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SERGEI AMSTISLAVSKY

Reproductive Biology and Embryo Technology in *Mustelidae*

Doctoral dissertation

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ABSTRACT

Mustelidae is the largest family in the order *Carnivora*, with 59 extant species and more than 400 subspecies. The present research project represents the first attempt to develop embryo technologies appropriate for use in the conservation of European mink. To study mustelid early embryonic development *in vivo*, a total of 100 embryos were flushed from 26- to 92-day-old female stoats (*Mustela erminea*), and a further 150 embryos were flushed from European mink. Embryos were either transferred or fixed for microscopic study; in parallel morphological changes in the *corpora lutea* in ovaries and the progesterone profile in faeces were monitored during early pregnancy of European mink.

Newborn stoat females entered oestrus during the first month of life and stayed in heat for up to several months. When mated, these females ovulated 3 - 4 days later. Embryos arrived in the uterus 11 - 12 days *post coitum* (dpc), slowly expanded and persisted as diapausing blastocysts until implantation 8 - 9 months later. European mink proved to be a seasonally polyoestrous species with no diapause. Embryos migrated into the uterine horns 6 dpc at the morula stage, and, in most, cavitation began within the first day of arrival. Blastocysts grew rapidly until implantation on day 12 pc. Prominent *corpora lutea* were observed in the ovaries throughout the preimplantation period and concentrations of progesterone reached their maximum around the day of implantation.

The transfer of 7 - 11 dpc European mink blastocysts to pseudopregnant honorik/nohorik females (interspecies hybrids between European polecat and European mink) resulted in term kits. This approach was successful since a reasonable survival rate (= live kits/transferred embryos) of 50 % was achieved on a repeatable basis. Although in the first trial only 56.3 % of term kits survived, the rate of postnatal survival in the second trial was higher, reaching 70 %. The results of these experiments with European mink and related species in the genus *Mustela* provide basic reproductive knowledge for incorporating embryo technology into the framework of conservation programmes for the European mink.

Universal Decimal Classification: 591.158, 591.16, 599.742.4, 636.082, 636.934.5
CAB Thesaurus: *Mustelidae*; *Mustela lutreola*; *Mustela erminea*; polecats; reproduction; embryos; embryo transfer; embryonic development; flushing; pseudopregnancy; embryo implantation; preimplantation period; oestrus; oestrous cycle; mating; ovulation; corpus luteum; progesterone; blastocyst; hybrids; kit production; microscopy; endangered species; wildlife conservation

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This thesis is a summary of my *Mustelidae* project which started in the early 1990s and ran along with many of my other projects in Russia and in Germany until recently. Initiated in Siberia, it continued through collaboration with the University of Kuopio, here in Finland, where I found excellent opportunities for work, learning, updating methods and…being a student again. This *Mustelidae* project has eventually become the closest to my heart.

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thorough and comprehensive review and our subsequent communication via email, but also for your own classic papers on embryo development of horse and cattle. These papers were my first “school” when I turned to embryotechnology in the early 1990s, and together with your more recent articles reviewing the milestones of embryotechnology are the “golden standard” for doing best quality science and writing brilliant scientific papers. I am very grateful to Professor Cecilia Luvoni for agreeing to be the opponent in the public defence of my thesis.

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keeping me updated with the latest and most interesting scientific achievements. Thank you!

Stoat was chronologically the first animal species studied in this Ph.D. project. It was a piece of real luck to start with such an amazing animal species like stoat; it revealed curious puzzles of animal reproduction. The first people who encouraged me and welcomed me at the starting point of the study, and whom I owe the very possibility to initiate my work with mustelids, were Dmitry and Julia Ternosvky. It was a pleasure to work together with such great scientists dedicated to their research, thank you both for sharing with me some of your knowledge about mustelids and for your high spirits! Valery Cindrenko, an outstanding photographer and wonderful person earned my deepest thanks for his help in documenting our results. Valery, thank you a lot for your excellent pictures! At that time I worked in yet another inspiring lab, led by Professor Arkady Markel. Arkady Lvovich, you have always been for me not only admirable scientist, but also a kind person and friend. I am proud to count myself a member of the Scientific School of Professor Markel.

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Soon after the stoat article was published, I started cooperation with the group of Professor Maija Valtonen, working with polecats and ferrets, aiming to develop a suitable package of reproductive technologies applicable for the conservation of European mink. This project "Ex situ conservation of endangered species by cryopreservation of gametes and embryos" sponsored by the Finnish Biodiversity Research Programme (FIBRE) developed rapidly and soon we got some necessary experience and technologies. During the early 2000s our work was funded mainly by the Finnish Academy of Sciences, enabling us to exchange visits and to start work with European mink in Siberia. The finalizing steps of my work on this thesis and writing the dissertation itself were financially supported by grants from the Finnish Cultural Foundation (FCF) and the Centre of International Mobility (CIMO). My thanks go to these Finnish Foundations, as their financial support made it possible to accomplish the thesis. I am also grateful to the Russian Foundation for Basic Research, for a grant Nr. 08-04-00147a, which makes it possible to continue our work on *Mustelidae* Research Station in Novosibirsk since 2008.

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counted among my good friends. Thank you all for creating an enjoyable working atmosphere. My deepest thanks go to the University of Kuopio, which provided excellent possibilities for improving methods, for doing some important parts of my *Mustelidae* project, for updating my knowledge through attending some courses, and finally for the possibility to write and publically defend this thesis. I wish to express my sincere thanks to Vivian Paganuzzi of the language centre of this university for the superb linguistic revising of my book.

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Kuopio, 30th August, 2009
Sergei Amstislavsky

ABBREVIATIONS

AI	artificial insemination
ART	assisted reproductive technology
B	blastocyst
CL	*corpora lutea*
DB	diapausing blastocyst
DI	delayed implantation
DIC	differential interference contrast
dpc	days *post coitum*
EB	early blastocyst
eCG	equine chorionic gonadotrophin
EG	early gastrula
ET	embryo transfer
ExB	expanded blastocyst
GPI	glucose-6-phosphate-isomerase
GRB	genome resource banking
GV	germinal vesicle
hCG	human chorionic gonadotrophin
hpc	hours *post coitum*
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
i.m.	intramuscular
IMP	implantation
i.v.	intravenous
IVF	in vitro fertilization
IVM	in vitro maturation
IU	international unit
IUCN	International Union of Conservation of Nature and Natural Resources
LH	luteinizing hormone
LN_2	liquid nitrogen
M	morula
M-II	metaphase two
ov	oviduct
PBS	phosphate-buffered saline
pc	*post coitum*
PF	preovulatory follicles
®	registered trademark
S.E.M.	standard error of mean
SSC	saline sodium citrate
s.c.	subcutaneous
™	trademark
ut	uterus
VEGF	vascular endothelial growth factor
zp	*zona pellucida*

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles which are referred to in the text by their Roman numerals I - IV:

I. Amstislavsky S, Maksimovsky L, Ternovskaya Yu, Ternovsky D. Ermine reproduction and embryo development (*Mustela erminea*). Scientifur 1993;17:293-298.

II. Amstislavsky S, Aalto J, Järvinen M, Lindeberg H, Valtonen M, Zudova G, Ternovskaya Yu. Transfer of European mink (*Mustela lutreola*) embryos into hybrid recipients. Theriogenology 2004;62:458-467.

III. Amstislavsky S, Kizilova E, Ternovskaya Y, Zudova G, Lindeberg H, Aalto J, Valtonen M. Embryo development and embryo transfer in the European mink (*Mustela lutreola*), an endangered mustelid species. Reprod Fertil Dev 2006;18:459-467.

IV. Amstislavsky S, Lindeberg H, Ternovskaya Yu, Zavjalov E, Zudova G, Klotschkov D, Gerlinskaya L. Reproduction in the European mink, *Mustela lutreola*: oestrus cyclicity and early pregnancy. Reprod Domest Anim 2009;44:489-498.

This thesis also contains previously unpublished data. Unpublished data from the experiments presented in III and IV are based on the materials and methods of the original publication and marked with superscript ^{u} (III^{u}, IV^{u}).

CONTENTS

1. INTRODUCTION

The subject of this study may be described as being on the border of two relatively recent emerging disciplines: reproductive biology and conservation biology. Species extinction rates are increasing, especially in mammals (Novacek and Cleland 2001) and conservation biologists all over the world are concerned. There are currently several approaches for conservation planning. One strategy is to conserve whole landscapes (Margules and Pressey 2000). Reserves alone are not adequate for nature conservation but they may sample and/or represent the biodiversity of each region and separate this biodiversity from processes threatening its persistence (Margules and Pressey 2000). There are many sub strategies and exciting findings on this path, such as the recently published data on wolves indicating that conserving top predators in ecosystems may be very important, since top predators can play unexpected but crucial roles in maintaining ecosystem integrity (Chapron et al. 2008).

All these strategies can be defined as *in situ* programs. They are very important, but are not always applicable for two main reasons. The conservation of the whole ecosystem is very expensive and needs a lot of political and organizational efforts (Lasley et al. 1994), but still might be useless in some cases, especially if any mammalian species is threatened by an invasive alien species all over its historical range (Macdonald and Harrington 2003, Macdonald et al. 2006, Salo et al. 2007). Eradication programs are very expensive and raise ethical concerns.

In cases where it is extremely difficult to conserve a mammalian species *in situ*, an *ex situ* approach may help (Wildt et al. 1992, Leibo and Songsasen 2002, Pukazhenthi and Wildt 2004, Andrabi and Maxwell 2007, Paris et al. 2007). So far, the most exploited modification of an *ex situ* approach in the case of European mink is breeding in captivity. However, the paradox of captive breeding is that the longer a population is maintained in captivity, the lower are the chances for successful transformation of this captive bred population into a self-maintained reproducing population in the wild (Frankham 2005). One common 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). A review of 116 re-introduction programs has suggested 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 important for survival in the wild, such as anti-predator behaviours, and these adverse effects increase with generations in captivity (Price 1984, Lickliter and Ness 1990, McPhee 2004).

Recently, the concept of a Gene Resource Bank (GRB) has been applied, although with limited success, for the purposes of conservation of nondomestic and wildlife species (Wildt et al. 1992, Leibo and Songsasen 2002, Loskutoff 2003, Pukazhenthi and Wildt 2004, Pukazhenthi et al. 2006, Andrabi and Maxwell 2007, Paris et al. 2007). Assisted reproductive technology/embryo technology/GRB are not substitutes for traditional methods in the *ex situ* and *in situ* conservation of any endangered species, but the careful incorporation of these modern techniques strengthens traditional conservation tools and provides an interdisciplinary approach based on the comprehensive study of species-specific reproductive biology (Wildt et al. 1992).

The present research project was the first attempt to address such key reproductive aspects of European mink (*Mustela lutreola*) as early pregnancy and preimplantation embryo development, with the use another mustelid from the same genera *Mustela*, stoat (*Mustela erminea*), as reference species. The final goal of this project was to develop specific range of artificial reproductive technologies for mustelids useful for conservation an endangered European mink in particular.

2. REVIEW OF LITERATURE

2.1 *Mustelidae*: general overview of the family

Mustelidae is the family of *Carnivora* that contains the largest number of species in this mammalian order (Bininda-Edmonds et al. 1999, Koepfli et al. 2008). The early classification of mustelids was based mainly on morphology and the descriptive analysis of external characters (Pocock 1921), a combination of morphological characters and "similarity in adaptiveness" (Simpson 1945), or a complex approach based on morphological analysis, verified by karyotyping and ecological studies (Ternovsky and Ternovskaya 1994). As a result, there was a great controversy over the number of subfamilies, genera and species in the *Mustelidae* family (Pocock 1921, Simpson 1945, Ternovsky and Ternovskaya 1994). The current approach is based on molecular methods which allow the analysis of mitochondrial DNA and/or cytochrome b sequences (Davison et al. 2000, Sato et al. 2003). However, these studies are limited to a small number of species which happened for some reason to interest the authors, so while these studies resolve some problems, they do not provide a classification. A significant milestone was the building of a phylogenetic supertree for the order *Carnivora* using parsimony analysis and the available molecular/life history data from different sources (Bininda-Edmonds et al. 1999). The classification of mustelids was significantly clarified with the analysis of about 12,000 base pairs of mitochondrial and nuclear DNA obtained from 22 gene segments (Koepfli et al. 2008). According to this classification, the *Mustelidae* family comprises 8 subfamilies; there are 59 extant mustelid species belonging to 22 genera (Koepfli et al. 2008). However, there are more than four hundreds subspecies of mustelids (Schreiber et al. 1989).

Mustelids are important for humans; some of them are used as pelt producers, some as pets and pest killers, and some for hunting. Besides, most mustelids have a great aesthetic value for man. The colour, shades and texture of their fur and the grace and beauty of their movements have a strong positive effect on human emotions. Mustelids were domesticated later than canids and felids: the dog (*Canis familiaris*) was domesticated 10 - 15,000 years ago (Savolainen et al. 2002), and the cat (*Felis catus*) at least 9,500 years ago, according to archaeological records (Vigne et al.

2004). However, the domestic ferret (*M. putorius furo*) has been domesticated for only about 2,000 years (Thomson 1959).

Ferrets are mentioned by Aristotle in his *Historia Animalium,* which was written about 320 - 350 B.C., and about one century earlier Aristophanes mentioned ferrets in one of his comedies (Thomson 1951, McKay 1995). There is actually some uncertainty over whether Aristotle and Aristophanes are referring to ferrets or polecats, but anyway these sources do not explain why the ancient Greeks domesticated the animal (Thomson 1951). About four centuries after Aristotle, both the Greek historian and geographer Strabo, in his book Geographica, and Pliny the Elder mention a "ferret", which was used for hunting rabbits (Thomson 1951, McKay 1995), although it is uncertain whether that "ferret" was the same as the animal known as a ferret nowadays (Thomson 1951). Whether or not it was an actual ferret or some other mustelid, it was beneficial for humans to have this smart animal domesticated, and not only for hunting purposes: it has also inspired works of art. For example, the animal in Leonardo's masterpiece the "Lady with an Ermine" is in fact not an ermine (stoat), but a domestic ferret (Rzepinska 1978). However, the presence of "Ermine" in the name of Leonardo's masterpiece is more symbolic, since stoats were associated with the aristocracy and ermine became an emblem of purity (Rzepinska 1978). In Europe, stoat furs were a symbol of royalty; the ceremonial robes of members of the UK House of Lords are still trimmed with ermine (http://en.wikipedia.org/wiki/Stoat).

The American mink (*Mustela vison*) was adapted to ranch rearing at the end of the nineteenth century in North America (Joergensen 1985), and gradually this species became the most highly valued of the mustelids as a pelt producer. The fur industry, particularly the breeding of farmed mink, developed successfully during the twentieth century, especially in the USSR, Nordic countries such as Finland and Denmark, and the USA (Joergensen 1985). Although the Russian Federation, Finland and the USA were the leaders in producing mink pelts in the 1970s (Schreiber et al. 1989), these countries have not expanded the fur industry since then; each of these countries still produces up to 3 million pelts annually, whereas in 2006 Denmark produced 13 million pelts and China 10 million. China is rapidly becoming the leader in the fur industry and as a pelt producing country. According to the International Fur Trade Federation (IFTF), global fur sales in 2005 amounted to $12.8 billion

(http://en.epochtimes.com/news/7-2-20/51905.html). In some countries, such as Russia, mink, sable and marten pelts harvested from the wild are among the most valuable products in wintertime. However, sable (*Martes zibellina*) was also introduced into farming, although later than American mink: the first sable farm was established in Russia in 1928. Captive breeding of sable developed successfully after the publication of the classic work of Manteifel (1934) led to a better understanding of reproduction in this species (Pavljuchenko et al. 1979). Before the collapse of the Soviet Union, sable farming was almost exclusively restricted to that country, although nowadays some sable farming is done elsewhere, e.g. in Finland and Denmark (http://www.agronews.ru/newsshow.php?NId=24058&Page=1077). Polecats or ferrets are sometimes used for pelt farming, e.g. in Australia, Finland, Russia and some other countries (McKay 1995), but the domestic ferret (*Mustela putorius furo*) is mostly kept as a pet or used as a laboratory animal (McKay 1995, Fox and Bell 1998).

The reproduction of ferrets has been thoroughly investigated for about a century (reviewed recently by Lindeberg 2008). Moreover, the ferret has become a popular model in biological and medicine-oriented studies (Donovan and ter Haar 1977, Li and Engelhardt 2003), because some physiological systems of the ferret resemble those of humans (Fox and Bell 1998). The reproductive biology of two other farmed species of *Mustelidae*, American mink (Hansson 1947, Enders 1952) and sable (Manteifel 1934, Ternovsky and Ternovskaya 1994) have also been thoroughly investigated and used to study delayed implantation (Baevsky 1955, 1970, Moreau et al. 1995, Desmarais et al. 2004, Lopes et al. 2006, Marks et al. 2006). The *Mustelidae* family contains the greatest number of species (among eutherian mammals) that exhibit delayed implantation (Renfree and Shaw 2000), and this has stimulated studies of the evolutionary origin of delayed implantation in mustelids (Lindenfors et al. 2003, Thom et al. 2004, Ferguson et al. 2006).

In addition to the domestic ferret, some other mustelids have also been domesticated. For example, there is a very tame population of farmed (American) mink in the Institute of Cytology and Genetics, Novosibirsk, selected by Oleg Trapezov (Trapezov 1997). There is also evidence that the indigenous people of South America domesticated the Tayra (*Eira barbara*), and this interesting mustelid is still kept as a

household pet in some Latin American countries, helping to protect houses against vermin such as rodents (Schreiber et al. 1989). The steppe polecat (*Mustela eversmanni*) and wolverine (*Gulo gulo*) have also been considered to be possible species for domestication (Ternovsky 1977). Of more than a dozen species once present on the Research Station in Novosibirsk the most resistant to domestication was the stoat (Ternovsky 1977, Ternovsky and Ternovskaya 1994).

Besides the three most commonly farmed mustelid species (the ferret, American mink and sable), some other members of the *Mustelidae* family have been shown to reproduce successfully in captivity on farms. McDonald and Larivière (2002) listed in their review the guidelines for the captive breeding of American martens (*Martes americana*), black-footed ferrets (*Mustela nigripes*), long-tailed and common weasels (*Mustela frenata* and *Mustela nivalis*) and American river otters (*Lontra canadensis*). There is also valuable information on the captive breeding of stoats (Ternovsky and Ternovskaya 1994, McDonald and Larivière 2002), although this species is far from easy to breed in captivity, as was shown in New Zealand (O'Connor et al. 2006), mainly because of its reproductive specificity and resistance to domestication. European mink, an endangered species, has been shown to reproduce successfully in captivity (Ternovsky and Ternovskaya 1994, Maran and Robinson 1996), and guidelines for their management are available (Maran and Robinson 1996). Eleven mustelid species have been bred in captivity on the *Mustelidae* Research Station in Novosibirsk (Ternovsky and Ternovskaya 1994, Amstislavsky and Ternovskaya 2000, Ternovskaya et al. 2006). Some of these species, like European pine marten (*Martes martes*), Siberian weasel (*Mustela sibirica*) and mountain weasel (*Mustela altaica*) have been bred for a number of generations on this farm, and knowledge of the reproduction of these species is available, mostly in Russian (Ternovsky and Ternovskaya 1994) as these species are very rare if ever have been bred in captivity throughout the world.

The distribution of mustelids is indeed global. They are endemic throughout the world, with the exception of Antarctica, Australia, Madagascar, New Guinea, most of the Philippines, Sulawesi, the West Indies, most Pacific Islands and New Zealand (McKay 1995), although they have been introduced in some of these places, e.g. into Australia and New Zealand (McKay 1995, Parkes and Murphy 2004). Some

mustelids, such as the least weasel (*Mustela nivalis*), are able to successfully reproduce as far north as the Taimyr Peninsula on the coast of the Arctic Ocean (Broekhuizen et al. 2007). At the other extreme, the African striped weasel (*Poecilogale albinusha*) inhabits sub-Saharan and a part of Equatorial Africa (Larivière 2001). Some species such as the least weasel (*Mustela nivalis*), stoat (*Mustela erminea*), wolverine (*Gulo gulo*) and sea otter (*Enhydra lutris*) are native Eurasian and North American fauna; the honey badger (*Mellivora capensis*) is a native of both Africa and Eurasia; the long-tailed weasel (*Mustela frenata*) lives in both North and South America (Koepfli et al. 2008).

