Conservation of the European Mink (*Mustela lutreola*): Focus on Reproduction and Reproductive Technologies

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Contents

The European mink (*Mustela lutreola*) is a small mammal, which belongs to the *Mustelidae* family (*Carnivora*). Earlier, the range of distribution of this species encompassed much of the European continent. During the 20th century, the numbers of European mink declined and the range of its distribution became reduced to three fragmented populations; today this species faces extinction. The urgent necessity for effective conservation efforts to protect the European mink is accepted by the governmental organizations as well as scientific communities of most European countries. In this paper, the reasons for the disappearance of European mink are reviewed and results of past conservation efforts based on captive breeding and reintroduction programmes are critically evaluated in the broad context of modern concepts of conservation genetics and reproductive biology. The data recently obtained on the reproduction and pre-implantation development of European mink and the prospects of incorporation of modern reproductive technologies into the conservation programme of this species are discussed.

Introduction

European mink (*Mustela lutreola*), which belongs to the *Mustelidae* family (*Carnivora*) is classified as an endangered species according to the IUCN Red list (http://www.redlist.org). The decline of European mink was first noted by Dmitri Ternovsky and Igor Tumanov in Russia (Ternovsky and Tumanov 1973), and in the Action Plan for the Conservation of Mustelids and Viverrids (Schreiber et al. 1989), the European mink was already considered as one of the most endangered mustelid species. Previously, this species was present in most of the European continent including the southern and central part of Finland, France and in adjacent provinces of north-western Spain, Germany, Hungary, in the countries of former Yugoslavia, northern Romania and Bulgaria and the European part of Russia (Youngman 1982; Schreiber et al. 1989). Nowadays, only three well-separated populations still exist in the wild. The northeastern (NE) population occupies the territory around the Russian city of Tver and extends to some other areas in Russia and Belarus (Sidorovich 2000; Michaux et al. 2005). The southeastern (SE) population occupies the Danube river delta in Romania (Michaux et al. 2005; Kranz et al. 2006). The western (W) population is known to exist in northern Spain and southwestern France (Michaux et al. 2005; Palazon et al. 2006).

More than 10 years have passed since the publication of Maran and Henttonen (1995), in which hypotheses explaining the disappearance of European mink throughout Europe were listed and critically evaluated. The new data on systematic, distribution, physiology and behaviour of this species have been accumulated, and an action plan of conservation efforts was approved at the 1st International Conference on the Conservation of European mink held in Spain in 2003.

There are two centres, in Russia and in Estonia, where the European mink had been bred in captivity since 1970s (Ternovsky and Ternovskaya 1994) and 1980s (Maran 2006) respectively. During the recent decades, new breeding facilities have been established in Germany (Festl et al. 2006) and in Spain (Mañas et al. 2006). Significant efforts have been undertaken to introduce/reintroduce this species into the wild: onto islands in the Russian Far East (Ternovsky and Ternovskaya 1994) and onto Hiiumaa Island in Estonia (Maran 2006, 2007).

Early embryonic development in the European mink has been a recent focus (Amstislavsky et al. 2006a) and the possibility of cryopreservation of mustelid embryos has been confirmed by successful development of frozen-thawed polecat embryos *in vitro* (Amstislavsky et al. 2000) and *in vivo* (Lindeberg 2003; Lindeberg et al. 2003). Also a method for semen cryopreservation and artificial insemination (AI) has been developed in ferrets (Howard et al. 1991; Wildt et al. 1992; Kidder et al. 1998).

The aim of this review was to evaluate the reasons for the disappearance of European mink and the systematic position of the species, taking into account new data, to summarize the *in situ* and *ex situ* conservation efforts undertaken so far, and to evaluate the prospects of cryobanking based on recent discoveries in the reproductive physiology of the European mink and on the first successful results of applying embryotechnological approaches to mustelids.

Systematic and Genetics of European Mink

Karyotype analysis revealed a close relationship between European mink and European polecat (*Mustela putorius*). In contrast, karyotypes of American mink (*Mustela vison*) and European mink (*Mustela lutreola*) indicate a much more distant relationship; the number of chromosomes in European mink (38) is characteristic for European *Mustelidae* species (38–44), whereas that of the American mink (30) is well outside of this range (Volobuev and Ternovsky 1974; Graphodatsky et al. 1976). Recent molecular phylogenetic analysis confirmed that European mink are most closely related to

the polecat species (Davison et al. 2000; Sato et al. 2003). Davison et al. (2000) analysed mitochondrial DNA in European mink, polecats (*M. putorius*; *Mustela eversmanni*) and polecat/mink hybrids captured from nature. Sequencing of the two separate mitochondrial DNA fragments (cytochrome *b* and D-loop), and phylogenetic analysis using kolonok (*Mustela sibirica*) and itatsi (*Mustela itatsi*) as outgroups, revealed a low level of sequence divergence in cytochrome *b* between European mink and polecats. This was interpreted as indicating a close molecular relationship possibly resulting from either reticulate evolution (hybridization) or relatively recent speciation of European mink in a close relation to polecat. Sato et al. (2003) confirmed the close relationship between European mink and polecat species. For phylogenetic analysis, these authors used sequencing of the nuclear gene encoding interphotoreceptor retinoid binding protein (IRBP) together with mitochondrial cytochrome *b* genes. Their study also confirmed the monophyly of the genus *Mustela*, which includes European mink (*M. lutreola*), subgenus *Putorius* (*M. putorius, M. putorius furo, M. eversmanni*), kolonokus (*M. sibirica*), itatsi (*M. itatsi*), stoat (*Mustela erminea*) least weasel (*Mustela nivalis*) and solongoi (*Mustela altaica*). American mink (*M. vison*) can essentially be considered as an outgroup for this clade (Sato et al. 2003).

