, 1994; Maran, Henttonen, 1995*). The European mink is nominated as the priority species for conservation (Schreiber et al., 1989). The continuous spread of American mink is recognized to be one of the main causes of extinction of the European mink (*Ternovsky, 1975; Ternovsky, Ternovskaya, 1994; Maran, Henttonen, 1995*). The rapidly reducing the number of European mink throughout the former's range strongly dictate the use of modern reproductive technologies and *ex situ* approach to prevent the total extinction of this aborigine species. On the other hand, the successful recovery of black-footed ferret (M. nigripes) in North America is encouraging application of reproductive technologies to mustelids (*Wildt et al., 1992*).

The traditional methods of *ex situ* preservation of animals (e.g. captive breeding of small populations of animals in zoos and other breeding facilities) have strong limitations as listed recently (*Lasley et al., 1994; Loskutoff, 1998*) and are often ineffective in reaching the ultimate goals of safeguarding targeted endangered species (*Lasley et al., 1994; Maran, 1996; Loskutoff, 1998*). The concept of the genetic resource bank, based in its technical part, on the modern reproductive technologies, has been developed (*Wildt, 1992*). Methods of *ex situ* preservation of gametes and embryos are recommended to be combined with the traditional approach of conservation of animal populations *in situ* and *ex situ* (*Wildt,1992; Wildt et al., 1992*). Excellent reviews summarizing the attempts of application of a genetic resource bank concept to wild animal species are available (*Wildt, 1992; Rott, 1996*). Interspecies embryo transfer of *in vivo* (*Stover et al., 1981*) and *in vitro* (*Johnston et al., 1994)* produced embryos in hormonally stimulated gaur (*Bos gaurus*), an endangered ungulate species, resulted in live offspring. In these cases domestic cattle recipients were used. It is well known that reproductive technologies and, especially, embryo transfer protocol was well-developed in cattle (*Calessen et al., 1998*). Moreover, basic knowledge on the reproduction and embryo development obtained in cattle was extrapolated to gaur (*Bos gaurus*), because this endangered species is closely related to domestic cattle (*Bos taurus*). Human IVF procedure used in clinics was recently successfully applied to western

lowland gorilla (*Pope et al., 1997*). Earlier, a baboon infant was obtained by transferring an It should be noted, however, that numerous applications of the genetic resource bank concept to nondomestic wildlife species, which are not related to cattle or humans, have yielded only infrequent births (see *Wildt et al., 1992; Rott,1996; Loskutoff, 1998* for review). Moreover, unsuccessful results of embryo transfer are not always published. A special concern is the possibility of interspecies embryo transfer, when the pool of available recipient females is limited. In most cases of interspecies transfer, even those which resulted in pups born, the pregnancy was compromised: abnormal histological architecture of the placenta, low numbers of placentomes and other complications of pregnancy were observed in these cases (see *Lasley et al., 1994* for review). In addition to these few successful attempts, many other cases were unsuccessful due to resorption or abortion of the foreign embryo. Successful and unsuccessful attempts of interspecies embryo transfer and the causes of good and bad luck were analysed in the special reviews (*Anderson, 1988; Lasley et al., 1994*). In some cases a special immunosuppressive treatment was used to prevent failure of interspecies embryo transfer (*Croy et al., 1985*). Very careful choice of a recipient species, which resembles the endangered species in its reproductive physiology is recommended (*Anderson, 1988; Lasley et al., 1994*). Nonetheless, in the majority of cases, when interspecies pregnancy resulted in pups born, embryo transfer was much more successful in one direction than the opposite one (*Anderson, 1988*). In our experiments embryo transfer between closely related ferret subspecies (*M. putorius and M. putorius furo*) resulted in pups born, but successful embryo transfer between different Mustelidae species is still unknown. High success rate of embryo transfer in polecats is good background for attempting to perform interspecies embryo transfer between European mink and closely related polecats. This study is in progress now.

in vivo produced embryo (*Kraemer et al., 1976*).

The Mustelidae family belong to the Carnivora order. In fact, methods of IVF, embryo transfer, embryo cryobanking and accompanied modern reproductive technologies have been adopted mainly to a single family in Carnivora: Felidae. The possibilities of cryopreservation and successful embryo transfer of *in vivo* (*Dresser et al., 1988*) and *in vitro* (*Pope et al., 1994*) produced embryos have been demonstrated in the domestic cat. Moreover, the nonsurgical method of embryonic transfer has been developed for this species (*Swanson and Godke, 1994*) and aimed to minimize surgical intervention. This is beneficial from the ethical point of view. Based on these technological achievements and investigation of Felidae reproductive biology with the use of the domestic cat as a model species, this technology has then been applied to wildlife felids (*Wildt et al., 1992*). Methods of assisted fertilization (mainly artificial insemination and semen cryopreservation) have also been successfully applied to black-footed ferret, whereby the endangered Mustelidae species were saved from extinction (*Howard et al., 1991; Wildt et al., 1992*). These studies were done in the framework of the American program aimed at conservation of the black-footed ferret (*M. nigripes*). Results described in this paper suggest that embryo technology might be also applied to other Mustelidae species and especially to European mink, which is a priority species for conservation in Europe *(Schreiber et al., 1989*).

European mink is in the same genus as polecats (*Youngman, 1982*). Genetically, European mink is most closely related to Siberian polecat (*Mustela sibirica*) which in the literature is also called Kolonocus sibirica (*Ternovsky; Ternovskaya, 1994*) and even Kolinsky mink (*Mead, 1989*). These two species have the same number of chromosomes (2n= 38) (*Grafodatsky et al., 1976*). Also, there are many genetic similarities between European mink , European polecat and domestic ferret (*M.putorius furo*). This genetic relation of

European mink with polecats has been confirmed by comparative analysis of karyotypes (*Grafodatsky et al., 1976*).

The reproductive pattern and gestation length of European mink resemble that of European polecat and domestic ferret. Hybrids between European polecat, Siberian polecat, domestic ferret and European mink have been obtained on a large scale in captivity (*Ternovsky, Ternovskaya, 1994*). Moreover, hybridization between European mink and European polecat occurs spontaneously in nature (*Tumanov, Zverev, 1986*). The majority of our research is done with European polecat, to be able to approximate the reproductive data to the closely related European mink. It is also promising to use European polecat or Siberian polecat (*M. sibirica*) as a recipient species in the continuation of an *ex situ* program aimed at conservation of the European mink.

It seems very timely to apply the concept of a genetic resource bank to European mink, because nowadays the extant population is still genetically viable enough to ensure adequate material available for conservation, but it is in rapid decline throughout much of its range (*Ternovsky, 1975; Schreiber et al., 1989; Ternovsky, Ternovskaya, 1994; Maran, Hettonen, 1995; Maran, 1996*). On the other hand, success of the previous projects for protecting European mink initiated in Russia (*Ternovsky, Ternovskaya, 1994*) and Estonia (*Maran, 1996*) has been limited. These projects were based only on the captive breeding and reintroduction of European mink in nature but the ultimate goal of safeguarding this species is not achieved yet. We agree with Wildt (*1992*), who recommends combination of the traditional approach based on captive breeding with the modern approach based on the concept of a genetic resource bank for conservation of endangered species. We hope the embryo cryobanking and embryo transfer technology adopted to European polecat will be useful in this concern.

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