There are a number of Eurasian, African, South American and North American species (Koepfli et al. 2008), and the historical range of some species such as the Indonesian mountain weasel (*Mustela lutreolina*) is restricted to only a few isolated islands (Schreiber et al. 1989). The origin of the great majority of mustelid species is Eurasia (Koepfli et al. 2008). For example, the modern mustelid fauna of Africa contains eight species and it has been confirmed that at least five of them are derived from Eurasia (Koepfli et al. 2008). Similarly, there is evidence indicating that mustelids colonized South America from North America (Hunt 1996, Koepfli et al. 2008), and that Eurasia was the origin of most American mustelids (Koepfli et al. 2008). Nowadays, Eurasia contains the majority of extant *Mustelidae* species, with 34 of the 59 known mustelid species being either exclusively endemic to, or having part of their distribution in, this continent (Schreiber et al. 1989, Koepfli et al. 2008).

Some mustelid species have expanded their historical range due to farm breeding, feralization and introduction as biocontrol agents. The domestic ferret as well as the stoat and weasel were introduced to New Zealand in an attempt to control pests, especially rabbits (McKay 1995, Parkes and Murphy 2004). These introduced mustelid species now create ecological problems in New Zealand (McKay 1995, King et al. 2001, King and Powell 2007) and, to avoid the total collapse of local bird fauna, control methods have been tested in a move to develop effective tools against the devastating effects of the introduced mustelids (Parkes and Murphy 2004, LaFalci and Molinia 2007). The most well-known mustelid species that expanded widely throughout the world, far beyond of its native range, is the American mink (*Mustela vison*) (Macdonald and Harrington 2003, Macdonald et al. 2006). Originally found

only in North America, farm breeding, feralization and sometimes even deliberate introduction, have spread this species throughout the whole American continent as far south as Patagonia, and throughout the European continent as far north as the British Isles and Iceland (Macdonald and Harrington 2003). American mink also spread eastward throughout Russia and other countries of the former Soviet Union (Ternovsky and Ternovskaya 1994). Macdonald and Harrington (2003) review the damage that this has caused to native fauna – to eider ducks in Iceland, terns in Scotland, water voles in Britain, and rodent fauna in Patagonia. This review and some other sources (Ternovsky and Ternovskaya 1994) stress the negative effects of this intruder on local populations of the native European mink.

European mink and the black-footed ferret are the most well-known endangered species among mustelids and some conservation efforts have been applied to these two species (see Schreiber et al. 1989, Beer et al. 2005 and Amstislavsky et al. 2008 for review). Schreiber et al. (1989) listed 17 threatened and endangered mustelid species in need of conservation efforts. Table 1 presents these endangered *Mustelidae* species/subspecies and indicates their current conservation status. Since the Big-Thicket hog-nosed skunk (*Conepatus mesoleucus telmalestes*) is considered to be already extinct in the IUCN 2007 red list, this species was not included in the table.

The first two species in this list, the European mink and black-footed ferret, are listed as highly endangered in the Encyclopaedia of Endangered Animals (Beer et al. 2005), and are being focused on by zoologists and conservation biologists. The recovery project of the black-footed ferret in North America is illustrative since it is so far one of the few to have resulted in a recovered, self-sustained, mammalian population (Grenier et al. 2007). It used a multidisciplinary approach, which involved comprehensive study of the reproductive biology of this species along with ART technologies (Wildt et al. 1992, Wolf et al. 2000, Howard et al. 2003, Santymire et al. 2006).

Table 1. *Mustelidae* endangered species/subspecies.

Name (Latin name)	Distribution	Status in the IUCN Red List
European mink (*Mustela lutreola*)	Europe, extinct throughout most of its range	"Endangered" , legally protected in some European countries
Black-footed ferret (*Mustela nigripes*)	North America, on the brink of extinction in the 1970-80's	"Extinct", successfully re-introduced into the wild in USA
European marbled polecat (*Vormela peregusna peregusna*)	Ukraine, Caucasus and Southern Europe	"Vulnerable", significantly reduced throughout its range
Tsushima marten (*Marten melampus tsuensis*)	Tsushima island (Japan)	"Vulnerable", legally protected on Tsushima island
Wolverine (*Gulo gulo)*	Palearctic and Nearctic Realms	"Vulnerable", declined in many parts of its range
Indonesian mountain weasel (*Mustela lutreolina*)	Tropical forests of Sumatra and Java at altitudes from 1000 m and higher	"Endangered"
Back-striped weasel (*Mustela strigidorsa*)	Nepal, Burma, Thailand, Laos China, Vietnam at altitudes from 1000 m and higher	"Vulnerable"
Formosan yellow throated marten (*Martes flavigula chrysospila)*	Mountainous districts of Taiwan	Legally protected on Taiwan
Javan yellow throated marten (*Martes flavigula robinsoni)*	Java (Indonesia)	"Endangered"
Nilgiri marten (*Martes gwatkinsi*)	Southern India	"Vulnerable", legally protected in India
Javan ferret badger (*Melogale orientalis)*	Java (Indonesia)	"Near threatened"
Kinabalu ferret badger (*Melogale everetti*)	Forests of mountain Kinabalu (island of Borneo)	"Vulnerable"
Colombian weasel (*Mustela felipei*)	Mountains of Colombia and Equador	"Endangered"
Tropical weasel (*Mustela africana*)	Forests of Northern Brazil, Colombia, Equador and Peru	"Data deficient"
Grey headed tayra (*Eira Barbara senex*)	Tropical forests of Mexico, Central America: Guatemala, Belize, Northern Honduras	"Vulnerable"
Pigmy spotted skunk (*Spilogale pygmea*)	coast of Mexican Pacific	"Lower risk"

Sources: Schreiber et al. 1989, IUCN (International Union of Conservation of Nature and Natural Resources): http://www.iucnredlist.org/; Koepfli et al. 2008

The possibility of captive breeding of the European mink has been comprehensively studied in Russia and elsewhere in Europe (Ternovsky and Ternovskaya 1994, Maran and Robinson 1996, Festl et al. 2006). Introduction/re-introduction programmes have been applied in the past (Ternovsky and Ternovskaya 1994, Maran 2006, 2007), but either they were only partly successful so far and are currently underway to improve success rates (Maran 2006) or there is lack of knowledge of the current status due to the lack of recent follow-up studies (Shvarts and Vaisfeld 1995, Ternovskaya et al. 2006). The latest attempt has been started only recently and it is currently underway (http://www.nabu-saar.de/lv/images/stories/nis/nis_073.pdf), thus it is too early to conclude whether or not it has achieved its goals.

2.2 European mink and stoats: why are these two *Mustelidae* species so important?

2.2.1 European mink – an endangered species

The European mink (*Mustela lutreola*) is a small mammal belonging to the *Mustelidae* family. Although it shares the name with the American mink (*Mustela vison*), it is systematically and phylogenetically much closer to polecats/ferrets (Ternovsky and Ternovskaya 1994, Bininda-Edmonds et al. 1999, Sato et al. 2003, Koepfli et al. 2008).

Karyotype analysis reveals a close relationship between the European mink and European polecat (*Mustela putorius*). In contrast, karyotypes of American mink and European mink indicate a much more distant relationship; the number of chromosomes in the European mink (38) is characteristic of European *Mustelidae* species (38 to 44), whereas that of the American mink (30) is well below this range (Volobuev and Ternovsky 1974, Graphodatsky et al. 1976).

Recent molecular phylogenetic analysis has confirmed that European mink are most closely related to the polecat species. Davison et al. (2000) analysed mitochondrial DNA in European mink, polecats and polecat/mink hybrids captured in the wild, and found a close molecular relationship between European mink and polecats, possibly resulting either from reticulate evolution (hybridization) or the relatively recent

speciation of European mink. Sato et al. (2003) confirmed these conclusions, and also confirmed the monophyly of the genus *Mustela*, which includes European mink (*M. lutreola*), the subgenus *Putorius* (*M. putorius, M. putorius furo, and M. eversmanni*), kolonokus (*M. sibirica*), itatsi (*M. itatsi*), stoat (*M. erminea*), least weasel (*M. nivalis*) and solongoi (*M. altaica*). The American mink (*M. vison*) was considered an outgroup of this clade (Sato et al. 2003). The most recent study based on an analysis of about 12,000 base pairs of mitochondrial and nuclear DNA data obtained from 22 gene segments confirmed that the closest relatives of European mink are polecats/ferrets, i.e. *M. sibirica, M. putorius, M. eversmanni, M. nigripes* (Koelpfi et al. 2008).

Earlier, the range of distribution of European mink encompassed much of the European continent, including the southern and central parts of Finland, France and adjacent provinces of north-western Spain, Germany, Hungary, the countries of the former Yugoslavia, northern Romania and Bulgaria and the European part of Russia (Youngman 1982, Schreiber et al. 1989, Ternovsky and Ternovskaya 1994, Maran 2007). During the 20th century, the numbers of European mink declined and this species is now considered to be on the brink of extinction (Schreiber et al. 1989, Ternovsky and Ternovskaya 1994, Sidorovich 1997, Macdonald and Harrington 2003, Beer et al. 2005, Maran 2007). The decline was first noted by Dmitry 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) it was already considered to be one of the most endangered mustelid species. The range of distribution of European mink has been reduced to three fragmented populations. The north-eastern (NE) population occupies the territory around the Russian city of Tver and extends to some other areas in Russia and Belarus (Sidorovich 2000). The south-eastern (SE) population occupies the Danube river delta in Romania (Kranz et al. 2006). The western (W) population is known to exist in northern Spain and south-western France (Palazon et al. 2006).

Michaux et al. (2004, 2005) studied the genetic background of the three extant populations of the European mink and discussed possible strategies for the conservation of the 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 was much more

diverse and was characterized by ten different mtDNA haplotypes. The SE population was considered to be intermediate between the W and NE in its haplotype diversity. Additionally, European mink were genotyped using six microsatellite markers. The lack of genetic heterogeneity in the W population led the authors to conclude that this population probably derives from a few animals that colonized western France and Spain relatively recently, possibly as a result of human introduction.

Often, the primary factors contributing to the extinction of a mammalian species are habitat destruction, over-exploitation or over-hunting, pollution and the adverse impact of introduced alien species (Macdonald et al. 2006). The history of European mink exemplifies this well. The main hypotheses that are discussed in relation to the extinction or decline of European mink in Europe include the adverse impact of the American mink, possible hybridization with the European polecat, introduced diseases, pollution, over-hunting and habitat loss (Ternovsky and Ternovskaya 1994, Maran and Henttonen 1995, Sidorovich 1997, Macdonald and Harrington 2003, Maran 2007).

Habitat destruction due to urbanization and the expansion of agriculture is probably the fundamental cause for the decline of most mammalian species, and the European mink is no exception. However, there are other specific factors that have influenced the numbers of European mink and led to its rapid decline even in places where the habitat is neither disrupted nor polluted (Sidorovich 1997, Maran 2007).

The role of polecats in the disappearance of European mink was proposed as early as in the 1980s (Granqvist 1981, Schröpfer and Paliocha 1989). According to one hypothesis, the hybridization of European mink with polecats may cause the assimilation and extinction of the former species (Maran and Henttonen 1995, Maran et al. 1998).

The possibility of producing hybrids between European mink and polecats in captivity was confirmed on a large scale in Novosibirsk (Ternovsky and Ternovskaya 1994). Moreover, in the Seugne River area, where relatively large populations of European polecats and European mink still co-exist, field observations reveal that hybridization between these species is possible although it rarely happens (less than 3 %; Lode et al.

2005). These observations are in good agreement with the earlier results of Tumanov and Zverev (1986), who suggested that 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 the risk of assimilation of the former species by the latter is not high. In some marginal cases, however, the frequency of hybridization may increase (Davison et al. 2000). For instance, during the last years of the existence of the European mink in Estonia, the proportion of suspected hybrids between European mink and polecats was much higher than the expected 3 % (Maran 2007).

The American mink is a "malign invasive species" according to the definition of Macdonald et al. (2006) and causes damage to ecosystems in different parts of the world, from Great Britain to Patagonia (Macdonald and Harrington 2003, Macdonald et al. 2006). The destructive role of the American mink on the existence of the populations of indigenous European mink has been emphasised by the majority of experts who have studied the decline of the latter in various 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. The European mink is still present only in south-western France and it has disappeared from the northern part of its former range (Lode et al. 2001, Maizeret et al. 2002). The American mink either never existed 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. The reasons for the extinction of European mink are often complex (Lode et al. 2001, Maran 2007). Lode et al. (2001) concluded that in north-western France the critical factors 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 several regions of Russia the decline of European mink was recorded long before the invasion of American mink. On the other hand, in some regions, such as Belarus and Estonia, the impact of the American mink on the disappearance of the aboriginal species is well documented (Maran and Henttonen 1995, Sidorovich 2006).

The negative influence of American mink may be indirect or direct (Macdonald and Harrington 2003, Macdonald et al. 2006). 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). In the Lovat river area (Belarus), 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 (Sidorovich 2006).

Based on the observation that, in captivity, American and European mink may mate, get pregnant, but then fail to produce offspring due to resorption of the fetuses, Ternovsky (1977) hypothesised that the same process may also occur in the wild. Whether or not European mink indeed copulate with American mink in the wild has never been properly studied, but some wild mammal species may easily mate with related domestic species, and thus hybridization, even without introgression, might cause wasted reproductive efforts and be highly detrimental (Allendorf et al. 2001). Moreover, feral populations of American mink are carriers of Aleutian disease (Mañas et al. 2001, Fournier-Chambrillon et al. 2004, Yamaguchi et al. 2006) and this disease may easily spread to European mink. However, this hypothesis still needs experimental verification before it can be accepted, and in earlier experiments in which European mink were exposed to American mink in captivity, there were no recorded transmissions of diseases between them (Maran et al. 1998).

American mink are generalist predators, able to feed on fish, invertebrates, birds, amphibians and small mammals, this species is highly adaptive and more versatile than the European mink in using artificial environments (Ben-David et al. 1997, Larivière 1999, Maran et al. 1998). A large-scale behavioural experiment performed in Tallinn Zoo, Estonia, found that American mink are markedly more active and socially interactive than are European mink (Maran et al. 1998, Maran 2007), which may explain why the former may be more adaptive to different wild environments. Such a socialization effect is a known consequence of domestication in some other *Carnivora* species, e.g. canids (Trut 1999, Hare et al. 2005).

The presence of American mink in the same region where European mink exist aggravates the situation and often makes it irreversible. Ternovsky and Ternovskaya (1994) stress that where these two species do co-exist; it is always the population of European mink that declines, while American mink increase in numbers. However, as has now become clear, American mink are not always the trigger for the decline of European mink in some areas.

2.2.2 Current status of conservation of the European mink

The conservation efforts focused on European mink have so far concentrated mainly on establishing captive bred units and attempting to transform captive bred populations into self-sustained fertile wild populations (introduction/re-introduction activities). One important issue is what should be considered an "evolutionarily significant unit" in any programme focusing on the conservation of European mink (Maran 2003). 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), Estonia (Maran 2006) and, more recently, Germany (Festl et al. 2006). These practical attempts assumed that the European mink can be regarded as a single "evolutionary significant unit". On the other hand, recent activity on the captive breeding of European mink at the El Port de Suert facility in Spain (Mañas et al. 2006) is based on a different concept: i.e. only the western population, rather than the whole species, is considered to be a discrete "evolutionary significant unit".

Captive breeding of European mink started in 1970 in Novosibirsk, Russia, on the Ternovsky *Mustelidae* Research Station. More than three decades of captive breeding in the Novosibirsk Research Station have confirmed its potential value in the preservation of European mink. About 500 litters of European mink have been produced during this period (Ternovsky and Ternovskaya 1994, Ternovskaya et al. 2006). The females are sexually mature as early as at 10 - 11 months of age, but only

a minority of males (about 30 %) achieve sexual maturity during the first year of life; the remaining males either participate at the age of two years or fail to breed successfully in captivity. The fecundity of females is dependent on their age, and at Novosibirsk the maximum litter size has been nine kits (Ternovskaya et al. 2006). Polygamy is characteristic of some (although not all) European mink males, some European mink males impregnating up to nine different conspecific females within one breeding season.

The second place where it has been possible to successfully breed European mink in captivity for a number of generations was established in Tallinn Zoo. Activities to establish a captive breeding population in Estonia were started in the 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 the 17 institutions involved, about half of the entire EEP captive bred population being maintained in Tallinn Zoo (105 - 120 of the EEP animals; Maran 2006). This is the largest captive bred population worldwide; however, according to Maran (2003, 2006) the size of a captive bred population needed to maintain 90 % of the heterozygosity of this species during 100 years is 364 - 693 animals.

The EuroNerz Foundation started in Osnabrück, Germany, in 1998 and is the third place where a European mink captive bred population is maintained on a regular basis. Currently the breeding stock in Osnabrück consists of about 40 individuals. This centre faces the problem of aggressive behaviour between adult males and females preventing them from mating. To overcome this problem, new litters have been maintained as a group until late autumn or winter, resulting in significantly improved socialization and subsequent reproductive success (Festl et al. 2006).

Recently, a captive breeding program has been initiated in Spain, based in El Pont de Suert (Lleida, Spain), covering an area of 2,970 square meters (Mañas et al. 2006). The aim of this program is to maintain the western stock of European mink in captivity to save 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 have riverbank vegetation and running water. The first kits born in captivity were documented during the 2005 season http://www.gencat.cat/mediamb/fauna/pdf/viso_informe_ang.pdf. As mentioned above, at this centre the western population, not the whole species, is considered to be a discrete "evolutionary significant unit".

A number of attempts to transform the captive bred population into a reproducing population in the wild have been made in Russia, Estonia, and Germany, the three most notable examples being the following. In the first, a total of 388 animals were released between 1981 and 1989 onto the two largest of the Kuril island chain, Kunashir and Iturup, in the Russian Far East (Ternovsky and Ternovskaya 1994). As a result of this large-scale action, a viable population of European mink was successfully established on each island, although there is some controversy about the density of the resulting population (Voronov 1992, Shvarts and Vaisfeld 1995). The latest follow-up study confirmed that populations still existed on these islands about ten years after the last release (Shvarts and Vaisfeld 1995), though the current state of these populations needs to be confirmed. In the second example, in Estonia, the practical efforts to re-introduce European mink have been so far restricted to Hiiumaa island. A total of 295 animals were released during the period 2000 - 2006, the same number of males and non-pregnant females (131 of each) plus 33 pregnant females (Maran 2006). Despite an initially high post-release mortality, regular breeding in the wild has been observed more recently on Hiiumaa island (Maran 2006, 2007), and it remains too early to draw final conclusions about the success of this currently active project. In the third example, a re-introduction experiment was initiated in Germany. Since 2006 a total of 68 European mink have been released from EuroNerz captive bred stock into the wild, not onto islands but on the mainland, in a specially selected protected territory in south-western Germany (Saarland). (http://www.nabu-saar.de/lv/images/stories/nis/nis_073.pdf).

In Novosibirsk, in contrast to the three places mentioned above, European mink and three different species/subspecies of polecats/ferrets have been successfully bred in captivity on the same farm for several decades (Ternovsky and Tenovskaya 1994). In our preliminary research, attempts have been made to transfer embryos between

ferrets and polecats and between European mink and polecats (Amstislavsky et al. 2006). The domestic ferret is considered to be an albino form of the European polecat, therefore embryo transfer between the domestic ferret and European polecat was considered as intraspecies (Amstislavsky et al. 2006). In five intraspecies embryo transfers between domestic ferrets and European polecats (Table 2), two pseudopregnant recipient females received only two embryos each, whereas in the other three cases 6 to 10 embryos per female were transferred. These three females whelped, whereas when only two embryos were transferred to the recipient dam, no kits developed to term. The overall live birth rate was 50.0 % (13 kits /26 transferred embryos).

Table 2. Intra- and interspecies embryo transfer in the European mink (*Mustela lutreola*) and polecat/ferret species (*Mustela putorius, Mustela putorius furo)* (from Amstislavsky et al. 2006).

Donor female species	Days after first mating	No of embryos transferred	Recipient female species	Days after first mating	Weight of recipient (g)	Kits born (%)
Intraspecies embryo transfer						
E.polecat	7	6	E.polecat	6	580	6(100)
D.ferret	7	6	E.polecat	6	520	2 (33.3)
D.ferret	7	10	E.polecat	6	435	5(50)
D.ferret	7	2	E.polecat	6	827	0
D.ferret	7	2	E.polecat	6	630	0
Total		26				13(50)
Interspecies embryo transfer						
E. polecat	7	9	E. mink	6	595	0
E. mink	9	8	E. polecat	7	629	0
E. mink	7	3	E. polecat	6	670	0
E. mink	8	2	E. polecat	7	617	0
E. mink	7,8[a]	6	hybrid ferret[b]	7	690	0
Total						0

[a] Two European mink females were used as donor females.
[b] A hybrid between a domestic ferret and a steppe polecat was used as a recipient.