Michaux et al. (2004, 2005) studied the genetic background of the three extant populations of the European mink and discussed possible strategies for conservation of this species. They investigated mitochondrial DNA (mtDNA) variations using the complete D-loop region sequences and found that a single haplotype predominates in the W population. The NE population of European mink was much more diverse and was characterized by ten different mtDNA haplotypes. The SE population was intermediate between W and NE. Additionally European minks were genotyped by using six microsatellite markers. The lack of genetic heterozygosity in the W population led the authors to conclude, that the western population probably derives from a few animals which colonized western France and Spain relatively recently, possibly as a result of human introduction.

Reasons for the Disappearance of the European Mink

In the great majority of cases, the primary factors contributing to extinction of a mammalian species are habitat destruction, over-exploitation/over-hunting, pollution and the adverse impact of introduced alien species (Frankham 2003). The history of European mink exemplifies this general suggestion well. The main hypotheses, which are discussed in relation to extinction/decline of European mink in Europe, include the adverse impact of the American mink, possible hybridization with the European polecat, introduced diseases, pollution, overhunting and habitat loss (Ternovsky and Ternovskaya 1994; Maran and Henttonen 1995; Sidorovich 1997; Maran 2007). During the last decade, some hypotheses have been tested and it is possible to clarify the picture based on the knowledge gained recently on

the biology, behaviour and ecology of European mink and its relation to other mustelid species.

Habitat destruction as a result of urbanization and the expansion of agriculture is probably the fundamental cause for the decline of most mammalian species, and the European mink is not an exception. However, there are some specific factors which have influenced the numbers of European mink and led to its rapid decline even in places where habitat is neither disrupted nor polluted (Sidorovich 1997; Maran 2007).

Possible hybridization with polecats

A role for polecats in the process of European mink disappearance was proposed already in 1980s (Granqvist 1981). According to one hypothesis, hybridization of European mink with polecats may cause admixture and extinction of the former species (Maran and Henttonen 1995; Maran et al. 1998). Interspecies hybridization of wild and domestic species occurs in the wild, and this process threatens a number of known wild species (Rhymer and Simberloff 1996; Allendorf et al. 2001). For example, feral housecats (*Felis silvestris catus*) can easily crossbreed with the African wildcat (*Felis silvestris libyca*), a process which has threatened the existence of this felid species (Stuart and Stuart 1991; Randi et al. 2001). Hybridization has also occurred between domestic dogs (*Canis familiaris*) and the Ethiopian wolf (*Canis simensis*), and this process is considered to be a substantial threat to this most endangered canid species (Gottelli et al. 1994).

Hybrids between male European polecats and female European minks have been obtained in captivity on the research farm in Novosibirsk (Russia) on a large scale for many years (Ternovsky and Ternovskaya 1994). More recently, the reverse hybrids have also been obtained on the same farm (Ternovskaya et al. 2006). Taking this into account, crossbreeding between European mink and European polecat may also occur in nature.

Possible natural hybridization between polecats and European mink has been addressed from evolutional, systematics and conservational points of view (Davison et al. 2000; Sato et al. 2003; Lode et al. 2005), and significant genetic introgression between populations of European polecats and European minks has been confirmed recently (Davison et al. 2000; Sato et al. 2003). However, in the Seugne River area, where relatively large populations of European polecat and European mink still coexist, field observations reveal that hybridization between these species is a very rare event (<3%) (Lode et al. 2005). These observations are in a good agreement with the earlier results of Tumanov and Zverev (1986), who suggested hybridization between these two species is possible in the wild, but normally occurs only occasionally. It seems that the European mink and the European polecat are able to live sympatrically, and risk of admixture of the former species by the latter is not high. In some marginal cases, however, the frequency of hybridization may increase. For instance, it has been reported from Estonia that, during the last years of existence of the European mink, the proportion of suspected hybrids between European

mink and polecats was much higher than the expected 3% (Maran 2007).

The role of the American mink

The destructive role of invasive species, the American mink, on the existence of the populations of indigenous European mink has been emphasized by the majority of experts who studied the decline of European mink in different countries (Ternovsky and Ternovskaya 1994; Maran and Henttonen 1995; Tumanov 1996; Sidorovich 2006). The recent disappearance of European mink from north-western France (Brittany) was carefully monitored (Lode et al. 2001), and this case provides the unique opportunity to test the potential role of the American mink in this process. Nowadays, the European mink is still present only in southwestern France, but it is extinct from the northern part of its former range (Lode et al. 2001; Maizeret et al. 2002). It was concluded that American mink either never occurred or has remained extremely rare in the area from which European mink disappeared in France (Lode et al. 2001). In this case at least, it would be unreasonable to attribute the main role in the European mink's decline to competition with the American mink. It was demonstrated that the reasons for extinction of European mink are often complex and Lode et al. (2001) concluded that in northwestern France, the critical factors in the extinction of European mink were the alteration of water quality, habitat modification and intensive trapping, rather than the introduction of American mink. Maran and Henttonen (1995) noted that in Moldova, Ukraine and in several regions of Russia, the decline of European mink was recorded long before the invasion of American mink. However, in some regions, like Belarus and Estonia, the impact of the American mink on the disappearance of the aborigine species is well documented (Maran and Henttonen 1995; Sidorovich 2006). It can therefore be concluded that the presence of American mink in the same region where European mink exists aggravates the situation and often makes it irreversible. Ternovsky and Ternovskaya (1994) stressed in their book that, where these two species do coexist, it is always the population of European mink that declines while American mink increase in numbers. The negative influence of American mink may be indirect, but there are a number of hypotheses also suggesting direct influence, as follows.