In five interspecies embryo transfers (Table 2), embryos were transferred from European mink into European polecats or *vice versa*. None of the pseudopregnant recipients gave birth to offspring after this straightforward interspecies embryo transfer (Amstislavsky et al. 2006). Since no term kits developed from the transferred embryos during this straightforward interspecies trial, this approach cannot be considered as a proper option for the European mink; this negative result was the starting point for investigating ways to overcome this interspecies transfer failure.

2.2.3 Stoats: a prosperous species challenging conservation biologists

The stoat is not an endangered species and still occupies its historical palearctic and nearctic realm in Eurasia and North America (Shreiber et al. 1989, Koelpfi et al. 2008). Since being introduced into New Zealand, it has become invasive and is currently considered one of the main pests in that country, having devastating effects on the endemic bird fauna, e.g. kiwi, kaka, mohua, yellow-crowned parakeet and dotterel (King et al. 2001, Parkes and Murphy 2004). The iconic New Zealand bird, the kiwi, is under severe threat and only a few kiwi survive their first 100 days of life, with less than 5 % reaching adulthood (Miles 1998).

To understand reproduction in the stoat it is important not only to find clues as to why females caught in the wild often fail to maintain pregnancy in captivity (O'Connor et al. 2006), but also to find the "Achilles' heel" in the reproduction of this dangerous pest so that effective biocontrols can be developed. The long-lasting obligatory implantation delay in the stoat was identified as the Achilles' heel, and studies of a model species (American mink) was undertaken to intervene in the delay with the final goal of finding a way of pregnancy termination by inducing implantation and parturition in stoats during unfavourable seasons (Marks et al. 2006). Moreover, several recent projects in New Zealand aimed at disrupting reproduction in stoats to control this pest have been reported (O'Connor et al. 2006, LaFalci and Molinia 2007, Molinia et al. 2007).

Thus, comprehensive study of early pregnancy and preimplantation embryo development in this mustelid species, which is puzzling in its reproductive specificity (Deanesly 1943, Ternovsky 1983, Ternovsky and Ternovskaya 1994, King et al. 2001), is also very important from the viewpoint of conservation biology (Parkes and Murphy 2004).

2.3 Reproductive Biology of *Mustelidae*

2.3.1 Oestrous cycle

The oestrous cycle has been comprehensively studied in polecats/ferrets based on observation of vaginal cytology, condition of the vulva, receptivity and hormonal

monitoring. As early as 1900, vaginal cytology was used by Walter Heape for the first classification of the mammalian oestrous cycle (Heape 1900, cited in Betteridge 1981), and since that time it has been widely accepted as one of the main tools for the study of the oestrous cycle in some mammals, including *Carnivora,* such as dogs (Johnston et al. 2001), and mustelids (Moshonkin 1981, Mead et al. 1990, Williams et al. 1992, Ternovsky and Ternovskaya 1994, Maran and Robinson 1996). Female polecats/ferrets are considered to be in oestrus if vaginal lavages contain more than 90 % cornified epithelial cells (Mead et al. 1990, Williams et al. 1992, Young et al. 2001). In the black-footed ferret, both the percentage of superficial cells in vaginal lavages and the size of the vulva correlate positively with faecal oestradiol concentrations (Young et al. 2001). This study, as well as the earlier report of Mead et al. (1990), who also compared the results of vaginal cytology and hormonal monitoring but in the steppe polecat, proved that the observation of vaginal histology may be considered a reliable tool for the study of the oestrous cycle in mustelids.

Different types of cyclicity can be described within the *Mustelidae* family. However, they can be classified into two major categories. Many mustelid species demonstrate prolonged oestrus during the breeding season, which is normally terminated by mating. If not mated, they stay in oestrus for many days or even several months. Ferrets/polecats are especially known to demonstrate a prolonged period of oestrus (Lindeberg 2008). Once in oestrus, domestic ferret females will remain so for more than three months (up to five months) until oestrus is terminated by mating or hormonal injection (Hammond and Marshall 1930, Williams et al. 1992). Other polecats/ferrets (e.g. steppe polecats, black-footed ferrets) demonstrate a similarly prolonged oestrus as confirmed by vaginal smear cytology and the possibility of fertile mating with an appropriate male (Mead et al. 1990, Williams et al. 1992). For example, oestrus in non-bred female black-footed ferrets lasts 30 - 40 days, and in steppe polecats for an average of 41 days (Williams et al. 1992). Based on the fact that domestic ferret females, if not mated, exhibit constant oestrus between late March and early August, Marshall (1905) classified this species as mono-oestrous. However, others suggest that the domestic ferret is seasonally polyoestrous (Lindeberg 2008)

based on the fact that females can enter a second oestrus during the same breeding season after being mated with a sterile male at the beginning of the breeding season, and undergoing a consequent period of pseudopregnancy. Some other mustelids also demonstrate prolonged oestrus. In stoats, for example, which differ from polecats genetically and physiologically despite belonging to the same genus *Mustela* (Koepfli et al. 2008), oestrus lasts several months until terminated by mating (Ternovsky 1977, Ternovsky 1983, Ternovsky and Ternovskaya 1994).

Some other mustelid species may demonstrate repeated oestrus during the breeding season (Kler 1941, Ternovsky 1977, Ternovsky and Ternovskaya 1994). It is known that female sables (*Martes zibellina*) and pine martens (*Martes martes*) after a short period of prominent oestrus and receptivity move into a period of non-receptivity with corresponding changes in their vaginal smear cytology. Then, 7 - 10 days (or even up to 20 days) later they usually demonstrate all the signs of oestrus again (Kler 1941). European mink as well as sable and pine marten are true seasonally polyoestrous mustelid species. Although data on European mink are scarce, their cyclicity resembles that of the genetically more distant marten and sable more than that of their closest relatives, polecats/ferrets (Moshonkin 1981, Maran and Robinson 1996). Moshonkin (1981), working on a captive bred population in the southern part of Russia (Astrakhan), estimated that the duration of oestrus in European mink is 1 to 10 days. If not mated the female enters dioestrus, but then may come on heat again one or two times during the breeding season. Moshonkin (1981) found that the period between the first and the second oestrus is about one month.

The majority of the mustelids studied so far, despite these differences (either one prolonged oestrus or a number of more short periods of oestrus), also share similar features. Seasonality of breeding is characteristic of virtually all mustelids living in a natural photoperiod, at least of those with ranges far from the Equator (King and Moody 1982, Ternovsky and Ternovskaya 1994). Another common feature is that if the litter is lost soon after birth, females of most mustelid species studied are able to return to oestrus (Mead et al. 1990, Ternovsky and Ternovskaya 1994).

Some mustelids demonstrate specific features of oestrous cyclicity, either similar to those found in other mammalian taxa or unique. The least weasel (*Mustela nivalis*

nivalis) demonstrates *postpartum* oestrus at least in conditions of captive breeding, enabling females of this subspecies to accomplish a second pregnancy while rearing the first litter (Sheffield and King 1994). Such *postpartum* oestrus (which is found in some other mammalian species, such as mice) does not occur in another subspecies of the least weasel (*M. nivalis vulgaris*; Frank 1974, Sheffield and King 1994). Due to this reproductive trait, the least weasel is able to produce multiple litters every year, at least in captivity, under natural photoperiod conditions (Frank 1974, Ferguson et al. 2006).

The domestic ferret, which is probably the best known mustelid from being a popular pet in some countries (McKay 1995), also demonstrates some reproductive peculiarities in its oestrous cycle. It is well known that if a jill comes into oestrus, she can remain oestrous for up to five months (Marshall 1905, Hammond and Walton 1934), until she is mated or made pseudopregnant by hormonal injection. If not terminated, continuous oestrus may depress bone marrow function, cause anaemia and even result in death (Sherrill and Gorham 1985). The close relatives of the domestic ferret, the European polecat and black-footed ferret, also demonstrate a prolonged period of oestrus, but much shorter than in the domestic ferret (Mead et al. 1990, Williams et al. 1992) and without depressed bone marrow function, anaemia and death.

Probably the most unusual reproductive feature among mustelids is the precocious maturation of newborn stoat females, which enter oestrus during the first two weeks of life. This phenomenon was discovered and comprehensively studied by Dmitry Ternovsky (Ternovsky 1983) in the captive stoat breeding colony in Novosibirsk. Some other authors also noticed that stoat females are able to come into oestrus when as young as five weeks, and to conceive if mated with an adult male (Müller 1970, cited in McDonald and Larivière 2002). Ternovsky noticed that stoat females even as young as 17 days, still blind, deaf and hairless, can conceive after mating with an adult male in the family nest (Ternovsky 1983). This phenomenon is now well documented (Ternovsky and Ternovskaya, 1994, King et al. 2001, McDonald and Larivière 2002).

The question arises whether mating of young suckling females with their own fathers is ethologically relevant and happens in the wild, or whether some behavioural patterns may effectively prevent such mating in natural habitats. This question was addressed experimentally by Ternovsky and Ternovskaya (1994), who kept five stoat litters in big cages containing not only the mother but also the father. They found that during the first 5 - 10 days after giving birth, the mother actively chased out the father, but then this behaviour ceased and the father was able to visit the litter, where he usually mated all the females and impregnated them. In all cases but one, the adult male interacted only with his daughters, ignoring his sons. However, in one litter (4 sons and 4 daughters) on days 13 - 15 after birth the father visited the nest and not only impregnated his daughters, but also killed all his sons. This infanticide behaviour toward sons may be considered an exception, as in the other four "families" the sons were not killed. The experimental situation described above, where an adult male stoat was forced to stay in the close vicinity of his litter, does not happen in the wild, where stoat fathers do not stay with their litters (Ternovsky and Ternovskaya 1994). This is in agreement with the view of King et al. (2001) that the lack of family pair bonds between adult male and female stoats, and the rapid turnover of stoat populations, minimizes the risk of an adult male impregnating his own young in wild.

2.3.2 Mating and ovulation

The great majority of the mustelids whose reproduction has been studied so far are induced ovulators, although some minor exceptions are known (Murphy 1989). In the comprehensive study of Carroll et al. (1985), an important question was addressed: which components of male mating behaviour trigger the LH concentration increase in plasma and subsequent ovulation? It has been shown that intromission induces both ovulation and plasma LH postcopulatory rise in domestic ferrets, and that neither the duration nor the number of acts were causative factors, even one short period of intromission being enough (Carroll et al. 1985), unlike the situation in the domestic cat (Wildt et al. 1980). Another important finding of this study was that if the male was allowed to express other components of mating behaviour, such as neck gripping, mounting and pelvic thrusting, these were not enough to induce either a plasma LH surge, or ovulation if intromission was missing (Carroll et al. 1985).

Some reproductive features characteristic of *Mustelidae* may well be explained by induced ovulation. The interval between copulation and ovulation is relatively long in mustelids: 33 - 72 hours in the American mink (Hansson 1947, Enders 1952), and 30-40 hours in the ferret (Hammond and Walton 1934, Chang and Yanagimachi 1963, Carroll et al. 1985). The existence of this lag phase and the relatively slow rate of the first cleavage explain why on the third day after copulation (72 hours *pc*) ferret embryos consist of only 4 - 8 cells (Robinson 1918, Hamilton 1934). The relatively long period between copulation and ovulation seems to be associated in mustelids with a prolonged period of sperm survival in the reproductive tracts of females. Chang (1965) demonstrated that spermatozoa retained their fertilising capacity in the female ferret reproductive tract for 126 hours. The time of sperm survival in mustelids is thus much longer than it is in most other mammals (Johnson and Everitt 2000).

The relatively long time of sperm survival in mustelids may be considered a specific reproductive characteristic of the family. In the common farm animals like cattle, pigs and sheep, the fertile life of spermatozoa in female reproductive ducts is shorter and does not exceed two days (Johnson and Everitt 2000, Senger 2003). This period is even shorter in common laboratory animal species, such as mice and rats (Johnson and Everitt 2000). However, horse sperm retain their fertilizing ability within the reproductive tract of the mare for 4 - 5 days (Johnson and Everitt 2000, Senger 2003), i.e. in equids and mustelids the fertile life of sperm within female is of about the same duration. However, in some bat species studied so far, the time of sperm survival in female reproductive ducts is much longer than in mustelids, up to 4 - 5 months (Bernard and Prolong 1988, Senger 2003).

The duration of coitus is rather long in most mustelids, although there is great interspecies and within species variability. In the extensively studied domestic ferret, the duration of copulation varies from 15 min to more than 3 hours (Hammond and Walton 1934, Ternovsky and Ternovskaya 1994, Fox and Bell 1998) and the same degree of variability is characteristic of other mustelid species in which reproductive physiology and behaviour have been studied in captivity (Ternovsky and Ternovskaya 1994). For example, in the Siberian weasel (*Mustela sibirica*), a close relative of the ferret, copulation lasts from 27 min up to 2 hours and 40 min (Ternovsky and Ternovskaya 1994). In the mountain weasel (*Mustela altaica*) the range is even more

variable, from 10 min up to 5 hours (Ternovsky and Ternovskaya 1994). At least in the domestic ferret the duration of copulation is not correlated with subsequent increase of LH and ovulatory response, and just one minute of copulation was enough to induce normal ovulation (Carroll et al. 1985). The duration of copulation of an adult male stoat with a young female is rather short (1 - 10 min) although it is much more variable when the male and female are both adult (2 - 59 min) (Ternovsky and Ternovskaya 1994).

Whether all species of mustelids are induced ovulators, and whether spontaneous ovulation ever happens in mustelids, are interesting questions. There is evidence that in some species, e.g. the ferret, ovulation is always induced either by mating with intromission (Carroll et al. 1985) or by simulating natural mating by injection of hCG (Chang 1968, Mead et al. 1988b), never occurs spontaneously, and is not induced by any encounter with a male that does not include intromission (Carroll et al. 1985). This contradicts the suggestion of Chang and Yanagimachi (1963), who argued that ferrets may sometimes ovulate spontaneously when they are handled. However, this postulate was based only on the observation that the vulvae of seven oestrous ferret females out of 29 transported to their facility started to shrink after arrival, and thereafter the vulvae started to swell again 42 - 48 days later, all seven eventually delivering kits. As neither an examination of ovaries nor hormonal monitoring was done in these seven females, it may simply be that they entered dioestrus and then came into heat again after some time.

Some other mustelid species such as stoats (*Mustela erminea*) are also known to ovulate exclusively after mating and are never found to ovulate spontaneously even if they stay in oestrus for a long period (Rowlands 1972, King and Moody 1982). In some mustelid species, in some circumstances, ovulation may occur spontaneously, especially if some elements of male copulative behaviours are expressed toward the female (Enders 1952, Møller 1974). Ovulation may occur in female American mink after only brief contact with a male, not necessarily involving intromission (Enders 1952). Møller (1974) observed that true *corpora lutea* have been formed in female American mink that were in the close vicinity of males, but without any contact with them.

2.3.3 Preimplantation embryo development

As in other mammals (Johnson and Everitt 2000, Sadler 2006) preimplantation embryo development in mustelids begins in the oviducts, then proceeds after the embryos migrate into the uterus. Embryo development and the accompanying events of early pregnancy have been most comprehensively studied in ferrets/polecats (Robinson 1918, Hamilton 1934), which are nondiapausing mustelids, although there are also some data from other *Mustelidae* species that are known to express implantation delay, e.g. American mink (Hansson 1947, Enders 1952), sable (Baevsky 1955), spotted skunk (Enders et al. 1986) and badger (Yamaguchi et al. 2006).

In a number of the mustelid species studied, asynchrony in blastomere cleavage has been observed from as early as the two-cell stage. This is characteristic of ferrets (Hamilton 1934) and stoats (LaFalci and Molinia 2007). The uneven number of blastomeres from the two-cell stage onward has also been reported in other *Carnivora* such as blue foxes (Valtonen et al. 1985), silver foxes (Lindeberg and Jalkanen 1993) and dogs (Metcalfe 1999), as well as in members of other orders, e.g. mice (Piotrowska et al. 2001) and horses (Betteridge et al. 1982, Betteridge 2007), but for ruminants the first cleavage cycles are well synchronized and 3-cell embryos are not common (Betteridge and Fléchon 1988).

Another characteristic feature of *Mustelidae* preimplantation embryo development is the large number of lipid droplets (Hamilton 1934), so mustelid embryos/oocytes look dark under a light microscope from the oocyte stage throughout the whole period of embryonic development (Enders et al. 1986). This is characteristic also of other families of *Carnivora* such as canids and felids, e.g. dogs and cats (Boyd and Hamilton 1952, Songsasen and Wildt 2007), whereas most other eutherian mammals are characterised by lighter-coloured embryos (Boyd and Hamilton 1952, McLaren 1982) with the notable exceptions of porcine (Kikuchi et al. 2002) and equine (Betteridge et al. 1982, Betteridge 2007) embryos.

Yet another characteristic feature of mustelid preimplantation development is that unimplanted embryos grow much larger than do the embryos of the great majority of other mammalian species that have been studied. Preimplantation embryos of ferrets reach a diameter of about 2 mm on day 11 pc (McRae 1992), one day before

implantation. The diameter of the embryos of diapausing mustelid species, e.g. the western spotted skunk, increase very slowly during the period of delayed implantation, but at the activation stage just prior to implantation they achieve a diameter of about 2 mm (Enders et al. 1986). The average number of cells in a ferret blastocyst two days before implantation, i.e. 10 dpc, is 6215 (Kidder et al. 1999a). For comparison, a mouse blastocyst in the peri-implantation period on day 5 pc consists of 110 - 119 cells (Handyside and Hunter 1986) and its diameter is about 100 micrometers (Enders 1971).

Unlike most farm animal species and many other mammals (Behringer et al. 2006), *Mustelidae* embryos are round or ovoid throughout the whole period of preimplantation development (Enders and Mead 1996, Renfree and Shaw 2000). In many other mammals the shape of embryos changes drastically before implantation and they become elongated (Wimsatt 1975, Blomberg et al. 2008). However, in some mammalian species, e.g. mice, rats and rabbits, the embryos do not become elongated (Wimsatt 1975, Enders 1971).

Preimplantation embryo development in the domestic ferret has been comprehensively studied for more than a century. In the domestic ferret (Daniel 1970) and Finnish farmed European polecat (Lindeberg and Järvinen 2003, Lindeberg 2003), cleavage stage embryos were found in oviducts on days 3 - 4 pc. The diameters of these were about 150 micrometers, and embryos containing up to 16 cells can be found (Daniel 1970, Lindeberg and Järvinen 2003, Lindeberg 2003). The first morulae in polecats/ferrets can be noticed on day 5 pc (Daniel 1970, Lindeberg 2003). Since the classic work of Robinson (1918), it has been known that in ferrets the embryos first enter the uterus on 6 dpc. In Finnish farmed polecats, all the embryos are still in the oviducts on 5 dpc, they migrate into the uterus 6 - 7 dpc, and on Day 8 pc nearly all embryos are in the uterus (Lindeberg and Järvinen 2003, Lindeberg 2003). Recently, Li et al. (2006a) recorded that in the domestic ferret most embryos are in the uterus by 7 dpc, and are already at the blastocyst stage, which is consistent with earlier observations.

Once ferret embryos are in the uterus, the sizes of blastocysts progressively increase, and this expansion is especially prominent after 8 dpc. (Daniel 1970, McRae 1992,

1994, Kidder et al. 1999a). Undisturbed migration of embryos from the oviducts into the uterus is necessary for the progress of blastocyst expansion in the ferret, as blastocysts retained in the oviduct by ligation of the utero-tubal junction fail to expand beyond 400 micron in diameter (McRae 1992). McRae described three phases of blastocyst expansion in the ferret, 200 - 400 micron, 400 - 700 micron, and 700 - 2000 micron, and partly described the endocrine environment needed to accomplish each of these phases successfully.