Hypothesis of Dmitry Ternovsky

This hypothesis postulates that in nature, mating of the American mink males with European mink females may result in pregnancy, but pregnancy then fails and resorption of the foetuses occur. More detailed information and corresponding illustrations have been depicted in two monographs (Ternovsky 1977; Ternovsky and Ternovskaya 1994). Three females of European mink were mated to stud American mink males of proven fertility. However, none of the hybrid embryos, developed to term. One pregnant female was surgically investigated, and visual observation of the uterus confirmed the characteristic uterine swellings indicating the presence of the foetuses inside; at the time of operation (day 33 *post coitum*) all the foetuses were in the process of resorption. Whether or not European mink indeed copulate with American mink in nature has never been properly studied, but it has been confirmed that some wild mammal species may easily mate with related domestic species, and that hybridization, even without introgression, might cause wasted reproductive efforts and be highly detrimental (Allendorf et al. 2001).

Interspecies agression hypothesis

Based on his own comprehensive field observations, Vadim Sidorovich proposed that the reason for the drastic decline of the European mink population may be the aggressive behaviour of the intruder – the American mink (Sidorovich 2000, 2006). Over several years, he registered intra- and interspecies interactions between these two species in the Lovat river area (Belarus), and observed that the majority of contacts between European and American mink were aggressive. He further suggested that the European mink were often attacked by the larger and more powerful American mink and consequently deserted the river area and sheltered in atypical and suboptimal habitats.

Transmission of New Infections

Infections carried by invading species, and to which an indigenous species is susceptible, are clearly likely both to remove competition and assist invasion. Aleutian disease virus (ADV) introduced by feral American minks, for instance, has been suggested as a possible cause of regional extinction of European mink (Mañas et al. 2001, Fournier-Chambrillon et al. 2004). However, this hypothesis still needs experimental verification before it can be accepted, and in earlier experiments in which European minks were exposed to American mink in captivity, there were no recorded transmissions of diseases between them (Maran et al. 1998).

Reproductive Superiority of American Mink

The brief review of hypotheses attempting to explain the decline of European mink leads us to conclude that none on its own can explain the dramatic disappearance of European mink throughout Europe during the twentieth century. Maran (2007) concluded that a number of interacting factors are involved in the decline of European mink, but emphasized the combined impact of American mink, fragmentation and loss of habitat, and over-hunting.

Our own hypothesis explains the competitive success of American mink by unique aconstellation of reproductive traits described in this species, which makes it possible to reproduce successfully in different habitats. Superfetation and delayed implantation have been observed in American mink (Shackelford 1952, Thom et al. 2004a). Furthermore, recent studies revealed that American mink females are actively polyandrous in captivity (Thom et al. 2004b). Reproductive biology of European mink is less studied, however, none of the reproductive traits mentioned above have been

described for this species so far, and it is known that there is no implantation delay in European mink (Ternovsky and Ternovskaya 1994; Amstislavsky et al. 2006a). The combination of superfetation, delayed implantation and female polyandry would clearly present an advantage in terms of reproductive assurance and enhanced genetic mixing, and, thereby, adaptation to new environments. One can predict the reproductive superiority of American mink over European mink. Reproductive superiority may be crucial for successful adaptation of the former species in Europe and elsewhere (Macdonald and Harrington 2003) and, on the other hand, may represent the ultimate cause of the decline of the latter species at least in those cases, when both 'minks' live sympatrically (e.g. Estonian and Belarussian cases).

Observations done in captivity are in a good agreement with our hypothesis. In equivalent conditions of captivity, the fecundity of American mink is greater relative to that of European mink (Ternovsky and Ternovskaya 1994); 5.4 ± 0.35 vs 4.3 ± 0.10 kits per litter respectively. In two conventional fur farms, where this was studied in detail, the fecundity of American mink was as high as 5.4 ± 0.10 (Klotchkov and Eryuchenkov 2003) or even 5.91 ± 0.14 (Experimental farm of the Institute of Cytology and Genetics, Novosibirsk; Kharlamova and Trapezov 1999).

Current Status of Conservation of the European Mink

The concept of an 'evolutionarily significant unit' first proposed by Ryder (1986) has since been discussed in the context of defining a useful 'management unit' for conservation purposes (Fraser and Bernatchez 2001). Current opinion is that western, eastern and southern animals have to be managed together (Michaux et al. 2005), and this approach is in agreement with the practical experience gained in conservation of the European mink in Russia (Ternovsky and Ternovskaya 1994; Ternovskaya et al. 2006) and in Estonia (Maran 2006). These practical attempts assumed that the European mink can be regarded as a single 'evolutionary significant unit'.

Captive Breeding of European Mink

Russia

More than three decades of captive breeding in Novosibirsk, Research Station has confirmed its potential value in the preservation of European mink. About 500 litters of European mink were produced during this period (Ternovsky and Ternovskaya 1994; Ternovskaya et al. 2006). Females are sexually mature and useable in a breeding programme as early as 10–11 months of age, but only a minority of males (about 30%) achieved sexual maturity during the first year of life; the remaining males either participated at the age of 2 years or failed to breed successfully in captivity.

The fecundity of females is dependent of their age, and in Novosibirsk, the maximum litter size has been nine kits (Ternovskaya et al. 2006). Polygamy is char-

acteristic of some (though not all) European mink males, some European mink males impregnating up to nine different conspecific females within one breeding season.

Estonia

Activities to establish a captive breeding population in Estonia were started in 1980s and regular breeding was achieved during the mid-1990s. The captive bred population maintained in Tallinn is a main nucleus for the European mink Endangered Species Program (EEP), in which more than 200 animals are kept in 17 Institutions involved, about half of the entire EEP captive bred population being maintained in Tallinn Zoo, accounting for 105–120 animals (Maran 2006). This is the largest captive bred population in Europe and worldwide, but, according to Maran (2006) the size of captive bred population needed to maintain 90% of this species's heterozygosity, is 364–693 animals.

Germany

The EuroNerz Foundation started in Osnabrück in 1998 and currently maintains a breeding stock of about 40 individuals. This centre faces a problem of hyper-aggressive behaviour between males and females. To overcome this problem, new litters have been maintained as a group until late autumn or winter, resulting in significantly improved socialization and reproductive success (Festl et al. 2006, Wolfgang Festl, personal communication).

Spain

Recently, a captive breeding programme has been initiated in Spain, based in El Port de Suert (Lleida, Spain), and covers the area of 2970 square metres (Mañas et al. 2006). The aim of this programme is to maintain the western stock of European mink in captivity to prevent it from extinction and to reinforce the wild population by new releases. The number of animals is currently 56, but capacity exists for 112 adults. Each enclosure has both outdoor and indoor areas (with nesting boxes); the outdoor installations including riverbank vegetation and running water. The first kits born in captivity were documented during the 2005 season (http://mediambient.gencat.net/Images/43_60896.pdf). The concept of this centre is different from other centres, that is the western population, not the whole species, is considered to be a discrete 'evolutionary significant unit'.

Island Introduction/Reintroduction and Release to the Wild

The Russian initiative

The largest attempt to transform a captive bred population into a reproducing population in the wild has been undertaken in the Russian Far East (Ternovsky and Ternovskaya 1994), where the two largest of the Kuril island chain, Kunashir and Iturup, were chosen

for introduction, being about 1550 and 6725 km² in area respectively. Both islands are sufficiently distant from the mainland and other islands to prevent the invasion of American mink.

Young European minks were released onto the islands, usually in September, and eight groups, a total of 388 animals, were released between 1981 and 1989. Before the release, animals have been trained to swim, to build a den, to explore their environment and to hunt (Ternovsky and Ternovskaya 1994). For this purpose, families selected for the release were initially placed into a special territory belonging to the Research Station, which resembled the natural habitat, and the husbandry of these animals after training was kept to a minimum. As a result of this large scale action, two viable populations of European mink were successfully established on Kunashir and Iturup (Voronov 1992; Ternovsky and Ternovskaya 1994; Shvarts and Vaisfeld 1995; Ternovskaya et al. 2006).

There was, however, some controversy about the resulting density of European mink population on the islands. According to Voronov (1992), the total number of the animals living on these two islands was around 500, and it was also suggested that the density of European mink ranged between 0.1 and 3.5 animals per kilometre of stream/river coastlines. According to another study (Shvarts and Vaisfeld 1995), however, the population density on both the islands never exceeded one animal per kilometre. The critical issues of the action have been discussed elsewhere (Shvarts and Vaisfeld 1995).

The paper of Shvarts and Vaisfeld (1995) provides an important follow-up study, because it was carried out 10 years after the first release of European mink onto Kunashir island, and they confirmed that a reproducing population of European mink then existed on this island. Recently, first hand information from Julia Ternovskaya, who, together with Dmitry Ternovsky, was an active participant of Kuril action plan, has become available in English (Ternovskaya et al. 2006).

The Estonian initiative

In Estonia, two islands (Hiiumaa, about 1000 km², and Saaremaa, about 2400 km²) were selected for the establishment of European mink populations from captive born founders. A feral population of American mink existed on Hiiumaa island, so an eradication operation was undertaken during years 1998–2000 (Maran 2006). Following this, a total of 295 animals were released during the period 2000–2006. Numbers of males and non-pregnant females were equal (131 of each), in addition to 33 pregnant females (Maran 2006, Tiit Maran, personal communication). It was hoped that if kits were born in wild, then their chances of survival would be higher the than would be the case for animals born in captivity, although there is a potential risk of higher mortality amongst the pregnant females. The animals selected for the release have been trained whenever possible, involving minimization of contacts with keepers, provision of live prey available in wild, training for swimming, diving and fishing, enrichment of enclosures with the natural objects, and exposure to domestic dogs to optimize the development of anti-predator behaviour.

During most of the years of action, however, the survival rate evaluated during the following spring after release was very low and did not exceed 10–15% (Maran 2006). Welcomed change in the situation occurred in the 2006 season in which tracks of European mink were detected along most of Hiiumaa island's streams and rivers. Litters born from matings in the wild were recorded and the population size was estimated as at least 25 animals (Maran 2006, Tiit Maran, personal communication).

Some innovative approaches arose during this Hiiumaa experiment, such as the release of pregnant animals, the release of females in their nest boxes, retrapping the released females in autumn and keeping them in captivity during winter, and constructing temporary enclosures where released animals can find shelter during extreme conditions.