2.3.4 Extracellular embryonic coats

The best known and basic embryonic coat surrounding the mammalian embryo is the *zona pellucida* (zp) (Rankin et al. 2000, Denker 2000, Menkhorst and Selwood 2008). In mice, the *zona pellucida* is composed of well characterized glycoproteins: ZP1, ZP2, and ZP3 (Wassarman 1998, Rankin et al. 2000). Recent studies with knockout mice lacking one of these principal ZP components have clarified the role of each in the fertilization process in the mouse. The most dramatic phenotype was observed in ZP3-null mice (Liu et al. 1996, Rankin et al. 2000). These and other studies indicate that the presence of ZP3 in the *zona* is important for normal fertilization in mice (Rankin et al. 2000, Bedford 2004). The thickness of this basic embryonic coat is estimated as 6 - 8 μm in rodents and some other mammals in which the egg/embryonic size is relatively small, and as 13 - 16 μm in species which have larger embryos/eggs (Bedford 2004). Besides being implicated in the process of fertilization, zp is known to participate in other processes during early development that vary with the species and stage (Rankin et al. 2000). One important characteristic of mammalian zp is its relative resistance to the effects of proteolytic enzymes (trypsin, chymotrypsin and pronase) (Bedford 2004).

During oviductal and uterine passage, embryonic coats in most, if not all, mammalian species acquire some extra layers or at least incorporate some new elements (Menkhorst and Selwood 2008). So-called “tertiary” membranes (mucoid coats, shells, egg jellies etc) acquired during egg passage through the oviduct and uterus are common in amphibian, avian, and reptile species as well as in monotreme and metatherian mammals (marsupials) (Betteridge 1989, Menkhorst and Selwood 2008). Evolution of viviparity seems to have caused reduction of these tertiary membranes

and they are much less prominent in the embryos of the eutherian mammals (Betteridge 1989). However, these membranes are still present during some stages of embryonic development in some species of lagomorphs, equids, and carnivores (Enders 1971, Betteridge 1989, Denker 2000, Menkhorst and Selwood 2008).

Horses and rabbits are known as models for extensive studies of extraembryonic coats (Böving 1957, Betteridge 1989, Denker 2000). In the horse, after day 7 post ovulation the blastocyst is encompassed only with so-called capsule as other coats disappear, but capsule persists for about two weeks more (Betteridge 1989). In the rabbit, a mucoprotein layer is laid onto the zp during passage through the oviducts. In the uterine environment this mucoprotein layer becomes thinner, and during the next two days the zp is dissolved and replaced with the so-called "neozona"; after 6 dpc a gloiolemma appears on the top of this complex well stratified structure (Betteridge 1989, Denker 2000). Early studies indicated that if cow embryos are transferred into ligated oviducts of the rabbit, a thin mucin layer appears outside their zp (Adams 1982); this is good confirmation that the oviduct and uterus are the sources of material for extra coating in the rabbit. On the other hand, it has been demonstrated by xenogenic grafting studies that the trophoblast is the principal source of material for forming the equine embryonic capsule (Albihn et al. 2003).

In carnivores, Enders (1971) described a "subzonal layer" in the fur seal blastocyst. Denker (2000) analysed Enders' results retrospectively and concluded that this "subzonal layer" of the seal seems homologous to the neozona of the rabbit (Denker 2000). Denker (2000) also analysed the results of Swanson et al (1994) who comprehensively studied *in vivo* development of domestic cat embryos. As emphasised by Denker (2000), it is very likely that new material is deposited upon the zp while cat embryos move through the oviduct into the uterus. The coats of morulae flushed from the uterus are indeed thicker than those of cleavage-stage embryos flushed from the oviducts (Swanson et al. 1994). Enders et al (1986) demonstrated the different texture of various layers in the blastocyst of the western spotted skunk. Although the authors still refer to this coat as zp, more detailed examinations in future studies may reveal that there are clearly defined extra embryonic coats in at least some members of *Mustelidae* family.

2.3.5 Implantation in mustelids

Embryo implantation is an event that divides embryonic development in mammals into two parts: the preimplantation and postimplantation periods of pregnancy. *Mustelidae* are characterized by central implantation, when the expanding embryo is first within the lumen of the uterus (Boyd and Hamilton 1952), then starts the invasion of endometrium by trophoblast cells penetrating between the uterine epithelial cells, and ends with the establishment of a zonary endotheliochorial placenta (Enders and Schlafke 1972, Schlafke and Enders 1975). Some other *Carnivora* families, e.g. *Canidae* and *Felidae,* also are characterized by a zonary placenta (Amoroso 1952), although in mustelids there are some marked differences in the formation of the placenta (Mossman 1987). Histologically, implantation and postimplantational events are well described in mustelids with continuous development, such as the ferret (Enders and Schlafke 1972, Gulamhusein and Beck 1973), and also in diapausing species such as the western spotted skunk (Enders and Mead 1996). Unlike in mice and rats (Pijnenborg and Vercruysse 2004) there is no prominent decidual formation in mustelids, and the term "adeciduate species" has been used at least for the ferret (Mead et al. 1988a).

Since the classic work of Böving (1954), it has been known that during the process of apposition embryos may well move between the uterine horns, and that there is a great species specificity for such migration (Boyd and Hamilton 1952, McLaren 1982, Denker 2000). There are species such as mice and rats in which the embryos rarely or never migrate into the contralateral horns (McLaren 1982). In mustelids, embryos easily move between the horns, as has been confirmed for the ferret (Chang 1968, Li et al. 2006a), sable (Baevsky 1960) and stoat (King 1990).

The key role of progesterone in implantation in mammals is well documented (Graham and Clarke 1997, Spencer et al. 2007). In species with diapause, an insufficient level of progesterone is implicated in maintaining blastocysts in a dormant state and a peak level of this hormone is observed at the activation stage before implantation (Lopes et al. 2004). It has been shown that progesterone levels in the diapausing stoat remain low during most of pregnancy and there are no differences in plasma progesterone concentrations between pregnant and unmated stoat females until

the time of blastocyst activation and implantation (Gulamhusein and Thawley 1974). In the ferret, the blastocysts do not undergo diapause in their preimplantation development, and plasma levels of progesterone increase dramatically during the first week of pregnancy and reach a maximum at about the time of implantation, after two weeks of pregnancy (Blatchley and Donovan 1976, Daniel 1976).

Experimental lowering of progesterone availability to the preimplantation embryo dramatically reduces the rates of embryo development in the ferret (Rider and Heap 1986, McRae 1992, 1994). When monoclonal anti-progesterone antibody was administered to ferrets during days 3 - 4 pc, embryos were arrested at cleavage stages; this condition also resulted in a tube-locking effect, i.e. the retaining of some embryos in the oviducts (Rider and Heap 1986). In these antibody-treated animals, the plasma level of progesterone was six-fold higher than in controls, which indicates that the embryos were arrested at cleavage stages and partly retained in the oviduct as a result of the antibody binding of progesterone in circulation. This experiment imitates the situation of progesterone deficiency during delayed implantation in mustelid species. In experiments on ovariectomy/progesterone replacement in pregnant ferrets (McRae 1992, 1994) and American mink (McRae 1994), it has been demonstrated that appropriate progesterone concentrations are necessary for blastocyst expansion, but additional factors besides progesterone are needed for the implantation of the mustelid embryo.

Luteal factor, which, together with progesterone, is needed for implantation in the ferret, has been partially characterized by Mead et al. (1988c) as a 60-kDA protein produced by CL between pregnancy days 6 and 9. More recently this factor has been further characterized and identified as the enzyme glucose-6-phosphate-isomerase (GPI) (Schulz and Bahr 2003, 2004). Molecular cloning and sequencing have identified that GPI is identical to AMF (autocrine motility factor) (Watanabe et al. 1996), which is known to promote metastasis during carcinogenesis (Shimuzu et al. 1999). The facts that GPI/AMF is necessary for implantation, and that the same factor is secreted by tumour cells (Niinaka et al. 1998), support the view that the invasive mechanism of implantation is similar to the metastasis processes. Although it has been clearly demonstrated that GPI (along with progesterone) plays a key role in

implantation in ferrets (Schulz and Bahr 2003, 2004), so far no studies on the role of GPI in implantation have been done in any other species other than the ferret.

The activation of blastocysts in mustelids with delayed implantation, such as the American mink, spotted skunk and stoat, depends on the photoperiod, and in all these species it has been confirmed either directly or indirectly that the lengthening of days influences the timing of implantation (Mead 1971, Murphy and James 1974, Gulamhusein and Thawley 1974). Prolactin, a gonadotrophin which is mainly synthesized and secreted by the lactotroph cells of the pituitary, provides an important link between the hypothalamus-hypophysis and the ovary, regulating the levels of progesterone (Lopes et al. 2004, Bachelot and Binart 2007).

The role of prolactin in reproduction has been comprehensively reviewed recently, including its regulation of implantation in various mammalian species, but mainly in rodents (Bachelot and Binart 2007). In mustelids, prolactin is known to be involved in the regulation of implantation (Renfree and Shaw 2000, Lopes et al. 2004), and administration of exogenous prolactin to American mink hastens implantation (Papke et al. 1980).

Unlike some other fur animals, which have only one annual pelage change, most of mustelids studied so far have two moulting periods yearly (in spring and in autumn) and they develop different summer and winter coats (Blomstedt 1998). In the stoat, snow-white winter hair is replaced by the brownish summer coat in April-May and the snow-white winter coat appears again during the autumnal moult. Long and short nights produce different pattern of melatonin secretion by the pineal gland. The levels of melatonin are highest at night (Lincoln 1984); thus the animal normally gets information about what season it is via melatonin levels. The seasonal pattern of melatonin secretion inversely reflects that of prolactin secretion, and for the mink prolactin is suggested to be a link between the melatonin (pineal) and the hair follicle for controlling pelage growth and moult (Rose et al. 1985). Prolactin is, in most mammals, the key hormone of implantation (Freeman et al. 2000), and it has been suggested that prolactin is the major component of the luteotrophic complex that terminates embryonic diapause in mink (Papke et al. 1980) and in stoats (King 1989). Spring moult and implantation are events that may happen more or less

simultaneously as both are part of the same chain: short nights cause a decrease of melatonin secretion and, as a consequence, an increase of prolactin levels which may be considered as the main hormonal trigger for both moult and implantation. The stoat is a good example of a mustelid species in which the reproductive and moulting events in both sexes are strictly controlled by light conditions (King and Moody 1982, Ternovsky 1883, King 1989), and it has been shown that in New Zealand the date of implantation and birth in this species strictly depends on latitude (King and Moody 1982). Under the conditions of the *Mustelidae* Research Station in Novosibirsk, the first dark spots on the white winter coat of the stoat normally appear at the beginning of April and parturition occurs about a month later (Ternovsky 1983). Thus, in some mustelids, including the stoat, the start of moulting is an indirect indicator of implantation. A similar phenomenon has been described for the American counterpart of the stoat, the long-tailed weasel (*Mustela frenata*) (Wright 1964 cited in King 1989). It has been suggested for both these species that all females remaining white unusually late in the season can be assumed to have lost their fetuses (King 1989).

Recently, in the laboratory of Bruce Murphy (Canada), the cross-talk between an embryo and the uterus was further deciphered. It has been shown that vascular endothelial growth factor (VEGF), which is necessary for implantation, is induced by the prostaglandin E_2 secreted by the activated blastocyst of the mink (Lopes et al. 2006). Prostaglandins have been shown to be implicated in the initiation of implantation in most of the mammalian species studied (Psychoyos 1973, Kennedy et al. 2007): for example, it increases vascular permeability at implantation sites in the ferret (Mead et al. 1988a).

2.3.6 Delayed implantation

The *Mustelidae* family contains 59 extant species (Koepfli et al. 2008) of which about one third are known to have obligatory delayed implantation in their embryonic development. Another third of the *Mustelidae* family members do not have this delay, and there is lack of reliable data for the remaining third (Lindenfors et al. 2003, Thom et al. 2004).

It is generally accepted that delayed implantation (DI) evolved (e.g. in the *Carnivora* order) to make it possible for mating to occur during the optimal season (when males and females are in their best condition), and for parturition to be in a season when resources for the rearing of young will be at their maximum (Sandell 1990). Sandell (1990) suggested that DI evolved independently at least 17 times in mammals, thus postulating a polyphyletic origin of this trait. On the other hand, Lindenfors et al. (2003) suggested a monophyletic origin of DI in carnivores.

Lindenfors et al. (2003) emphasised that in species with short life spans, those with unpredictable mortality, and those which produce several litters per year, delaying any aspect of reproduction will bring them no evolutionary advantage, and may be detrimental. Because small body size normally unites the above-mentioned characteristics, they hypothesized that species lacking DI are smaller than their sister species which have delayed implantation (Lindenfors et al. 2003). Thom et al. (2004) explored some correlations of DI with such important life-history characteristics of mustelid species as longevity (life span). They found that with few exceptions longevity is positively associated with the presence of DI. This conclusion is in good agreement with the basic concept of both Thom et al. (2004) and Lindenfors et al. (2003) that the time costs imposed by DI may prohibit its occurrence in short-lived species, since the number of reproductive events that can occur in a lifetime is the most crucial factor for a species' survival.

Ferguson et al. (2006) did not find any relation between body size and the presence or absence of DI in mustelids, but suggested that DI is associated with the adaptation of mustelids to seasonality in high latitudes. Indeed, the temporal separation of mating and parturition provided by DI is unnecessary at the equator, where seasonal variations are minimal, but brings real benefits at higher latitudes. This hypothesis has been confirmed by a number of studies which emphasise that DI is more common at greater distances from the equator (Ferguson et al. 2006, Thom et al. 2004). Ferguson et al. (2006) further provided evidence that mustelids living at high latitudes are characterized by larger individual home ranges, low population density and DI. The latter most characteristic allows mating during the optimal season and provides a better opportunity for females to choose mates, because seasonal environments at high latitudes generally reduce these opportunities.

Delayed implantation in mammals, when it happens, always occurs at the blastocyst stage (Baevsky 1970, Renfree and Shaw 2000, Lopes et al. 2004), although the embryos may already be at the hatched blastocyst stage, as in roe deer (Aitken 1975), or still blastocysts within the embryonic coats, as in mustelids (Baevsky 1970, Enders et al. 1986, Lopes et al. 2004). The main formal division of DI is into facultative and obligatory diapause (Renfree and Shaw 2000). Facultative diapause (implantation delay) may happen in some situations, for example in mice and rats mated at the *postpartum* oestrus during active lactation, when blastocysts are quiescent until the lactation stimuli cease.

If a species has obligatory diapause, it is unconditionally present and may encompass long periods of up to 9 - 10 months, as in the sable, spotted skunk or badger (Baevsky 1955, Enders et al. 1986, Mead 1993, Yamaguchi et al. 2006). In contrast to those of some other animal groups, diapausing blastocysts in mustelid species increase in size with time (Mead 1993, Renfree and Shaw 2000). Slow growth of the blastocyst, still encompassed within the embryonic coats, has been reported for the spotted skunk (Enders et al. 1986), sable (Baevsky 1955), and badger (Cresswell et al. 1992, Yamaguchi et al. 2006).

Recently, some genes that are differentially expressed in activated and delayed blastocysts in mice have been identified (Hamatani et al. 2004). However, even in the mouse model, only "candidate genes" that may be responsible for the phenomenon have been nominated, and further verification is needed (Hamatani et al. 2004). Because DI is a complex phenomenon in many mammalian groups, different mechanisms may be responsible for it (Mead 1993, Renfree and Shaw 2000, Lopes et al. 2004) and sometimes the same mechanisms are used for different purposes (Lopes et al. 2004).

2.4 Reproductive technologies relevant to the genome resource bank in *Carnivora*

There are enormous differences between mammalian species in embryonic development and in placentation as well as in gestation length (Enders 1971,

Betteridge 2001, Behringer et al. 2006). So it is not surprising that reproductive technologies work perfectly with some animals, e.g. most farm animals and laboratory rodents (Betteridge 1981, Hogan et al. 1986, Dobrinsky 2002) but are very laborious or sometimes hard to adapt to other animals, such as wildlife and endangered species (Wildt et al. 1992, Leibo and Songsasen 2002, Loskutoff 2003, Andrabi and Maxwell 2007, Paris et al. 2007). The efficiency of assisted reproductive technology (ART) applied to non-domestic and endangered species remains extremely low in most cases, and often the positive result reported the first time with any species is the only instance when ART was successful with that species (Loskutoff 2003, Paris et al. 2007).

Despite some complications in the use of reproductive technologies for the propagation and conservation of companion animals and non-domestic and endangered species (Paris et al. 2007), there are a number of successful examples of using ART for genome resource banking (GRB) in several non-domestic and/or endangered mammals (Leibo and Songsasen 2002, Pukazhenthi and Wildt 2004, Andrabi and Maxwell 2007). Andrabi and Maxwell (2007) have drawn up a list of 22 GRB facilities worldwide that collect and preserve biological material from threatened or endangered animal species and are devoted to preventing the loss of genetic diversity. Of these 22 centres, seven are located in North or South America, nine in Europe/UK, two in Africa, and the other four centres are in China, India, South Korea and Saudi Arabia. In most cases these GRB activities are focused on wild relatives of bovine, ovine, caprine and porcine species, i.e. relatives of farm animals which represent the mainstream of ART development (Andrabi and Maxwell 2007).

Although there is a significant demand for the development of reproductive technologies with such traditional companion animals as cats and dogs (Long et al. 2003), ART and embryo technology (including embryo cryobanking) are more advanced with felids than with canids (Farstad 2000a, b). This is not surprising if we take into account the fact that most *Felidae* species are endangered (Myers 1984, Beer

et al. 2005), and some programmes originated to develop reproductive technologies for their conservation (Swanson and Brown 2004).

One of the reasons why embryo technologies are, in general, less advanced for canids than for felids is that there is less bounteous funding for threatened and endangered *Canidae* species (Farstad 2000a, b, Long et al. 2003). Another reason is that, unlike those of most other mammals (Johnson and Everitt 2000, Sadler 2006), canid oocytes are ovulated relatively immature, at the germinal vesicle (GV) stage (prophase I) of meiotic maturation (Pearson and Enders 1943, Renton et al. 1991, Farstad 1993, Valtonen and Jalkanen 1993, Farstad et al. 2001), which makes it particularly difficult to adapt some reproductive technologies for this family, especially those based on *in vitro* maturation of oocytes (Songsasen and Wildt 2007). However, AI (artificial insemination) and semen cryopreservation are routinely used for dog and fox species (Linde-Forsberg and Forsberg 1989, Linde-Forsberg et al. 1999, Farstad 2000a, b).

Although there are many families in the order *Carnivora* (Valtonen 1992, Bininda-Edmonds et al. 1999), all the successful published attempts to apply ART have been restricted to three families: *Felidae, Canidae, Mustelidae* apart from a single experiment with *Ursidae*. The main achievements in reproductive technologies, embryo technology in particular, are listed below for felids, canids and ursids to provide a context for more detailed description of studies done with mustelids. Advances as well as challenges in adapting reproductive technology to *Carnivora* have recently been extensively reviewed, including those techniques which are beyond of the scope of this thesis and are not included in this review (Farstad 2000a, b, Swanson and Brown 2004, Luvoni et al. 2005, Luvoni 2006, Pope et al. 2006a, b, Songsasen and Wildt 2007).

2.4.1 Genome Resource Bank oriented technologies in *Felidae*

Kittens have been born after AI with frozen-thawed semen of the domestic cat (Platz et al. 1978, Tsutsui et al. 2000). Protocols for the freezing-thawing of ejaculated semen and epididymal sperm in cats have been established with acceptable rates of sperm motility and acrosome integrity after cryopreservation (Luvoni 2006). More recently, semen cryopreservation and AI have been applied to a number of wildlife

felid species (Swanson and Brown 2004). There are protocols established for the cryopreservation of cat oocytes (Luvoni and Pellizzari 2000, Luvoni 2006) although this technology is still considered "experimental" because the survival rate of cat oocytes after freezing is still poor and so far no successful attempts to get kittens born from frozen-thawed oocytes have been reported (Luvoni 2006).

Freshly collected cat embryos were successfully transferred as early as in 1978 (Schriver and Kraemer 1978). Ten years later, it was demonstrated that *in-vivo*-derived cat embryos are able to resume development after conventional cryopreservation, and, when transferred to appropriate recipient females, result in term kittens (Dresser et al. 1988). Johnston et al. (1991) generated cleavage stage "hybrid" embryos *in vitro* by fertilizing leopard (*Panthera pardus*) and puma (*Felis concolor*) oocytes with frozen-thawed domestic cat (*Felis catus*) semen. Domestic cat embryos generated *in vitro* have also been successfully frozen and thawed, developing into term kittens after transfer (Pope et al. 1994). More recently, live kittens were born from frozen-thawed embryos developed from cat oocytes matured and fertilised *in vitro* (Gómez et al. 2003). Embryo transfer/IVF/cryopreservation technology has been successfully adapted to some wild felids as well, for example the tiger (*Pantera tigris*), which is an endangered species (Beer et al. 2005); tiger embryos produced *in vitro* have given rise to term kittens after transfer (Donoghue et al. 1990, Gjørret et al. 2002). Other tiger (*Pantera tigris altaica*) embryos produced *in vitro* have resumed their development *in vitro* after vitrification and subsequent warming (Crichton et al. 2003).