Embryo and Semen Cryobanking and Associated Reproductive Technologies

Collection and cryopreservation of semen

Methods for semen collection and cryopreservation/artificial insemination with either fresh or frozen-thawed material for mustelids have been developed mostly in domestic and black-footed ferrets (Howard et al. 1991, 2006; Wildt et al. 1992, 1992; Kidder et al. 1999; Santymire et al. 2006). The crucial role of such methods in the recovery of the black-footed ferret from the brink of extinction, based on the last 18 animals, is well known (Biggins and Godbey 2003; Howard et al. 2003, 2006). Most of the fundamental and applied studies on mustelid semen have relied on the domestic ferret as a convenient model species, including those that were incorporated into the black-footed ferret recovery plan. It has been shown that intravaginal insemination is ineffective in ferrets (Wildt et al. 1992; Kidder et al. 1998), but the relatively non-traumatic laparascopic intrauterine insemination (Wildt et al. 1992), or the more sophisticated transcervical nonsurgical deposition of semen into the uterus/uterine horns (Kidder et al. 1998), does result in relatively high (66.7–79%) pregnancy rate. In the study of Kidder et al. (1998), for instance, female ferrets were successfully inseminated transcervically either into the uterine body or into the uterine horn. The latter site proved to be the most successful approach, however, with all five females inseminated by this route becoming pregnant, whereas only two out of five inseminated into the uterine body doing so. Application of these protocols to the black-footed ferret produced offspring from otherwise breeding-incompatible animals, especially from genetically valuable, but non-breeding males (Howard et al. 2006).

Methods for semen cryopreservation have been developed using the domestic ferret (*M. putorius furo*) and steppe polecat (*M. eversmanni*). Frozen-thawed semen of the former has been used for intrauterine insemination, resulting in 70% pregnancy rates and the birth of 31 kits (Howard et al. 1991). The same procedures have been performed with steppe polecat semen (83.3 % pregnancy, 26 kits born) (Howard et al.

2003). AI with frozen-thawed semen unfortunately did not meet with the same success in black-footed ferrets (Howard et al. 2003). Recently, however, semen and sperm have been under intensive investigation in this species with the aim of ultimately providing a successful semen bank for this endangered species (Santymire et al. 2006).

In the farmed European polecat (*M. putorius*), electroejaculation has been used for semen collection, and kits have been born after AI with fresh semen both in Novosibirsk (Russia, two litters, four inseminated females) and in Kuopio (Finland, one litter, two inseminated females) (our unpublished observations). Semen was surgically deposited into the uterine horns using a sharp capillary which we use to transfer farmed European polecat and European mink embryos. Use of the electroejaculation procedure for semen collection in the European mink very often results in ejaculates of poor quality or failure to respond to electroejaculation at all. However, European mink semen did, in one case, prove successful after stimulation by injection of gonadotropin and subsequent electroejaculation, in which a good quality ejaculate was obtained. Use of hormonal stimulation to facilitate semen collection in this species clearly, therefore, warrants further investigation, and could represent a valuable option in overcoming species-specific problems of electroejaculation. When electroejaculation protocols were applied to European and American minks caught from the wild, it was noticed that most of the latter's males produced semen of relatively good quality, whereas fewer European mink males responded positively, and, when they did, semen quality was poor (George Maksudov, personal communication).

Collection, cryopreservation and transfer of embryos

A method for surgical embryo flushing that preserves the reproductive function of the resource females has been developed by Jussi Aalto in Kuopio, Finland and was applied first to farmed European polecat (Lindeberg 2003), and subsequently to European mink (Amstislavsky et al. 2004). This surgical method of embryo flushing resulted in collection of 7.8–9.5 embryos per farmed European polecat resource female (Lindeberg et al. 2003; Piltti et al. 2004) and 5.3 embryos per European mink resource female (Amstislavsky et al. 2004). A non-surgical transcervical embryo recovery method has also been reported (Kidder et al. 1999) in which the transfer equipment consisted of a sophisticated catheter and endoscope with a halogen light source connected to a video camera for visualizing the cervix. The yield of embryos from the use of this procedure was encouraging; 8.76 embryos per donor animal (Kidder et al. 1999).

Administration of gonadotropins results in superovulation and increase in embryo yields in ferrets. Under optimal combinations, the use of equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) has resulted in an average of more than 19 oocytes or embryos per resource female (Li et al. 2001). However, because the effect of exogenous gonadotropins on sperm transport, and on the migration and

development of embryos, in mustelids has not been properly studied so far, most of the studies (such as our own with European mink; Amstislavsky et al. 2004, 2006a) have relied on embryos produced from natural matings.

In mustelids, embryo transfer has mostly been studied in ferrets or farmed European polecats (Kidder et al. 1999; Amstislavsky et al. 2000; Lindeberg et al. 2002; Lindeberg 2003; Li et al. 2006a), and in all of these studies pseudopregnant females were used as recipients. Ovulation was induced by mating recipients by either surgically (Lindeberg et al. 2002; Lindeberg 2003; Li et al. 2006a) or genetically (Amstislavsky et al. 2000) sterile males, although administration of hCG can be used for the same purpose (Kidder et al. 1999). Embryos were successfully transferred either surgically (Amstislavsky et al. 2000; Lindeberg et al. 2002; Lindeberg 2003; Li et al. 2006a) or non-surgically (Kidder et al. 1999) into the uteri of anaesthetized recipient females.

Recently, pre-implantation embryo development has been studied in the European mink, and now it is known that embryos achieve morula stage on Day 5 after mating, migrate from the oviducts into the uterus on Day 6 and cavitate on the same day with implantation finally occurring on Day 12 (Amstislavsky et al. 2006a). As mentioned earlier, it is possible to produce hybrids between European polecat and European mink, hybrids between male European polecat and female European mink being known as 'honoriks', and reverse hybrids 'nohoriks' (Ternovsky and Ternovskaya 1994; Ternovskaya et al. 2006). Both honorik and nohorik females are fertile (Ternovskaya et al. 2006) and may be used as recipients for transfer of European mink embryos (Amstislavsky et al. 2004, 2006a).

The success rates of transfer of fresh embryos in farmed European polecats, ferrets or European mink have usually varied between 40–50% (Amstislavsky et al. 2000, 2004, 2006a; Lindeberg et al. 2002; Lindeberg 2003), although rates as high as 90% (Li et al. 2006a) or as low as 26% (Kidder et al. 1999) have been reported. In the latter case, a less invasive, transcervical non-surgical method was used, indicating that a delicate balance may need to be maintained between pregnancy success and danger to recipient mothers.

Unilateral uterine horn transfer has been reported to be at least as efficient as bilateral embryo transfer in ferrets (Li et al. 2006a), although, implantation does not normally occur if less than three embryos are transferred per recipient female (Amstislavsky et al. 2000, 2006a). However, the transfer of embryos from two or three different resource animals into a single recipient female seems to increase the effectiveness of the procedure (Lindeberg 2003). Kits born after transfer of *in vitro*-generated domestic ferret embryos have been obtained (Li et al. 2001), and, the same group has recently reported as the world's first cloned ferret (Li et al. 2006b).

All the published attempts of cryopreservation of embryos of *Mustelidae* have been performed by our group. We began experiments with cryopreservation of stoat (*M. erminea*) embryos (Amstislavsky et al. 1993), and subsequently concentrated on cryopreservation of embryos of farmed European polecat (Amstislavsky

et al. 2000, 2006b; Lindeberg 2003; Lindeberg et al. 2003). By using a modification of a conventional bovine embryo freezing protocol, and ethylene glycol as a cryoprotectant, it was possible to successfully cryopreserve embryos of farmed European polecat. The cryopreserved embryos developed *in vitro* after thawing (Amstislavsky et al. 2000), and live kits have been born after transfer of conventionally frozen-thawed embryos into pseudopregnant recipients (Lindeberg 2003; Lindeberg et al. 2003). Vitrification and subsequent transfer of polecat embryos after warming also resulted in birth of kits (Lindeberg 2003). These encouraging results have now prompted us to begin experiments on cryopreservation of European mink embryos using the same protocol as proved successful for farmed European polecat embryos, these experiments being currently underway.

Discussion

The wide distribution of feral American mink throughout Eurasia is probably one of the principal hazards for both the survival of remnant populations of indigenous European mink, and for reintroductions. The two species represent example of the competition-exclusion, or 'Gause's' principle which holds that, where two ecologically similar species occupy the same niche, the less adapted or less competitive species will move to reduced abundance or extinction (Gause 1934). The ecological similarity of American and European mink is therefore most probably precipitating a direct conflict for survival when they coexist in any natural biotope. Why American mink always come to predominate over the aboriginal European species is not only an interesting ecological question, but also is highly pertinent to conservation measures for the latter. Recent findings, for instance, on how the success of new invading or artificially introduced species can be explained in part by reduced parasite burdens of the invaders may be relevant here (Clay 2003; Torchin et al. 2003) – it could be that the American species benefits from a reduced parasite load in the environment of a new continent. Moreover, when two species are sufficiently closely related for inter-species infection transfer to occur, there is clearly a danger to one of them. Aside from the above mentioned case of ADV, there is as yet insufficient information about the parasites (viral, bacteria, protozoal or helminth) of American and European mink in Eurasia for any conclusions to be drawn about the role of infections in the decline of the European species. There are, however, a number of other specific issues pertinent to the European mink/American mink case that will be discussed here in addition to potentially important aspects of the reproductive strategy and physiology of European mink, and the prospects for new reproductive technologies relevant to their conservation.

Unlike European mink, (*Meles meles*) American mink have been deliberately selected by fur farmers for larger body size over many generations (Ternovsky and Ternovskaya 1994). The resulting great size disparity alone will result in injury or displacement from territories of European mink following interspecies aggressive encounters (Sidorovich 2006). This may already be having a selective effect on indigenous mink in that European mink that do manage to survive in areas where the two species coexist: they tend to have larger body sizes than average (Sidorovich 2006). Also, large-scale behavioural experiments performed in Tallinn zoo indicate that American minks are markedly more active and socially interactive than European minks (Maran et al. 1998). Such a socialization effect is a known consequence of domestication in canids (Trut 1999; Hare et al. 2005), and captive breeding of mustelids on fur farms for more than 100 years has probably resulted in altered behavioural patterns that positively influence their reproductive success. Captive breeding usually causes loss of reintroduction capacity of a species (McPhee 2004), yet feral American minks demonstrate the opposite trend, possibly because of a selective advantage conferred by body size and behavioural changes acquired during captive breeding.

We consider that the reproductive behaviour and physiology of American mink also contributes strongly to their competitive success, particularly notable in this regard being multiple mating and superfetation, both of which have been described for American mink (Shackelford 1952) and might also occur in the badger (*Meles, meles*) (Yamaguchi et al. 2006). In American mink, which is actively polyandrous in captivity (Thom et al. 2004b), superfetation occurs when a female mates with different males over a period of six or more days, at which time a second ovulation can occur. Delayed implantation, which is characteristic for American mink (Thom et al. 2004a), makes it possible the synchronous development of embryos from two separate mating (Yamaguchi et al. 2006). Another unusual feature is that, in American mink, ovulation is not necessarily associated with copulation and may occur spontaneously under certain conditions (Gustaffson et al. 1987; Dunstone 1993). This constellation of unique reproductive features may significantly promote adaptation of American mink in different habitats.