In December 2007, three live ocelot kittens were born in Cincinnati Zoo after the transfer of 24 *in-vitro*-derived frozen-thawed embryos to eight synchronized ocelot recipients. These embryos had been frozen in 1999 - 2000 and kept in LN_2 for 7 - 8 years (Swanson 2008). Another wild felid species in which embryos have been successfully cryopreserved is the caracal (*Caracal caracal*). *In-vitro*-derived embryos of the caracal were conventionally frozen with a propylene glycol/sucrose/dextran70-based solution as cryoprotectant, and then 109 thawed embryos were transferred to nine caracal recipients. Two live term kittens were born (1.8 % success rate) (Pope et al. 2006a, b). Along with these achievements with cryobanking, the domestic cat has been cloned by two independent groups (Shin et al. 2002, Yin et al. 2005). Relatively

recently, the birth of cloned African wildcat kittens to a domestic cat recipient female was reported (Gómez et al. 2004).

2.4.2 Genome Resource Bank oriented technologies in *Canidae*

The history of assisted reproduction in dogs started in 1780, when the Italian monk Spallanzani performed an artificial insemination in a bitch, which is considered the first scientifically recorded use of ART in mammals (cited in Metcalfe 1999). Nowadays, artificial insemination with fresh and frozen-thawed semen is widely used in dog and red/silver fox breeding, although blue fox semen cryopreservation still meets with limited success (Farstad 1998, 2000a, b, Linde-Forsberg et al. 1999, Miller et al. 2005). There are also some positive results in semen cryopreservation in other canid species, e.g. wolves, although the success is much more limited than in dogs and red/silver fox (Leibo and Songsasen 2002).

The history of embryo transfer in dogs started in the same year as for cats. In 1978, the first puppies to develop to term after embryo transfer were reported (Kinney et al. 1979, Kraemer et al. 1979). Since that time, significant progress has been made in the transfer of *in-vivo*-derived embryos from dogs (Tsutsui et al. 1989, Tsutsui et al. 2001), silver foxes (Jalkanen and Lindeberg 1998) and blue foxes (Liu et al. 2008). So far only limited success has been reported on *in vitro* oocyte maturation and fertilization in *Canidae* (Metcalfe 1999, Otoi et al. 2000, Rodrigues and Rodrigues 2002), and only one study resulted in pregnancy after the transfer of *in-vitro*-derived dog embryos, which, however, did not develop to term pups (England et al. 2001).

2.4.3 Genome Resource Bank oriented technologies in *Ursidae*

Successful embryo transfer in the American black bear (*Ursus americanus*) has been reported (Boone et al. 1999). A non-frozen embryo was transferred to a pregnant recipient, which delivered two cubs. As was confirmed by DNA analysis, one of cubs developed from the transferred embryo and the other from the recipient's own embryo. Although the cubs succumbed to flooding in the den, necropsy results indicated that the neonates lived for 6 to 8 weeks before this accident happened. This

result is especially valuable if we take into account the fact that bears feature delayed implantation and a long period of hibernation in the den.

2.4.4 Genome Resource Bank oriented technologies in *Mustelidae*

Methods for semen collection and cryopreservation/artificial insemination with either fresh or frozen-thawed material for mustelids have been developed mostly for domestic and black-footed ferrets (Chang 1968, Wildt et al. 1992, Howard et al. 1991, Kidder et al. 1998, Santymire et al. 2006, Howard 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.

Nowadays, electroejaculation is the preferred method to collect semen from ferrets/polecats (Howard et al. 2003, Santymire et al. 2006); the animals are not killed and their reproductive performance is not compromised. *Post mortem* collection of spermatozoa from the epididymis and/or *vas deferens* was practised in early studies (Chang 1965, 1968) and this method can still be used.

In the first experiments on artificial insemination in mustelids, semen was deposited either into the uterine horn in ferrets (Chang 1965) or into the bursa surrounding the ovary/oviduct (Chang 1968). In the latter case, an experiment was performed not only with ferrets, but also with American mink. The subsequent improvement of the method of AI in mustelids was directed toward minimizing the surgical invasion. The simplest way to avoid surgery is to insert semen into the vagina. However, intravaginal insemination is ineffective in ferrets (Kidder et al. 1998).

Transcervical (nonsurgical) deposition of semen into the uterus/uterine horns with the use of a specially designed catheter (Kidder et al. 1998), does result in a 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 24 hours after administration of hCG. The latter site

proved to be the more successful, however, with all five females inseminated by this route becoming pregnant. 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 (*Mustela putorius furo*) and steppe polecat (*Mustela eversmanni*) as model species. 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). Artificial insemination with frozen-thawed semen unfortunately did not meet with the same success in black-footed ferrets (Howard et al. 2003). Recently, however, black-footed ferret semen and spermatozoa have been under intensive investigation with the aim of ultimately providing a successful semen bank for this endangered species (Santymire et al. 2006, Santymire et al. 2007). There have also been a few attempts to apply a semen cryopreservation protocol to diapausing mustelid species such as the western spotted skunk (Kaplan and Mead 1992) and stoat (LaFalci and Molinia 2007), but no live kits have been reported so far.

The collection, cryopreservation and transfer of mustelid embryos were already practised about forty years ago. Since the classic work of Chang (1968), the simplest way to collect embryos (*post mortem* from the excised reproductive tract) was exploited extensively in ferrets/polecats and in some other mustelids (see Adams 1982, Kraemer 1983 for reviews). A method for surgical embryo flushing that preserves the reproductive function of the donor females has been developed by Jussi Aalto in Kuopio, Finland and was applied first to farmed European polecats (Lindeberg 2003), and subsequently to European mink (II). The surgical method of embryo flushing resulted in the collection of 7.8 - 9.5 embryos per farmed European polecat donor female (Lindeberg et al. 2003, Piltti et al. 2004). A non-surgical transcervical embryo recovery method has also been reported (Kidder et al. 1999b) 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. 1999b).

The administration of gonadotrophins, studied mostly in ferrets, has had two aims: to provoke ovulation and to induce superovulation. Hormones are routinely used to provide superovulation in a majority of farm animals, with the notable exception of horses (Betteridge 1981, Scherzer et al. 2008). The first method of hormonally inducing ovulation in mustelids was invented in the classic studies of Chang (1968), who used 90 IU of hCG to induce ovulation in domestic ferrets and American mink. Mead et al. (1988b) later studied different doses of hCG in ferrets and found that a dose of 100 IU is optimal to induce ovulation in ferrets, since smaller doses (50 and 75 IU) resulted in a lower percentage of females ovulating, while higher doses (150 and 300 IU) resulted in fewer CL/female being formed compared with natural ovulation induced by mating. Both eCG and hCG are used sometimes to improve the outcome of breeding in American mink: to spur anoestrous mink into the breeding season, and to improve fecundity (Wehrenberg et al. 1992, Klotchkov and Eryuchenkov 2003).

Li et al. (2001) tried to find optimal doses of eCG and hCG to provide not only ovulation but superovulation in ferrets. The administration of 100 IU of eCG and, 72 hours later, 150 IU hCG resulted in an average of more than 19 oocytes or embryos per female, or 18.9 embryos/female in the case of hormonally treated females that were also mated (Li et al. 2001). This may, indeed, be considered a superovulatory response, since in the same study mated females that received no hormonal treatment ovulated an average of 8.9 oocytes (Li et al. 2001). Despite this visible success in inducing superovulation in ferrets, Li et al. (2001) noted that neither the overall rate of blastocyst formation *in vitro,* nor the birth and/or kit survival rates, were optimal after superovulation, and suggest the further refinement of superovulation strategies in mustelids. Most recently, an eCG dose of 10 IU was tried in an effort to induce superovulation in adult, wild-caught, acclimatized, female stoats (LaFalci and Molinia 2007) but no superovulatory effect was achieved.

So far only Li et al. (2001) have achieved superovulation in mustelids. However, the hormonal treatment used for superovulation adversely affects the development potential of ferret oocytes/embryos (Li and Engelhardt 2003, Li et al. 2006b), and Li et al. (2006b) considered the change from hormonally induced superovulation to

natural ovulation provoked by mating to be a crucial factor in their final success in producing cloned ferrets.

In mustelids, embryo transfer has mostly been studied in ferrets/polecats and sometimes American mink (Chang 1968, 1969, Kidder et al. 1999b, Amstislavsky et al. 2000, Lindeberg et al. 2002, Lindeberg 2003, Li et al. 2001, 2006a). In all of these studies pseudopregnant females were used as recipients. Ovulation was induced by mating recipients with 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. 1999b). Embryos were successfully transferred either surgically (Amstislavsky et al. 2000, Lindeberg et al. 2002, Lindeberg 2003, Piltti et al. 2004, Li et al. 2006a) or nonsurgically (Kidder et al. 1999b) into the uteri of anaesthetized recipient females.

The pregnancy rates following the transfer of fresh embryos in polecats/ferrets usually are in the range 40 - 50% (Amstislavsky et al. 2000, Lindeberg et al. 2002, 2003, Lindeberg 2003), although rates as high as 90 % (Li et al. 2006a) or as low as 26 % (Kidder et al. 1999b) have been reported. In the latter case, a less invasive transcervical method was used, and the low success rate indicates that a delicate balance may need to be maintained between successful pregnancy established after ET and danger to the 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). However, the transfer of embryos from two different donor animals into a single recipient female seems to increase the effectiveness of the procedure as the number of transferred embryos is increased (Lindeberg 2003). Kits born after the transfer of *in vitro*-generated domestic ferret embryos have been obtained (Li et al. 2001), and the same group has recently reported the birth of two of the world's first cloned ferrets (Li et al. 2006b).

In vitro embryo development has been reported for Finnish farmed European polecat, a species that does not exhibit delayed implantation (Lindeberg and Järvinen 2003). Recently, successful *in vitro* embryo development has been reported for the stoat (LaFalci and Molinia 2007), a species with obligate delayed implantation. Of 88 normal

1 - 4-cell-stage eggs or embryos recovered from stoat oviducts 5 to 6 days after mating, 56 (63 %) developed into blastocysts and 8 % even hatched *in vitro*. However, the same study demonstrated that diapausing embryos failed to develop in culture. Co-culture of diapausing embryos of another mustelid species with implantation delay, the American mink, with a Buffalo rat liver cell layer enabled these embryos to develop and sometimes even hatch (Moreau et al. 1995).

Experiments on the cryopreservation of stoat (*M. erminea*) embryos (Amstislavsky et al. 1993) preceded the experiments on the embryo cryopreservation in farmed European polecats (Amstislavsky et al. 2000, Lindeberg et al. 2003, Lindeberg 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 polecats. 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 et al. 2003). Vitrification of polecat embryos using open pulled straw (OPS) technology developed by Gabor Vajta, and their subsequent warming and transfer has also resulted in the birth of kits (Piltti et al. 2004). Recently, Sun et al. (2008) reported relatively high birth rates after transfer of vitrified-warmed ferret embryos. Compact morulae and early blastocysts after vitrification-warming, *in vitro* culture and subsequent transfer resulted in 71.3 % birth rate if cultured for 2 h or in 77.4 % birth rate if cultured 16 h before ET (Sun et al. 2008).

3. AIMS OF THE STUDY

The primary aim of the present study was:

To address the species-specificity of reproductive biology (i.e. early pregnancy events, embryonic development until implantation and oestrous cyclicity) in European mink (*Mustela lutreola*), and to develop a species-specific model of embryo transfer. Another mustelid species of the same *Mustela* genus, the stoat (*Mustela erminea*) was used as a reference species.

The following issues were chosen as specific objectives of the work presented in this thesis:

To investigate early pregnancy events with special emphasis on preimplantation embryo development in the stoat, a diapausing mustelid species (I);

To explore the applicability of a surgical embryo recovery technique for European mink donor females and the possibility of transferring European mink embryos into hybrid recipient females (hybrids between the European polecat and European mink, i.e. honoriks and nohoriks) (II);

To investigate the *in vivo* embryo development in European mink from ovulation/fertilization until implantation (III);

To study the rate of postnatal survival of kits born after transfer of more advanced stage European mink embryos into honoriks/nohoriks (III);

To investigate oestrous cyclicity and early pregnancy events in European mink, in particular the development of *corpora lutea* in ovaries, and to confirm the time of implantation (IV).

4. MATERIALS AND METHODS

4.1 Conditions of captive breeding on the farm

The experiments were carried out on the research farm of mustelids at the Institute of Systematics and Ecology of Animals, Russian Academy of Sciences, Siberian Division, Novosibirsk, Russia, at 82°45' E, 55°10' N. This *Mustelidae* Research Station was founded by Dmitry and Julia Ternovsky as early as 1970. All animals were kept in individual wire mesh cages measuring 150 x 150 x 200 cm (length x width x height), and each included a nest box measuring 120 x 35 x 35 cm. Throughout the year, the animals were exposed to the outdoor temperature and given wet food prepared in a local kitchen and water *ad libitum*. Additionally, the animals were fed with live mice and fresh mice and rat corpses on a regular basis. Mean monthly temperatures in Novosibirsk are presented in Table 3.

Table 3. Annual temperature in Novosibirsk.

Month	Mean temperature °C
Jan	-18.8
Feb	- 17.3
Mar	-10.1
Apr	1.5
May	10.3
Jun	16.7
Jul	19.0
Aug	15.8
Sep	10.1
Oct	1.9
Nov	-9.2
Dec	-16.5
Average	0.2

Data were taken from Russian site: http://www.seu.ru/programs/ecodom/book/02.htm

4.2 Mustelid species and experimental design

The total number of all donor females of the various mustelid species was 45 and the total number of recipient females involved in embryo transfer experiments was 12, thus altogether 57 females were involved in embryo transfer and/or embryo

development experiments. Together with 12 test females used to confirm the sterility of sterile males, 14 females monitored for progesterone concentration throughout pregnancy, and an additional 17 European mink females monitored for their oestrous cycles (together with some of European mink females from the donor group mentioned above), there were 100 mustelid females in different experimental groups described in the original articles I - IV (Figure 1). The experiments with stoats were performed in 1991 - 1992, and the experiments with European mink and honoriks/nohoriks in 2002 - 2007.

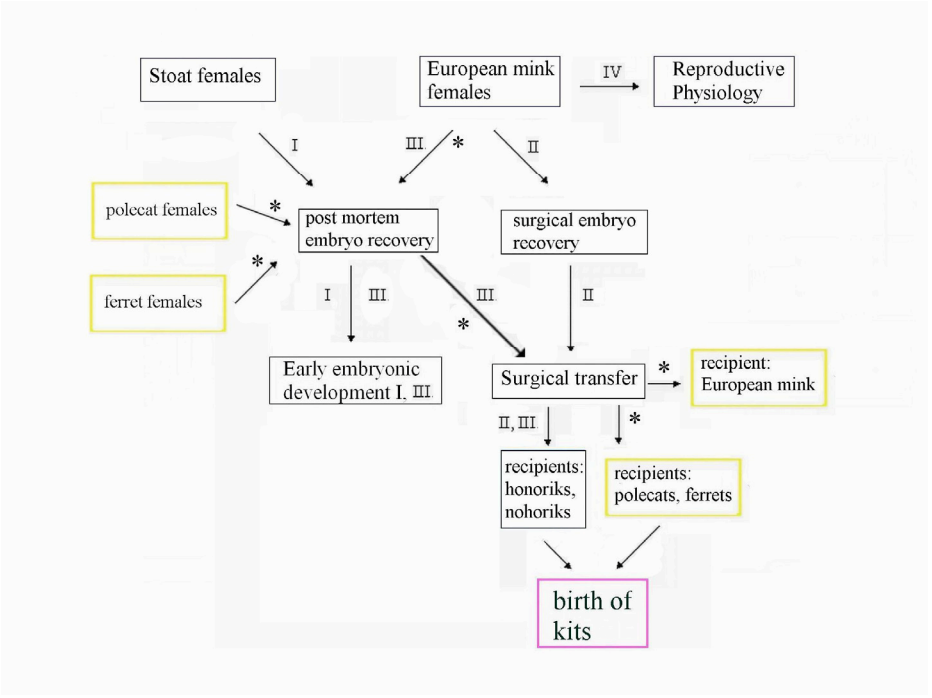

Figure 1. Experimental design: black boxes present results published in the original articles I - IV. Yellow boxes present results published in Amstislavsky et al. 2006 (a pilot study), which are marked with "*".

As discovered by Dmitry Ternovsky (1983), newborn stoat females normally achieve oestrus after the second week of life. A total of 13 stoat females were monitored from the age of two weeks and were mated at the age of 26 - 92 days during May - July of 1991. The uterine horns and oviducts of these females were investigated from 1 dpc until 270 dpc.

European polecat (*Mustela putorius*) females were used as a single donor animal (n=1) and as test females (n=12) to check the sterility of sterile males (II - III). During

the 2007 breeding season, an additional group of 4 European polecat breeding females were monitored by measuring progesterone in their faeces throughout pregnancy (IV^u).

European mink (*Mustela lutreola*) females were used as donor animals (n=31) (II, III). All the donor females were mated to conspecific (European mink) fertile males and flushed embryos were either transferred or studied as described below. During the breeding season of 2004 a further 17 European mink females were monitored for their oestrous cycle (in addition to some females from the above mentioned group of 31 which were monitored for their oestrous cycle while being used as donors) (IV). During the 2007 breeding season, an additional group of 10 European mink breeding females were monitored by measuring the progesterone concentration in their faeces throughout pregnancy (IV).

Captive hybrids of European mink females and European polecat males (Figure 2) have been obtained on a large scale at the *Mustelidae* Research Station in Novosibirsk. The name "honorik" was invented at the farm for this type of hybrid, and was then accepted and widely used in the Russian literature. The name honorik is a combination of the words "horek", which is the Russian name for polecats/ferrets, and "norka", which is the Russian name for mink (Ternovsky and Ternovskaya 1994). Reverse hybrids originating from mating male European minks to female European polecats were first obtained at the research farm in Novosibirsk in 2001 and were termed "nohoriks". The name nohorik, like the name honorik, reflects the origin of this particular type of hybrid (Ternovskaya et al. 2006). It has been shown that female honoriks (Ternovsky and Ternovskaya 1994) and nohoriks (both females and males) (Ternovskaya et al. 2006) are fertile. A total of 9 honorik and 3 nohorik females were used as recipients for the transfer of European mink embryos (II, III).

Figure 2. A female honorik, which was born in captivity in Novosibirsk.

4.3 Detection of oestrus and mating of females

Newborn stoat females were observed from postnatal day 10 since it has been shown that as early as 17 days after birth some suckling stoat females already show full oestrus and are able to mate with adult males (Ternovsky 1983, Ternovsky and Ternovskaya 1994). European mink and honorik/nohorik females were observed two to three times per week at the beginning of the breeding season. Nearer to the peak of the breeding season, the females were examined daily for swelling of the vulva to detect prooestrus. Prooestrus was defined as the period when there was an increase in the size of the vulva, and oestrus as the period of its maximum size, when it was reddish-pink in colour (Ternovsky and Ternovskaya 1994, Maran and Robinson 1996). When the vulva achieved its maximum size and vaginal smear samples indicated about 90 % cornification, the females were considered to be in oestrus (Ternovsky and Ternovskaya 1994). The technique for oestrus detection in juvenile stoat females was similar, but their oestrus was more predictable; smears were first taken after these females reached day 10 of their postnatal life and then on a regular basis when the females reached the age of two weeks.

Every European mink or stoat female chosen as a donor animal and in which oestrus was confirmed by the vaginal smear test was paired with a conspecific male of proven fertility. The same was also done to the breeding females on the farm. In most of the cases, a mating session occurred soon after the pairing of the female with a male. Whether or not there was a fertile mating after a mating session was decided by taking a vaginal sample and checking for the presence of spermatozoa using light microscopy (x 200). On the *Mustelidae* Research Station in Novosibirsk, the normal practice is that females are mated with a male on the first day of oestrus, and in almost all the cases there was only one mating session. During the breeding season of 2004, 13 European mink donor females were not paired with males during their first oestrus in order to study whether or not they are able to enter a subsequent oestrus. One of these females did not re-enter oestrus and so was not mated; the remaining 12 were mated at their second oestrus with conspecific males and were used as donor females. During the experiment of 2002, three European mink donor females were mated twice instead of once.

4.4 Induction of ovulation in recipient females by sterile mating (II, III)

The honorik/nohorik females designated to serve as recipients for embryo transfer had one or two mating sessions with genetically (III) or surgically (II, III) sterilised males. One genetically sterile male along with three vasectomised males were used to induce pseudopregnancy in recipient females. The genetically sterile male was a hybrid, the result of mating a European polecat male to a nohorik female.

For vasectomy (II, III) anaesthesia was induced by intramuscular injection of 0.15 mg/kg medetomidine hydrochloride (Domitor® 1 mg/ml, Orion-Farmos, Turku, Finland) and 10.0 mg/kg ketamine hydrochloride (Ketalar® 50mg/ml, Parke-Davis, S.A., Barcelona, Spain). The skin was incised on the ventral edge of the scrotum. The spermatic cord from one side was exposed through the incision and the *ductus deferens*, separated bluntly with a concave needle from the adjacent testicular artery, vein and nerve, was tied with nonabsorbable silk (FS-1, Ethicon, Germany) sutures in two places 0.3 cm apart. A piece of duct was then excised from between the two ligatures. The procedure was repeated on the second *vas deferens*. The scrotal fascia

and muscles were sutured with soluble 3-0 Vicryl (Ethicon, Germany) and the skin with nonabsorbable 1-0 silk (FS-1, Ethicon, Germany). The males were treated with 0.5 ml of postoperative antibiotic (Duplocillin LA, Intervet, Gist-Brocades NV Delft, Holland) and aerosol wound bandaid (Hansaplast®, Beiersdorf AG, Hamburg, Germany). The animals were kept in a warm place inside the building for 24 - 48 hours after the operation and then moved back to their home cages. After two weeks the stitches were removed from the scrotal skin.

The sterility of the sterile males was verified by the mating of 12 test European polecat females (2 - 4 per each male) before starting to use these males in the embryo transfer programme. None of the 12 test females delivered kits. Another indicator of their sterility was that spermatozoa were never detected in vaginal smears after mating them to recipient females. The genetically sterile male was euthanized after the experiment, and the testicles examined histologically. There was complete disruption of spermatogenesis in this male and no spermatozoa were present in either the epididymides or the seminiferous tubules (III^{u}).

4.5 Embryo collection from the donor animals

4.5.1 Collection of embryos from stoats (I)

Mated stoat females were surgically flushed under anaesthesia at various times between the first day of pregnancy and implantation nine months later. The 13 females were investigated twice, the first time by surgical excision of the ovary, oviduct and uterine horn on one side, and the second time by *post mortem* collection of the remaining ovary, oviduct and uterine horn. A total of 25 oviducts and uterine horns of 13 females (one uterine horn was not investigated) were flushed for eggs/embryos, which were prepared for further studies.

To induce general anaesthesia for the surgery, donor females were injected with ketamine hydrochloride (15.0 mg/kg body weight, i.m., Ketamine 50 mg/ml, Moskovskii Zavod Endokhrinnih Preparatov, Moscow, Russia) followed, a few minutes later, by 50 mg/kg nembutal (i.m., Nembutal®, Dainabot, Osaka**,** Japan). The hair coat was shaved at the *linea alba*, the skin washed and disinfected with iodine

(Solutio iodi spiirituosa 5 %, Novosibirsk, Russia) followed by 70 % alcohol. The females were placed on a clean thick towel on the operating table, the skin incised at the *linea alba*, the muscles and fat opened bluntly, and the peritoneum incised. The fat covering the ovary was lifted with forceps and the whole uterine horn exposed. Two sterile silk ligatures (3-0 Prolene, Ethicon**,** Hamburg**,** Germany) were placed at the proximal part of the uterine horn as close as possible to the bifurcation. Two more ligatures were positioned above the ovary to avoid bleeding after extirpation. Then the uterine horn was cut between the two ligatures, the ovary was cut off between the two ligatures, and the whole horn together with the ovary and oviduct was separated from the fascia with scissors.

In these excised halves of the reproductive tracts, the ovaries were separated from the oviducts and were processed for examination. The oviducts were cut close to the uterotubal junction, but on the uterine side. The uterine horns were each flushed with 20 ml of medium 199 (Vector, Novosibirsk, Russia) introduced through a blunted needle inserted into one end and collected from the other end into a Petri dish. Oviducts were each flushed into a Petri dish with 10 ml of the same medium .

After the operation the peritoneum and muscle layers were sutured with soluble material (3-0 catgut, B. Braun, Melsungen AG, Germany) and the skin with sterilized silk (3-0 Prolene, Ethicon, Hamburg, Germany). The skin was protected with antibiotic powder (Streptomicine, Kurgan, Russia) and aerosol wound bandaid spray (Septonex®, Spofa Co., Prague, Czech). After surgery, the females were treated with antibiotics (Bicilline®-1 s.c., Sintez, Russia) and observed in cages inside the house for the next 24 to 96 h.

At different periods of time covering the period 1 - 272 dpc (I), the animals were euthanized by cervical dislocation after being anaesthetized as described above, the second uterine horn together with the second oviduct and ovary were removed *post mortem*, and embryos were flushed as described above.

4.5.2 *Post mortem* embryo collection in European mink (III)

A total of 31 European mink donor females in which mating was confirmed by a vaginal smear test were humanely sacrificed between 1 and 16 days after mating (1 to 4 animals per day of pregnancy) as described below. The embryos were flushed from the uterine horns and oviducts of these animals *post mortem*, then either processed and evaluated (see Section 4.6) or transferred to recipients (4.7). Ovaries from 25 females were removed and processed for further examination (4.8). The reproductive tract of one pregnant female (12 dpc) was removed and photographed to show implantation sites. The implantation site of another female (13 dpc) was sectioned for further examination (4.8).

For flushing, the animals were anaesthetized with 0.15 mg/kg medetomidine hydrochloride (Domitor® 1 mg/ml i.m., Orion-Pharma, Espoo, Finland) and 10.5 mg/kg ketamine hydrochloride (Ketalar® 50 mg/ml i.m., Pfizer Oy, Helsinki, Finland). The ovaries, uteri and oviducts were removed and inspected and the reproductive ducts were flushed as described below (Valtonen et al. 1985, Lindeberg 2003).

The ovaries were separated from the oviducts and the oviducts were cut close to the uterotubal junction, but on the uterine side. The uterine horns were flushed with 20 ml of flushing medium (Complete Embryo Flushing Solution, Emcare™, Immuno-Chemicals Ltd., Auckland, New Zealand) introduced through a cannula inserted into the distal end of one horn and collected from the distal end of the second horn into a Petri dish. Both oviducts were flushed into a Petri dish, each with 10 ml of the same medium. The embryos were further monitored and selected either for embryo transfer or for more detailed study by light and fluorescent microscopy (4.6).

If flushing the uterine horns was not possible, and prominent uterine swellings were observed, the female was designated as being pregnant with implanting embryos. After surgical removal of the reproductive tract and ovaries, the animals were euthanized by cervical dislocation while still anaesthetized.

4.5.3 Surgical embryo collection in European mink (II)

Nine donor European mink females were surgically flushed with the flank method of Jussi Aalto, as used in the Finnish farmed European polecat (Lindeberg 2003). They were anaesthetized with 0.15 mg/kg medetomidine hydrochloride (Domitor® 1 mg/ml i.m., Orion-Farmos, Turku, Finland) and 10 mg/kg ketamine hydrochloride (Ketalar® 50 mg/ml i.m., Parke Davis, S.A., Barcelona, Spain), supplemented by further ketamine if necessary. Both lumbar flanks were shaved, then washed and disinfected with iodine (Betadine® 75 mg/ml, Leiras Ltd., Turku, Finland) followed by ethyl alcohol (70 %). The eyes were covered with gel drops (Oftagel® 2.5 mg/g, Santen Ltd., Tampere, Finland) and the animal placed in a lateral position on a sterilized towel on the operation table. The skin was incised at the lumbar flank (1.5 cm); the muscular layer separated bluntly, the peritoneum cut with scissors and further opened bluntly with forceps. The fat covering the ovary was lifted to expose the tip of the uterine horn. By means of the long ends of a 2-0 vicryl suture placed into the fat covering the ovary, the uterine horn was held without being touched while gently pushing a sterile i.v. cannula (22G, 0.8/25mm) from the tip of the horn towards the corpus. The cannula had 3 openings made in advance with a surgical blade. The cannula was then fastened to the fat suture by threading one end of the vicryl through the hole in the wing of the conus of the cannula and sutured to the caudal edge of the flank incision using the same vicryl, leaving only the conus of the cannula outside the closed incision. A plastic tube was placed into the conus of the cannula, the operated flank was covered with a gauze sponge and the female was gently turned over to expose the nonoperated flank for surgery.

The second flank was processed as for the first and a total of 20 ml of warmed (37ºC) flushing medium (Complete Embryo Flushing Solution, Emcare™, Immuno-Chemicals Ltd., Auckland, New Zealand) was slowly flushed through the plastic tube, through both uterine horns, and out into a Petri dish via the opposite side tube without touching either the female or the uterus. This flank method of embryo collection did not allow the counting of *corpora lutea*, nor the flushing of embryos from the oviducts.

After flushing, the tube, cannula, and suture were removed and the uterine horn was placed back into the abdominal cavity. The peritoneum and muscle layers were sutured with soluble material (Vicryl, Ethicon) and the skin with stainless steel (Monofilament wire, B. Braun Melsungen, Germany). The skin was protected with antibiotic powder (Bacibact®, Orion, Finland) and aerosol wound bandaid (Hansaplast®, Beiersdorf AG, Hamburg, Germany). The female was then gently turned over to close the initially operated flank similarly.

All donors were treated with antibiotics (Duplocillin LA, Intervet, Gist-Brocades NV Delft, Holland) and observed in cages inside the house for the next 24 to 96 h. Two of the 9 donor females were euthanized by cervical dislocation for *post mortem* embryo recovery: one immediately after surgery (a low number of embryos was recovered during surgical flushing), the other before flushing due to problems in inserting the cannulae into the uterine horn. The remaining donor females experienced no complications during or after surgery. One of them delivered 4 kits after mating again during the second oestrus of the same breeding season. All the other females of this group came into oestrus the next year and participated in the breeding program.

4.6 Processing and evaluation of embryos

Both stoat (I) and European mink embryos (III) were evaluated by light microscopy. Fluorescent microscopy was applied only to European mink embryos (III).

4.6.1 Light microscopy (I, II, III)

All the embryos were briefly evaluated on the spot for the stage of development using stereomicroscopy (x 70) and either transferred or further examined as described below.

All the stoat embryos were designated for more detailed evaluation. European mink embryos were divided randomly, either for transfer or for more detailed evaluation with light and fluorescent microscopy. The embryos designated for transfer were placed into a Petri dish with a fresh drop of holding solution, and the dish was covered with a nontransparent cover, so that the embryos were kept in the dark until the recipient was ready. The European mink embryos designated for the study of preimplantation embryo

development were examined in basically the same way as stoat embryos with one important difference: stoat embryos were evaluated while live (not fixed) whereas those of European mink were evaluated after fixation (see below).

For light microscopy evaluation, stoat (I) embryos were picked up from the flushing medium and placed in a drop of Dulbecco's PBS (Sigma, USA) and European mink embryos (III) to a drop of holding solution (Emcare™, Immuno-Chemicals Ltd.) with a fine glass capillary sterilized by heat in advance. Stoat embryos were evaluated fresh using an inverted microscope (Wild Leitz, Germany). European mink embryos were transferred from holding solution into 1 ml of Dulbecco's PBS (Sigma, USA) containing 2.5 % glutaraldehyde (Fluka, Switzerland) at pH 7.4 - 7.6 for fixation for 1 h at room temperature, then washed in DPBS twice for 15 min and evaluated with an inverted microscope 1X71 (Olympus, Japan).

Both stoat and European mink embryos were evaluated at 30 - 300x magnification depending on their size. The embryos were photographed, the stages of development were estimated and the mean diameters of the embryos including the coats were measured with an ocular micrometer. Phase-contrast microscopy, Nomarsky optics and DIC were used for the evaluation and photography.

4.6.2 Fluorescent microscopy (III)

After the European mink embryos had undergone light microscopic evaluation, they were exposed to 2 µg/ml DAPI (Sigma, USA) in SSC (saline sodium citrate) for 7 min at room temperature and then washed three times in SSC. DAPI has an excitation maximum at 345 nm and an emission maximum at 455 nm. The *zona pellucida* was removed either manually or by a micromanipulator (Labovert, Germany) with an inverted microscope (Leitz Labovert, Germany) at 50 - 125 x magnification. The stained embryos were placed in a drop of mounting medium (Vectashield, Fisher, USA) on a clean glass microscope slide. The slides were analysed and the cell numbers were counted using an inverted microscope (Axioscop 2 plus, Carl Zeiss, Germany), with filters suitable for DAPI staining (359 nm/461 nm) under magnifications of 100, 200, 400 and 1000, depending on the size of the embryos. The

embryos were imaged with the AxioCom HRc and analysed using Axiovision 3.1. software.

4.7 Surgical embryo transfer (II, III)

The recipients were anaesthetized similarly to donors but at a slightly reduced dosages (0.13 versus 0.15 mg/kg medetomidine hydrochloride and 9.5 versus 10 mg/kg ketamine hydrochloride). After a 10 - 15 min induction interval, the animals were checked and a subsequent ketamine dose was administered, if necessary. The embryos selected for transfer were aspirated into a pipette with a minimal amount of flushing medium and transferred into the uterine horn via a skin incision made in the right lumbar flank. The transfer instrument was a thin haematocrit capillary, sharpened and then sterilized by heating in advance, and connected to a plastic tube containing a mouthpiece. The diameters of the distal sharp ends of the transfer pipettes were variable and were adjusted to the sizes of embryos to be transferred. Embryos were aspirated into the instrument with a minimal volume of flushing medium and gently transferred into the ovarian third of the uterine horn without insufflating air. From 1 to 10 embryos were transferred into the right uterine horn. All the transferred embryos in each transfer session were from one or two donor animals of the same species (European mink), with one exception: one honorik recipient female received a mixture of European polecat and European mink embryos, total number of transferred embryos was ten (5 of each species). Very occasionally, some localized uterine bleeding was observed after transfer. Incision closure and postoperative treatment and care were as described for donors. The sutures were removed from the skin within two weeks and recipients were checked at least three times a day around the date of expected parturition.

4.8 Processing and evaluation of stoat and European mink ovaries, and of European mink implanted embryos (I, IV)

The ovaries were removed from the oviducts, separated from fat, fixed in Bouin's solution, and stored in a refrigerator at + 4ºC before further processing. During processing the ovaries were embedded in paraffin, sectioned at 8 μm and stained with haematoxylin and eosin. Sections were analysed and photographed with an Olympus AX 70/AX7 microscope with a fully automatic exposure photo tube (Olympus, U-

CMAD-2, Japan) supplied with a soft imaging system analySyS (Soft Imaging System GmbH,Germany) (European mink, IV) or with a Wild Leitz microscope (Germany) (stoat, I). Implantation sites of one European mink at 13 dpc were processed and analysed in the same way as ovaries.

4.9 Monitoring of progesterone during early pregnancy (IV)

Faecal samples were collected from ten European mink (*Mustela lutreola*) breeding females and four European polecat (*Mustela putorius*) breeding females. Every female was mated with conspecific male of proven fertility during April 2007. The first day of oestrus (which coincided in all cases of this experiment with the day of mating) was considered as Day 0. Samples were collected on 0, 8, 12 and 40 dpc. Only 3 of 10 European mink females were followed up until 40 dpc. Females selected for hormonal monitoring were housed in their home cages and received the same diet as the other animals on the farm. Special care was taken to clean their cages at 6 p.m. the previous day, so that fresh faeces would be readily visible next morning, when they were collected at 11 a.m.

Faecal samples were dried at 30 - 40°C and stored in a closed tube at room temperature. The concentrations of progesterone in dry faeces were measured by radioimmunoassay using Sigma antibodies (Rabbit Anti-Progesterone) and Amersham labelled hormones ([1,2,6,7-H^3] Progesterone). Steroids were extracted from faecal samples using the procedure described earlier (Gerlinskaya et al. 1993). A total of 50 mg of dry faeces were homogenized with distilled water (5 ml) in a glass grinder and then 1 ml was extracted with 4 ml ethyl ether; 3 ml of extract was removed, transferred into a new tube, and vacuum dried at 37°C before adding 100 µl of phosphate buffer (pH=7.0). The concentrations of progesterone were measured according to the recommendations of Sigma. The extraction yield was checked for every assay using [^{3}H]-labelled progesterone and varied from 70 to 76 %. The sensitivity of the assay, determined from the 95 % confidence limits of zero standards, was 5 pg/tube. The inter- and intra-assay variations were 11.6 % and 7.9 %, respectively. To determine the parallelism, a five-point two-fold dilution series of faecal samples in phosphate buffer was prepared and compared with the standard

curves of progesterone. There were no significant differences between the slopes of standard curves and the slopes of lines generated from faecal samples of European mink.

4.10 Monitoring kit survival and development (II, III)

The recipient females that received European mink embryos were checked at least three times a day around the date of expected parturition. When kits were found, their condition, sex and weight were recorded within 12 h after birth, as were their weights on Days 10 and 90. The rate of kit survival until adulthood (day 90) was monitored. The same characteristics were recorded for the offspring of the naturally bred European mink females present on the farm and used as the control group for embryo transfer experiments. These controls, however, were not included in the total number of 100 mustelid females in the different experimental groups mentioned above.

4.11 Statistics

Changes in the progesterone concentrations for pregnant European mink were analysed using factorial analysis of variance (one-way ANOVA) and differences between non-pregnant levels and levels on certain days of pregnancy were identified using *post hoc* analysis (Fisher LSD test) (IV). Differences in weights between the same or different sex kits produced by embryo transfer versus natural breeding at birth, at 10 days of age, and at 90 days of age were compared using Student's t-test (II, III). Where appropriate, results are presented as mean (M) ± standard error of mean (S.E.M.) (I - IV). All statistical analyses were performed using the STATISICA computer program (StatSoft, version 6.0) (I - IV). Level of significance was set at P=0.05 level for each analysis.

5. RESULTS

5.1 Preimplantation embryo development in the stoat – delayed implantation

The young female stoats used as donors in this study were born during the last week of April and the first week of May 1991. In all of them, oestrus had occurred when they were 10 - 20 days of age. Vaginal swelling was observed and cornified cells were a predominant cell type in the vaginal smears of these oestrous stoat females. The majority of females came into oestrus on postnatal days 17 - 18. Copulation occurred after pairing with adult males, as evidenced by a short (1 - 2 min) period of *coitus* and the presence of spermatozoa in the vaginal smears after mating (Figure 3). Under the conditions of the *Mustelidae* Research Station in Novosibirsk, the adult stoat mothers reached their oestrus only when they finished lactating (after weaning).

Figure 3. Adult stoat male mating with young stoat female (25 days old). (Courtesy of Dmitry Ternovsky).

There were no ovulated oocytes in the reproductive ducts of mated females within the first two days after mating. Large preovulatory follicles were present in the ovaries (Figure 4A). Figure 5A illustrates oocytes aspirated from these follicles. At this stage the oocyte was surrounded by numerous cumulus cells, representing the classic cumulus-oocyte complex. Fresh ovulation sites were seen in the ovaries of animals

that had ovulated 72 - 96 hpc. Later, *corpora lutea* were formed although these CL retained relatively small size (Figure 4B).

Oviductal flushing of female stoats 72 - 96 hpc yielded oocytes as well as live spermatozoa. In one of the donor females, ovulation occurred three days (72 hpc) after mating, in another, one day later, 96 hpc (Table 4, Figure 5B). Ovulated eggs flushed from the oviducts on days 3 and 4 were characterized by a dark ooplasm encompassed by *zona pellucida*, were already free of follicle cells, and showed the first polar body which had been already extruded into the perivitelline space (Figure 5B).