In contrast to the American mink, reproduction in the European mink is stress-sensitive (Rozhnov and Petrin 2006). It has been reported that, at least in captivity, aggressive behaviour may well predominate over sexual motivation in both males and females (*Meles meles*), even when physical condition indicates readiness to mate (Festl et al. 2006). Whether or not superfetation exists in European mink has not been addressed experimentally so far, but, because this species has no implantation delay, the oestrus period is very short, and once established, pregnancy develops rather rapidly (Moshonkin 1981; Ternovsky and Ternovskaya 1994; Amstislavsky et al. 2006a) and it appears rather unlikely that European mink exhibit superfetation.

The reproductive strategy and behaviour of the European mink therefore appear to place it at a competitive disadvantage to the American species, even before other factors are taken into consideration. Although good records exist only from captivity, for which American mink have been selected, fecundity is measurably higher in American than in European mink (Ternovsky and Ternovskaya 1994; Kharlamova and Trapezov 1999; Klotchkov and Eryuchenkov 2003).

At present, conservation efforts of European mink have concentrated on an 'ex situ' (captive breeding) and an 'in situ' approach (reintroduction into the wild). But, both these approaches are dependent on high level funding (Balmford et al. 1996; Von Schmalz-Peixoto 2003). Thus, the development of genome resource banks in the form of cryopreserved embryos and/or semen (discussed below) is likely to be a key element in a multi-disciplinary European mink conservation, programme complementing the important in situ and ex situ approaches.

The most crucial problem in captive breeding is a loss of genetic diversity because breeding colonies tend to be founded and maintained with small numbers of animals (Frankham 2003, 2005). This is compounded by an increase in homozygosity that allows fixation of dele-terious alleles, and is expressed as a loss of evolutionary potential, susceptibility to diseases, reproductive prob-lems, high rate of infant mortality and low life expect-ancy of captive born individuals (Amos and Balmford 2001; Von Schmalz-Peixoto 2003). It seems, though, that inbreeding depression does not affect certain species of Carnivora as seriously as was once thought (Kali-nowski et al. 1999). Our own observations on a captive population of stoat at Novosibirsk revealed that repro-ductive and general health status of the animals bred there since 1968 has not changed significantly, even though there has been close inbreeding (Amstislavsky and Ternovskaya 2000; and our unpublished observa-tions). Nevertheless, it is generally accepted that appro-priate genetic management is a major issue in captive breeding programmes, and that captive bred popula-tions must be used as a support for ongoing in situ conservation programmes and may replace wild popu-lations in research and education (Jalme 2002; Von Schmalz-Peixoto 2003; Maran 2006). It is also clear that captive bred populations may provide the last resort for species whose wild populations cannot be maintained by other means, until more favourable conditions prevail.

Results of introduction/reintroduction of European mink onto isolated islands were less impressive than expected in both the Russian and Estonian trials, although these ventures provided valuable new know-ledge on the biology of European mink. The Estonian case is most illuminating because of the high level of post-release monitoring that was carried out, in addition to the attempts at pre-release survival training, and the policy of promoting birth in the wild from the outset. The exercise made it clear, however, that the process of re-establishing a wild population from captive stocks of European mink is laborious, and, despite the rigour of pre-release training, post-release fitness in the wild remains problematic (Maran 2006).

A particular problem in the reintroduction of Euro-pean mink is that the majority of suitable sites available are already occupied by American mink. Where there are islands free of American mink, they are usually either out of the historical range of European mink in the first place (as with the Russian Kuril islands) or there exist potentially vulnerable species that European mink may predate and endanger, such as crayfish on Saaremaa island in Estonia. In both the Russian and Estonian cases, there were a number of legal and/or conservational concerns both prior to or following the action, and public and scientific opposition pointed to the possibility that European mink may change the ecology of the island to the detriment of existing species biodiversity (Ternovsky and Ternovskaya 1994; Shvarts and Vaisfeld 1995; Maran 2006; Ternovskaya et al. 2006). On the other hand, where the territory or island is already occupied by American mink, the removal of an existing population of American mink and the intro-duction of the European species may be a net benefit, although the cost and technical and ethical problems of elimination are not trivial (Macdonald and Harrington 2003).

A general problem associated with transforming a captive bred into a reproductively successful wild population is adaptation to captivity (Von Schmalz-Peixoto 2003; McPhee 2004; Frankham 2005). The most recent review of 116 reintroduction programmes suggests that only 25% may be classified as successful over time (Fisher and Lindenmayer 2000). Breeding in captivity has been shown to deleteriously affect many behavioural and morphological traits (Price 1984; Lickliter and Ness 1990; McPhee 2004), and inconsis-tency in survival trait such as anti-predator behaviours increased with generations in captivity. These effects clearly militate against successful reintroductions (Frankham 2005). For example, reintroductions of red wolves (Canis rufus) and African wild dogs (Lycaon pictus) demonstrated that individuals born in captivity are unable to capture their own prey adequately, or avoid their natural predators, or escape accidental death by road vehicles and other causes (Moore and Smith 1990; Frantzen et al. 2001).