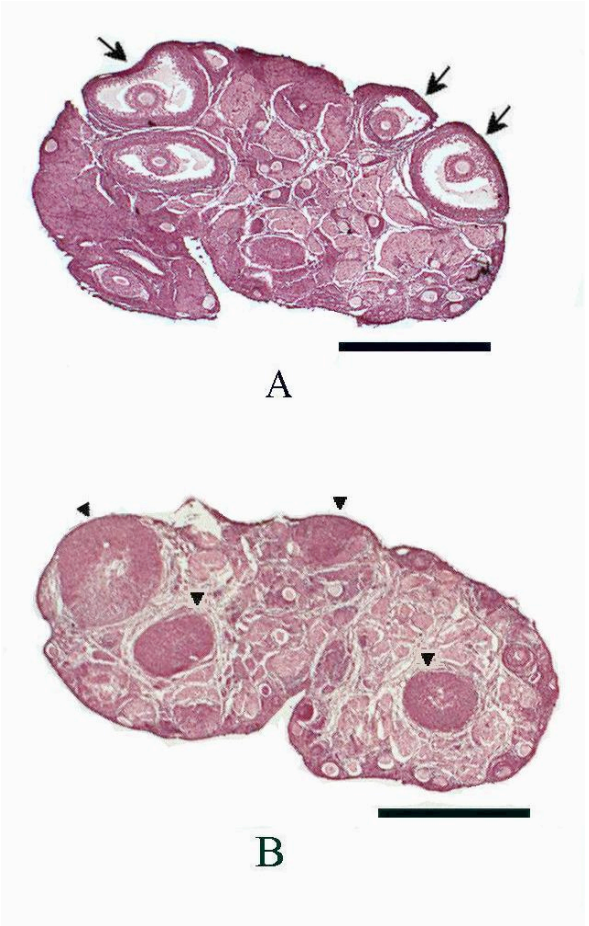

Figure 4. Sections of ovaries from (A) a 33-day-old stoat female on 2 dpc and (B) a 53-day-old stoat female on 9 dpc. Arrows indicate preovulatory follicles, arrowheads indicate *corpora lutea*. (Haematoxylin and Eosin stain; Scale bar = 1 mm)

One stoat female was studied one hour after mating. The right ovary and corresponding oviduct together with the most of the right uterine horn were removed and flushed. Although no ovulated eggs were recovered, motile spermatozoa were already present in oviductal and uterine flushings. The remaining reproductive tract of this female was investigated 8 months later. Eight degenerating eggs and two normal blastocysts were recovered. Old unfertilised eggs were embedded in a mass which was most probably of tubal or uterine origin as it was clearly an extra layer outside the *zona pellucida*, sometimes of irregular shape, and visible during routine light microscopy.

Cleavage stage embryos (3- to 4-cell) were recovered from the oviducts 7 dpc (Figure 5C). Morulae were first seen in oviductal flushings 9 dpc (Figure 5D). It seems that in the stoat the beginning of cavitation and embryo migration from the oviducts into the uterus occur on day 11 pc, since on this day the flushings yielded both morulae (Figure 5D) and early blastocysts (Figure 5E), and the embryos were evenly distributed between oviducts and uterine horns (Table 4). However, day-12 embryos were flushed from the uterine horns but not from the oviducts.

Embryos persisted in the uterine horns at the blastocyst stage from day 12 pc. until the following spring, i.e. 8 - 9 months (Table 4). Delayed stoat blastocysts contained a single layer of trophoblast cells surrounding a blastocoele. A disk consisting of round dark-coloured cells, i.e. the inner cell mass (ICM), protruded into this blastocoele cavity (Figure 5F). Although no signs of cell differentiation were noticed during the period of delayed implantation, the size of the embryos had increased and by the time of implantation the embryos were larger than at the beginning of delay (Figure 6).

To study the timing of implantation two females were investigated at the beginning of April. The first female was examined on April 2nd, which was day 234 of its pregnancy. Only one uterine horn of this female was investigated, the other remaining intact. The first black spots appeared on the winter fur of this female five days after the operation and it whelped 27 days after the operation, on May 5th. One of the four embryos flushed from its removed horn was fixed and sectioned (Figure 5G). A distinctly separated population of cells could be distinguished on the inner surface of the ICM (Figure 5H), indicating the start of gastrulation by the specialization of

primary endoderm. Another female was investigated 270 dpc, on April 6th immediately after the appearance of the first black spots on the winter fur of this female. Flushing of the embryos was impossible, and implantation sites were recorded.

Table 4. Embryo recovery results in the 13 female stoats during nine months of pregnancy (n= 25 uterine horns and oviducts, one uterine horn and oviduct was not investigated).

Days pc	Live spermatozoa in uterus/oviducts	PF or CL in ovaries	Embryos/eggs in uterus/oviducts	Nr of recovered eggs/embryos	Nr of horns/ oviducts
1-4	ut,ov	PF[c], CL[b]	absent[a], ov: oocytes[b]	11[b]	7
7,9	nd	CL	ov: Cleav, M	15	3
11	nd	CL	ov and ut: M, EB	3	1
12	nd	CL	ut: EB	5	1
21-251	nd	CL	ut: DB, AB[d]	66	12
272	not studied	not studied	ut: IMP	IMP	1
Total[e]				100	25

pc, *post coitum*; PF, preovulatory follicles; CL, *corpora lutea*, ov, oviduct; ut, uterus; Cleav, cleavage stages; M, morula; EB, early blastocyst; DB, diapausing blastocyst; nd, not detected; AB, blastocyst in activation; IMP, implantation

[a] Four females were studied days 1 - 4 pc (one female each day). Ovulation happened only on day 3 pc (one female) and on day 4 (one female)

[b] Detected only in ovulated females

[c] Detected only in non ovulated females

[d] Blastocysts with signs of activation were registered only in one female whose one uterine horn was flushed on April 2 and another uterine horn was left intact. This female gave birth 27 days after operation

[e] All flushed embryos were encompassed with intact embryonic coats

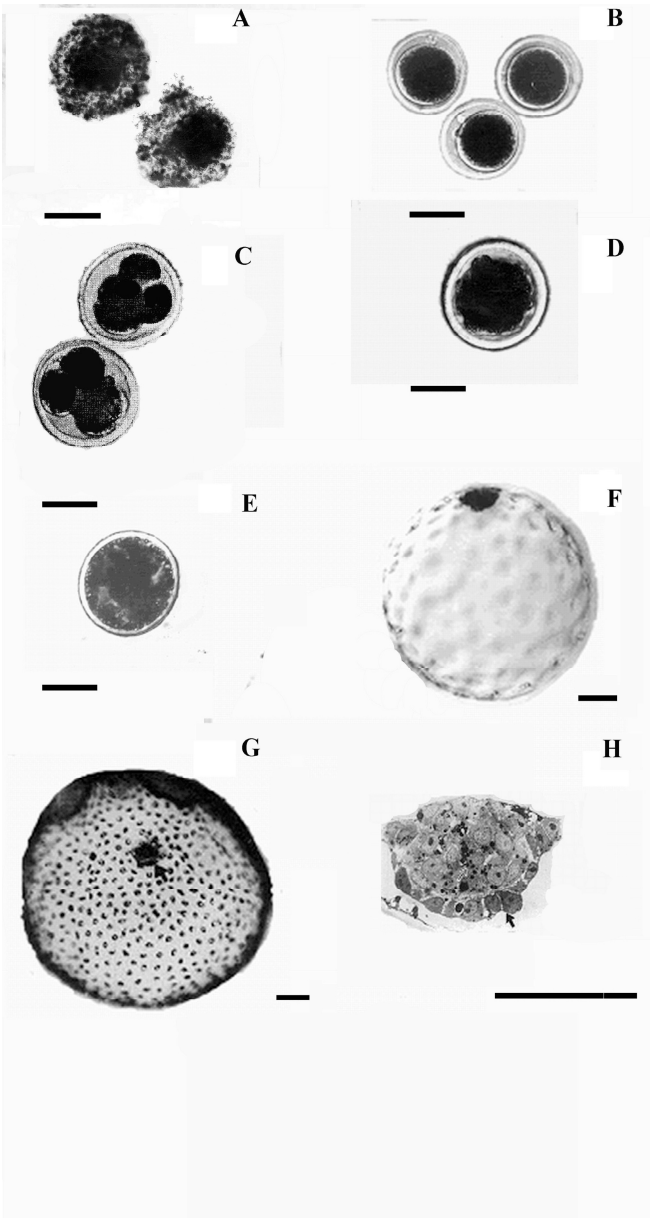

Figure 5. Preimplantation embryo development in the stoat from ovulation until implantation (representative examples). A. Oocytes before ovulation extracted from preovulatory follicle on day 2 pc. B. Ovulated oocytes, oviducts, 3 dpc. C. Cleavage-stage embryos, oviducts, 7 dpc. D. Morula, oviduct, 9 dpc. E. Early blastocyst, uterine horn, 12 dpc. F. Diapausing expanded blastocyst, uterine horn, 34 dpc. G. Expanded blastocyst at activation stage, a few days before implantation with initial stages of endoderm differentiation, uterine horn, 234 dpc.; arrow indicates ICM. H. Section through the ICM of the same blastocyst; arrow indicates the distinct layer of cells indicating the initial stage of endoderm differentiation. Modified from I. (Unstained; light microscopy; scale bar = 100 µm)

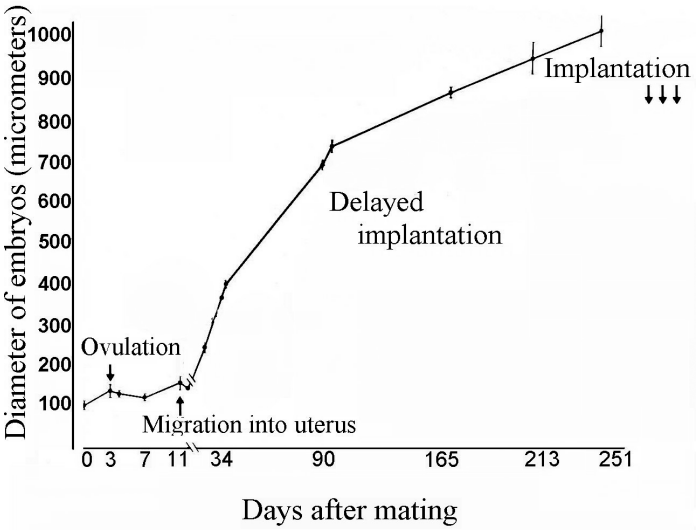

Figure 6. Embryonic growth in the stoat during the preimplantation period. Diameter of embryos at each day of pregnancy is represented as M ± SEM of the sample of embryos flushed on this day. More details on the numbers of embryos flushed during different periods of pregnancy are presented in Table 4. Modified from I.

5.2 Oestrous cycle in European mink

The time distribution of 39 females in oestrus during the 2004 season is shown in Figure 7. Fitting a Gaussian distribution to the data provided an estimate of peak activity at day 38.2, if we assume that day 1 was 1st March 2004. During the 2004 breeding season the first female was mated on 18th March and the last one on 10th May.

Data on the cyclicity of European mink females during the 2004 breeding season are listed in Table 5. The duration of the first oestrus was between 1 and 12 days. With one exception, all the females that were not mated at their first oestrus entered a second oestrus within 12 - 55 days. Most of these females were mated within the first three days of the second oestrus. Two of the females were not mated during the second oestrus and both then came into a third oestrus, one seven days later and the second 22 days after the second oestrus.

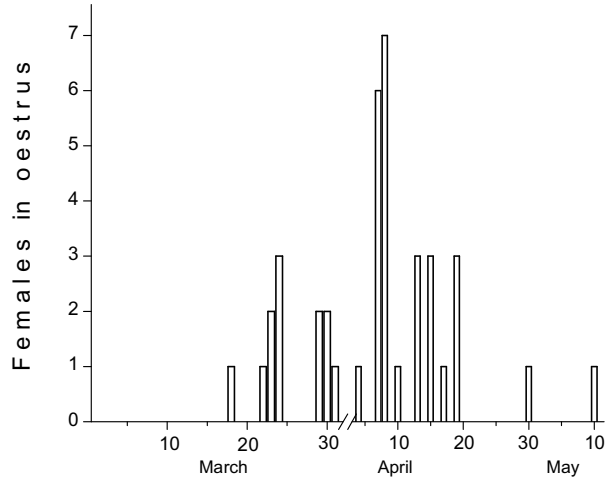

Figure 7. Frequency distribution of European mink females in first estrus during March – May of 2004, where day 1 is the 1st of March. Only females which reached oestrus the first time were counted. Modified from IV.

Table 5. Number of cycles and duration of oestrus in 13 European mink females that were not mated during their first oestrus in 2004. Values are means ± S.E.M.

Female number	Duration of 1st oestrus	Interval between 1st and 2nd oestrus	Duration of 2nd oestrus[a]	Interval between 2nd and 3rd oestrus	Duration of 3rd oestrus[a]
1	7	55	mated[b]		
2	5	21	12	22	mated
3	3		no oestrus[c]		
4	12	26	mated		
5	7	36	mated		
6	6	26	mated[b]		
7	6	12	mated		
8	7	28	mated		
9	10	42	mated		
10	5	12	mated		
11	1	30	mated		
12	9	26	1	7	mated
13	6	29	mated		
Average	6.5±0.8	28.6±3.4			

[a] Females were mated on the first day of their oestrus
[b] Females were mated on the third day of their oestrus
[c] Female did not exhibit second oestrus

5.3 Early pregnancy in European mink

5.3.1 Preimplantation embryo development in European mink

Preimplantation development in European mink is continuous, without implantation delay. Table 6 summarises the observations of its progress in the females examined during the 2004 breeding season when 26 embryos flushed from the females on days 2 - 6 of pregnancy and seven embryos flushed from females on days 7 - 11 were not used for embryo transfer, but instead were randomly chosen to investigate preimplantation development. In addition, two trophoblastic vesicles were flushed from one female on day 12 pc (already at implantation) and one of these vesicles was also processed and measured.

Ova or embryos were readily flushed from oviducts and uterine horns in all the pregnant females studied between 2 and 11 dpc, but on the first day pc no embryos/eggs were flushed and on day 12 pc flushing of the uterine horns was usually no longer possible as implantation occured.

No embryos or ova were flushed from the reproductive tract on day 1 pc, although motile spermatozoa were observed in both the oviducts and uterine horns. However, on day 2 pc, live spermatozoa were flushed from the oviducts, but not from the uterine horns. On this day, ovulated eggs and two-cell embryos were observed in the oviductal flushings (Figure 8A, Figure 9A). The absence of ovulated eggs in one animal on day 1 pc and their presence in one animal on day 2 pc suggests that ovulation in this species occurs during the night between the first and the second days pc.

On days 3 and 4 pc, there were no motile spermatozoa in the oviducts and uterine horns. Cleavage-stage embryos, mostly containing eight cells, were flushed from the oviducts on day 3 pc (Figure 8B, Figure 9B). On day 4 pc, embryos of different cleavage stages (up to 16 cells) were flushed from the oviducts (Figure 8C, Figure

9C). On day 5 pc, morula-stage embryos were flushed from the oviducts (Figure 8D), with cell numbers ranging from 19 to 43 (Figure 9D).

Table 6. Changes in the reproductive tract of the European mink females during first two weeks of pregnancy.

Days pc	Live spermatozoa in uterus/oviducts	PF or CL in ovaries	Embryos/eggs		Nr of recovered eggs/embryos	Number of females
			in oviducts	in uterus		
1	ut,ov	PF,CL			0 (ovulated eggs absent)	1
2	ov	CL	1-2 cell		4	1
3,4,5	nd	CL	Cleav, M		5; 7; 4	3
6	nd	CL	M	EB	2; 4	2
7	nd	CL		M, EB, B	4; 6; 10	3
8	nd	CL		B, ExB	5; 6; 3; 5; 6	5
9	nd	CL		ExB	5	1
10	nd	CL		ExB, EG	5; 4	2
11	nd	CL		EG	3	1
12	nd	CL	IMP		np*	3
13	nd	CL	IMP		np	1
16	ns	ns	IMP		np	1
Total					88	22

PF, preovulatory follicles; CL, corpora lutea; ov, oviduct; ut, uterus
Cleav, cleavage stages; M, morula; EB, early blastocyst; B, blastocyst
ExB, expanded blastocyst; EG, early gastrulation; IMP, implantation;
nd – not detected; ns – not studied; np – not possible
* In one donor animal two trophoblastic vesicles were accidentally flushed by extra pressure

Migration of European mink embryos from the oviducts into the uterus occurs on day 6 of their development. Two females were flushed on day 6 pc and, in both cases 50 % of the embryos were still in the oviducts and 50 % had already migrated into the uterine horns (two and two and one and one, respectively). The stage of the embryos on this day was either morula or early blastocyst (Figure 8E), with the number of cells ranging from 60 to 70 (morula) and from 76 to 108 (blastocysts) (Figure 9E).

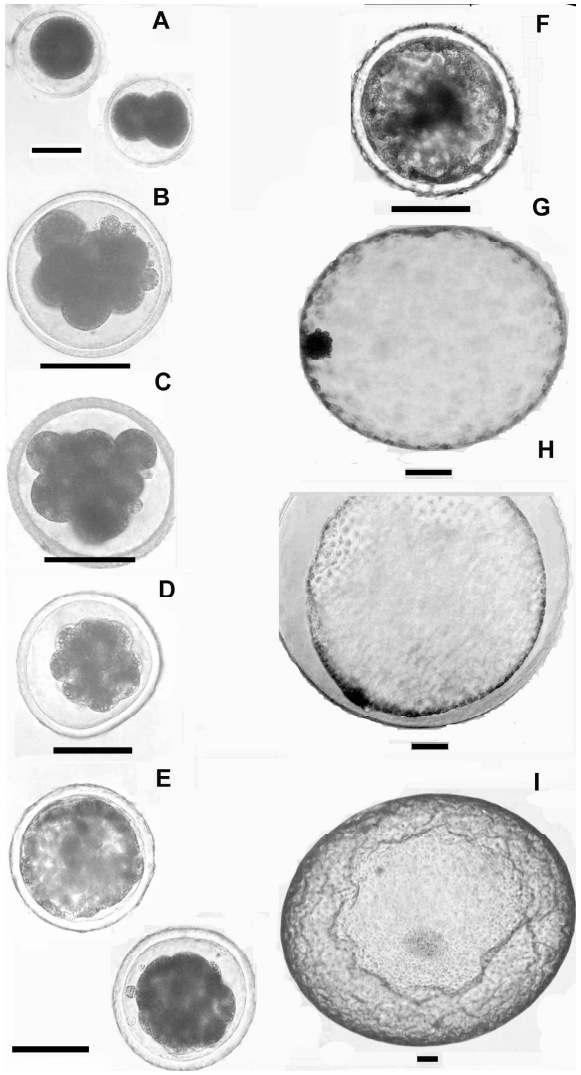

Figure 8. Preimplantation embryo development in the European mink from ovulation until implantation. A. Ovulated egg and two-cell embryo, oviduct, 2 dpc. B. Cleavage-stage embryo, oviduct, 3 dpc. C. Cleavage-stage embryo, oviduct, 4 dpc. D. Morula, oviduct, 5 dpc. E. Bottom: morula, oviduct; top: early blastocyst, uterine horn, 6 dpc. F. Blastocyst, uterine horn, 7 dpc. G. Expanding blastocyst, uterine horn, 8 dpc. H. Blastocyst with initial stages of endoderm differentiation, uterine horn, 10 dpc. This blastocyst is slightly collapsed with a visible gap between the *zona pellucida* and trophoblast layers. I. Blastocyst with continuous endoderm differentiation, uterine horn, 11 dpc. (Unstained; light microscopy; scale bar = 100 μm). Modified from III.