To alleviate the deleterious effects of adaptation to captivity, Frankham (2005) recommended the minimi-zation of generations in captivity and avoidance of any selection, conscious or unconscious, before ultimate release into the wild, and it was suggested that cryopre-servation of gametes and embryos can contribute to this end. Embryo and semen banks may also decrease the cost of captive breeding and reintroduction pro-grammes. For example, the maintenance of genetic diversity in the form of cryopreserved embryos will provide more flexibility in breeding management – females that are of limited value because of inbreeding could contribute to the expansion of valuable stocks as recipients of embryos from more valuable animals.

Moreover, cryopreservation of embryos and semen (Holt and Pickard 1999; Leibo and Songsasen 2002), in addition to cloning (Holt et al. 2004) and other new reproductive technologies (Pukazhenthi and Wildt 2004), are clearly important considerations in the conservation of wild and endangered species. Such approaches, however, require a thorough and detailed study of the reproductive physiology and behaviour of any target species (Wildt et al. 1992; Loskutoff 2003), especially if to take into account, that distinct differences are often evident between even closely related species.

The successful application of embryo cryobanking to European mink is encouraging. Pre-implantation em-bryo development has been studied in European mink and suitable methods for embryo collection and transfer have recently been published (Amstislavsky et al. 2004,

2006a). This, together with the use of polecats as a species for method testing, led to the development of an embryo freezing protocol applicable to mustelids (Lindeberg 2003; Lindeberg et al. 2003; Amstislavsky et al. 2006b), thereby opening the possibility of embryo cryobanking and embryo transfer towards the preservation of the genetic diversity of European mink. The use of less invasive techniques is of special concern given that European mink is an endangered species and the reproductive capacity of resource females has not to be compromised. A non-surgical transcervical embryo recovery method has already been reported in the domestic ferret (Kidder et al. 1999), although the expensive videoendoscope equipment required may be unrealistic under farm conditions. A surgical method recently developed by Jussi Aalto to flush mustelid embryos applied recently to European mink (Amstislavsky et al. 2004) is another option that preserves the reproductive capacity of resource females. However, despite all this significant progress, future studies may yet provide more advanced approaches that will be both efficient and practical and will present minimal risks to the resource females. The use of honoriks∕nohoriks as recipients for transfer of European mink embryos (Amstislavsky et al. 2004, 2006a), for instance, offers an advance that circumvents the problems associated with interspecies embryo transfer discussed elsewhere (Pukazhenthi and Wildt 2004).

Methods of semen collection and cryopreservation, and suitable protocols for artificial insemination, have been developed with the use of ferrets∕polecats as a model species (Howard et al. 1991, 2003, 2006; Wildt et al. 1992; Kidder et al. 1998). Artificial insemination was used extensively in the black-footed ferret recovery plan during the period 1991–2005, when 128 black-footed ferret kits were produced by AI by using either fresh or frozen semen (Howard et al. 2006). Using similar or adapted methods, this success can hopefully be achieved in future for European mink. The main obstacle at this stage, however, is the failure in European mink of electroejaculation methods that work well for polecats∕ferrets and American mink (our unpublished observations; George Maksudov, personal communication). Choosing of electroejaculation for semen collection is not, however, without its limitations, as discussed elsewhere (Pukazhenthi and Wildt 2004). Future studies are clearly required to overcome the problems of poor quality∕lack of electroejaculate in European mink so that semen cryobanking can properly play a part in conservation programmes for European mink.

The success of the rescue programme for the endangered American black-footed ferret (*Mustela nigripes*) supports the idea that the quality, not the quantity, and experience of the released animals is a key point in re-establishing wild populations (Biggins et al. 1998; Biggins and Godbey 2003). The probabilities for both short-term and long-term survival were three- to fourfold higher in animals reared in the quasi-natural conditions of large open pens as compared with animals reared in standard cages. Moreover, follow-up studies 1–2 years post-release have shown that the progeny with ancestors born in pens thrived, whereas the progeny of cage-reared animals virtually disappeared because of poor survival and reproduction rates (Biggins and Godbey 2003). Proper management of population, recapture of released animals to breed within special pens partly alleviated, but did not entirely overcome, the problem of captivity adaptation, as has been demonstrated by red wolf reintroduction programme in USA (Moore and Smith 1990) and by European mink reintroduction in Estonia (Maran 2006, 2007).

Experimental studies on laboratory and farmed animals have demonstrated that the weaning period is crucial to the development of adequate responses to environmental challenges, and that this depends on the condition of the suckling mother (Braastad 1998; Francis et al. 1999). These observations, in addition to follow-up studies on released black-footed ferrets (Biggins et al. 1998; Biggins and Godbey 2003), both suggest that an optimal reintroduction strategy would involve the rearing of European mink in quasi-natural conditions before their release. This strategy, however, significantly increases the costs of captive breeding. Collection and cryobanking of embryos, sampling from the full geographical range of the species, have considerable potential in retaining the genetic diversity of European mink until an appropriate level of captive breeding quality is achieved. Another lesson from the black-footed ferret programme was that extensive research of species-specific reproductive biology, and careful integration of reproductive technologies into the programme, greatly enhances the chances of success (Wildt et al. 1992; Wolf et al. 2000; Howard et al. 2003, 2006).

To conclude, we see the success of European mink conservation programme through four complementary priorities, namely the study of species-specific reproductive and behavioural patterns, captive breeding, reintroduction and, lastly, the exploitation of new reproductive technologies as cost-effective tools for preservation of genetic diversity and therefore adaptability to a changing ecological environment.

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