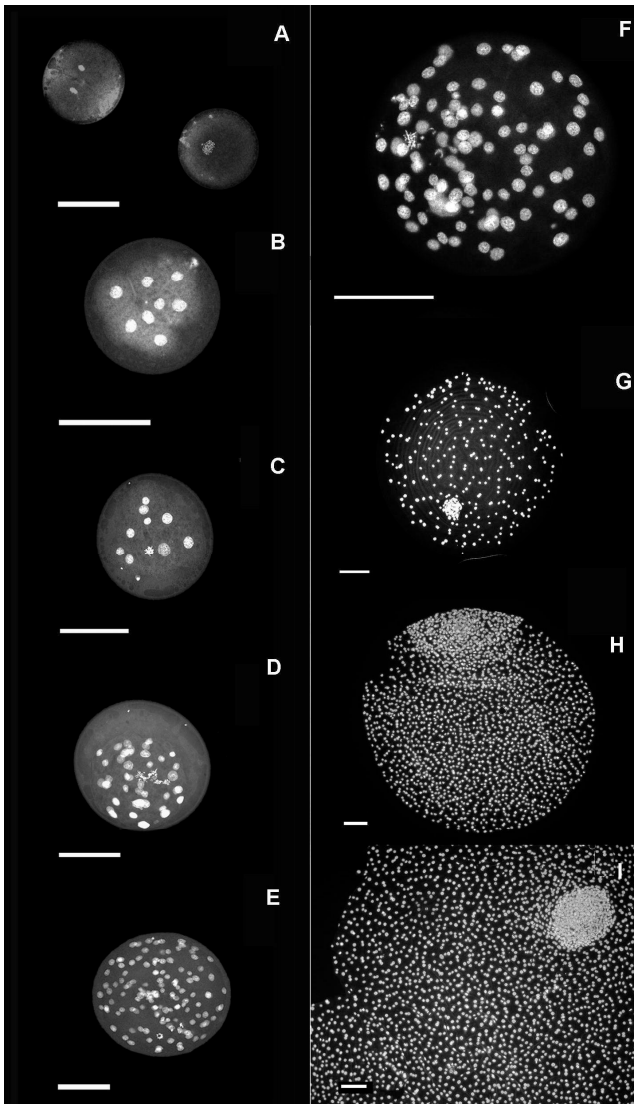

Figure 9. Preimplantation embryo development in the European mink from ovulation until implantation. A. Ovulated egg and two-cell embryo, oviducts, 2 dpc. B. Cleavage-stage embryo, oviduct, 3 dpc. C. Cleavage-stage embryo, oviduct, 4 dpc. D. Morula, oviduct, 5 dpc. E. Early blastocyst, uterine horns, 6 dpc. F. Blastocyst, uterine horn, 7 dpc. G. Expanding blastocyst, uterine horn, day 8 dpc. H. Blastocyst with initial stages of endoderm differentiation, uterine horn, 10 dpc. I. Blastocyst with continuous endoderm differentiation, uterine horns, 11 dpc. (DAPI: 4′,6′-diamidino-2-phenylindole staining (2 μg mL^{-1}), fluorescent microscopy. Scale bar = 100 μm). Modified from III.

Blastocysts of different sizes were recovered in the European mink from the uterine horns on day 7 pc (Figure 8F) and onwards. The number of cells was slightly higher than 100 for day-7 blastocysts (Figure 9F). On day 8 pc (Figure 8G) the blastocysts markedly expanded in size, although they still contained ICM without signs of differentiating. The number of cells was approximately 300, sometimes even more (Figure 9G). On day 10 pc some differentiation started and the formation of a distinct layer of cells in the ICM was observed (Figure 8H, Figure 9H), this process being more prominent on day 11 pc (Figure 8I, Figure 9I).

The diameters of cleavage-stage embryos, morulae and early blastocysts did not differ until day 7 pc. On day 8 pc, there was visible about two-fold increase in the size of blastocysts, as until 7 dpc the embryos were about the same size as were ovulated eggs (Figure 10). The size of the embryos continued to progressively increase on days 9 - 10 and particularly on day 11 pc, when the blastocysts had achieved a size of 1.5 mm. From day 12 pc, signs of implantation were evident in pregnant European mink females. However, trophoblastic vesicles (without ICM) were flushed from the uterine horns of one of these (12 dpc) females when the flushing pressure was increased. It was not possible to flush the uterine horns of the other two females, and implantation was confirmed by studying the fixed uterus. For all three day-12 females, the sites of implantation were clearly visible (see section 5.3.3 for more details).

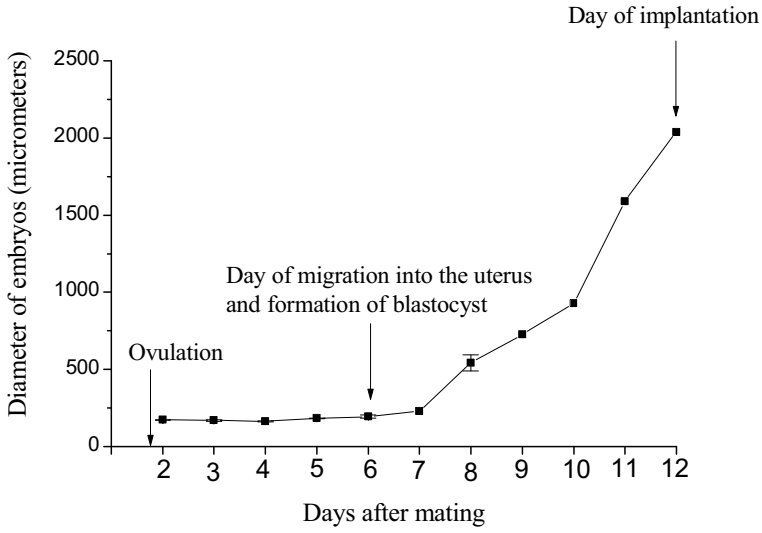

Figure 10. Embryonic growth in the European mink during the preimplantation period. All flushed embryos were measured and studied on days 2 - 6 pc. One randomly selected embryo from day-7 donor was studied. Three randomly selected blastocysts from three different donor females were studied on day 8 pc. One randomly selected embryo was studied per donor female on each of days 9 - 11. One trophoblastic vesicle which was accidentally flushed from donor female at implantation (12 dpc) was studied. Diameter of embryos at each day of pregnancy is represented as M ± SEM of the sample of embryos as it was on days 2 - 6 and 8 pc. See Table 6 for more details on the numbers of embryos/donor females. Modified from III.

All the European mink blastocysts flushed during the preimplantation period were enveloped by intact embryonic coats. Even one day before implantation, 11 dpc embryos were well inside of the coats and no signs of hatching were observed. Shrinking of the trophoblastic layer away from the coats, i.e. partial collapse of the blastocyst, was observed in some (although not all) of these blastocysts when they were flushed from uterus (Figure 8H).

5.3.2 Functional status of *corpora lutea* during the beginning of pregnancy

Large preovulatory follicles, smaller follicles and the development of a new *corpus luteum* (CL) were observed in European mink ovaries on day 1 pc (Figure 11A). Figure 11B illustrates the same ovary at a larger magnification, showing the details of a preovulatory follicle, exemplified by clear evidence of a developed antrum, theca, corona radiata, and oocyte. During the more advanced stages of pregnancy (day 8 pc) some developing follicles can still be found, but luteal cells have already proliferated and occupy the ovulated follicle (Figure 11C), with a blood clot still visible in the centre of the CL (Figure 11D). On day 12 pc, when implantation occurs, developing follicles are still in evidence, along with a completely formed CL (Figure 11E). The development of luteal cells was progressive throughout the entire 12-day period of observation. On day 12 pc those cells are circular in cross-section and resemble fully developed functioning luteocytes (Figure 11F).

The overall mean values of European mink faecal progesterone concentrations for oestrus/day of mating (day 0) and midpregnancy (day 8, day 12) as well as the very end of pregnancy (on day 40) animals are presented in Table 7. Individual progesterone levels in the same females are presented on Figure 12. ANOVA demonstrated a significant effect of the stage of pregnancy/day of observation ($F[3.29]=8.75$, $P < 0.001$). *Post hoc* comparisons revealed that the faecal progesterone concentration was significantly higher on day 8 of pregnancy than on day 0 ($P<0.05$), and this elevation was even more pronounced on day 12 ($P<0.001$). At the end of pregnancy, on day 40 pc, the progesterone concentration did not differ significantly from the initial peri-copulatory levels (Table 7, Figure 12).

Faecal progesterone concentrations were compared between European mink and European polecat females on two critical days, day 0 (day of oestrus = day of mating) and day 12, when implantation occurs in both species. At oestrus, on the day of mating, the faecal progesterone concentration was more than four times lower in European mink than in the European polecat, and the comparative difference persisted at day 12 (day of implantation) (Figure 13).

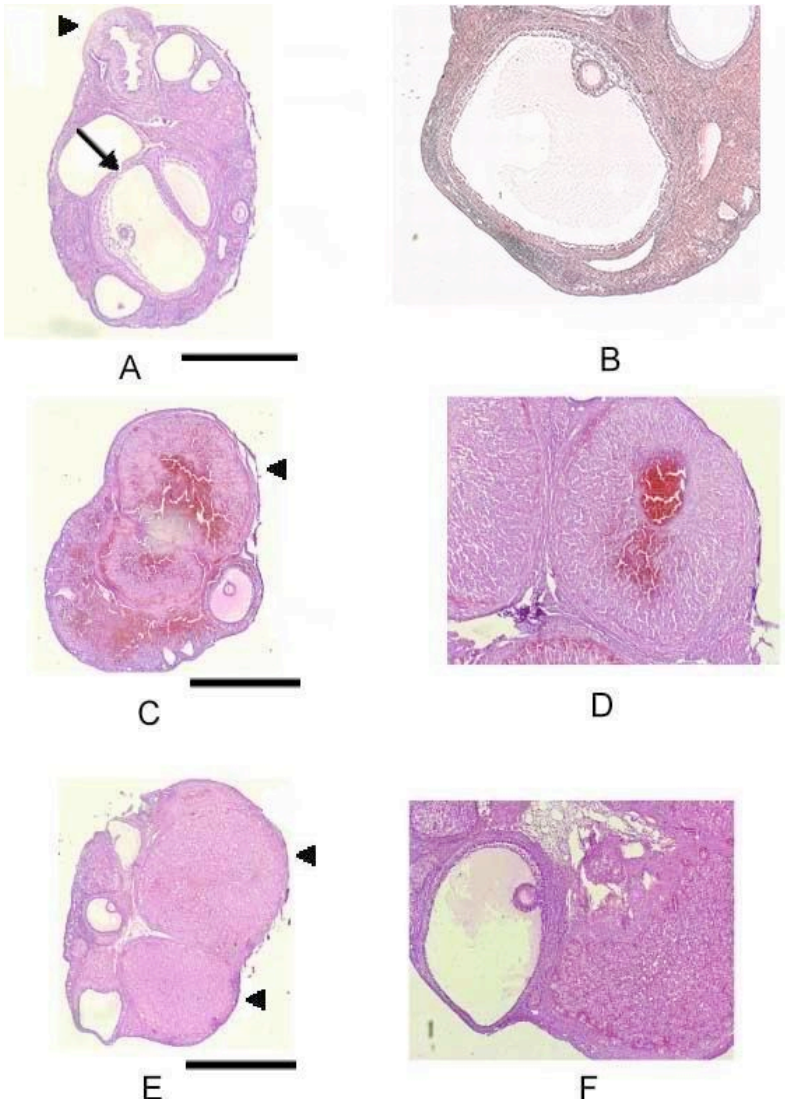

Figure 11. Histology of European mink ovaries. Arrow indicates a preovulatory follicle. Arrowheads indicate *corpora lutea*. A. Section of ovary from European mink on day 1 pc. B. Higher power view of a different section of the same ovary with details of the preovulatory follicle. C. Section of an ovary on day 8 pc. D. Higher power view of the same ovary with a *corpus luteum.* E. Section of an ovary on day 12 pc. F. Higher power view of the same ovary with details of a *corpus luteum* (right) and an antral follicle. Haematoxylin and Eosin stain; Scale bar = 1 mm. Modified from IV.

Table 7. Faecal progesterone concentration (ng/g dry weight) of oestrous and pregnant European mink and European polecat females during the breeding season of 2007. Values are means ± S.E.M.

Days post coitum	Number of European mink	Average progesterone concentration	Number of European polecats	Average progesterone concentration
0	10	42.70±14.79	4	177.7±30.3
8	10	107.68±22.82*	4	222.1±122.9
12	10	176.44±23.00**	4	1051.7±438.6
40	3	103.18±20.05	4	263.2±70.0

*P<0.05; **P<0.001 as compared to females at day 0

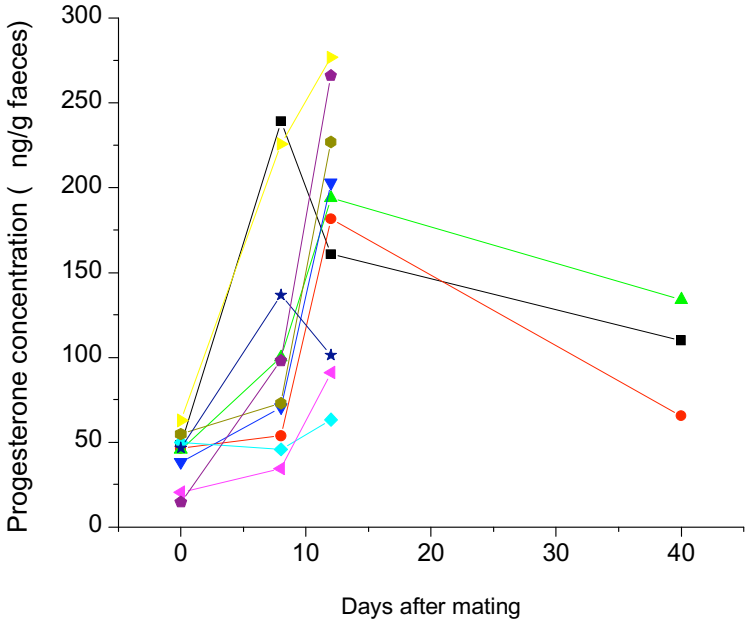

Figure 12. Individual faecal progesterone concentrations in ten European mink females on days 0, 8, 12 and, in three of them, on day 40.

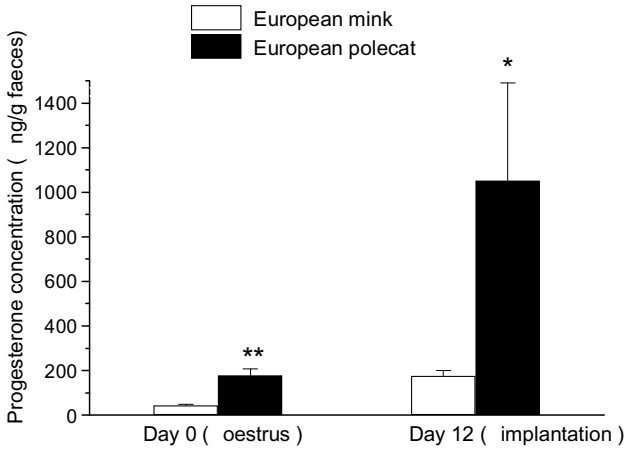

Figure 13. European mink and European polecat faecal progesterone concentrations at two critical points: oestrus and the day of implantation. *P<0.05; **P<0.001 as compared to European mink females. N = 10 European mink and 4 European polecat females.

5.3.3 Implantation in European mink

As described above, embryos or eggs were readily flushed from oviducts and uterine horns in all pregnant European mink females analysed from day 2 pc until day 11 pc. Attempts were made to flush the uterine horns of five females at about the time of implantation (3 females on day 12, one female on day 13 and one female on day 16). On or after day 12, however, flushing of embryos was found to be no longer possible, although in one of three females analysed on day 12 pc extra pressure made it possible to flush out two trophoblastic vesicles from one uterine horn. The females had swellings along the uterine horns indicative of implantation sites already on day 12 pc (Figure 14). The trophoblast is closely apposed to the uterine epithelium at this stage (Figure 15).

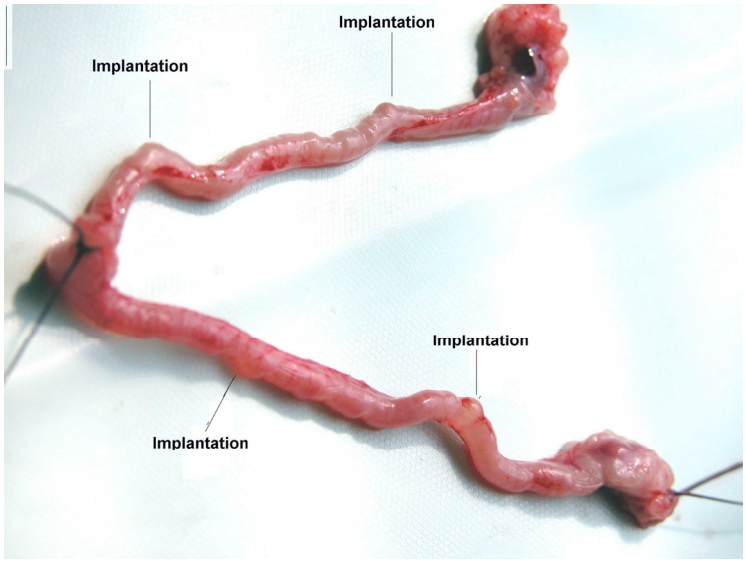

Figure 14. The reproductive tract of a European mink at day 12 of pregnancy. Sites of implantation are indicated.

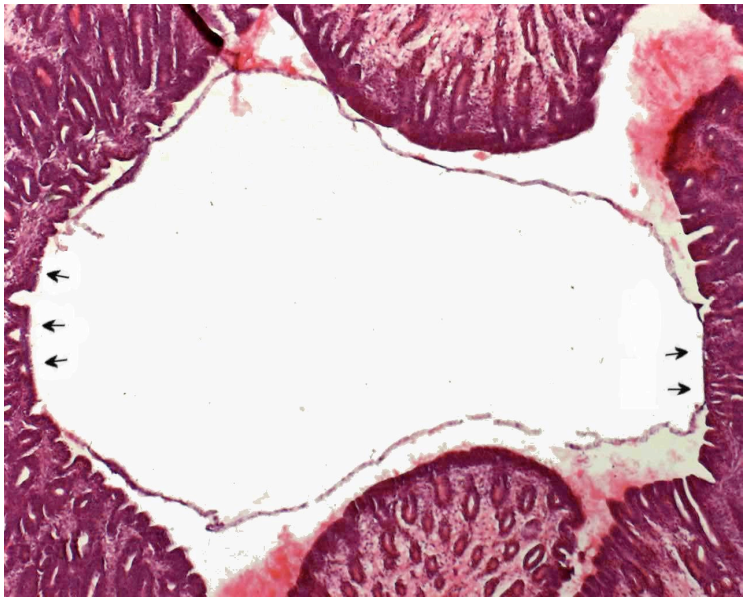

Figure 15. Implantation chamber with an implanting European mink embryo 13 dpc. Trophoblast is closely apposed to the uterine epithelium. Areas of cytotrophoblast formation are indicated with the arrows. No signs of decidualization are seen in uterine cells.

5.4 Embryo technological approach for European mink conservation

5.4.1 Overcoming the interspecies barrier: transfer from European mink to honoriks/nohoriks

A total of 72 European mink blastocysts were transferred into 12 hybrid recipients (Table 8). Two recipients (numbers 9 and 14) received one and two embryos respectively, and failed to whelp, while the other 10, which each received from 4 to 10 embryos, whelped (Figure 16). The overall live birth rate was 50% (36 kits/72 transferred embryos). The average weight of the 12 recipients was 769.1±30.4 g (range 584 to 930 g), and the average litter size of pregnant females (n=10) was 3.6±0.6 (range 1 to 6 kits per litter).

In 2002, the average weight of the 10 normally bred European mink (control group) was 748±27 g (range 555 to 890 g), and the average litter size of pregnant females was 3.8±0.5 kits (range 1 to 6 kits per litter) (Table 9). The average weight of 6 recipient females was correspondingly 801±41g and the average litter size of 5 pregnant females was 3.2±0.7 kits (range 1 to 5 kits per litter). Neither the average weight of the females nor the litter size differed between the embryo transfer and control groups during the spring of 2002.

In 2004, the average weight of the 10 normally bred European mink (control group) was 690±16 g (range 607 to 760 g), and the average litter size of pregnant females was 4.8±0.5 kits (range 3 to 7 kits per litter) (Table 9). The average weight of 6 recipient females was correspondingly 737±45 g and the average litter size of 5 pregnant females was 4.0±0.9 kits (range 1 to 6 kits per litter). Neither the average weight of the females nor the litter size differed between the embryo transfer and control groups during spring of 2004.

In 2004, five day-8 European mink blastocysts were transferred together with five day-7 European polecat blastocysts into the same recipient honorik female (Table 8). Five live kits (one polecat and four minks) were born, three of which survived (Figure 17).

Table 8. Summary of embryo transfers from European mink to European polecat - European mink hybrids (honoriks and nohoriks) during the breeding seasons 2002 and 2004. Values are means ± S.E.M.

Donor female number	Days after first mating[a]	No of embryos transferred	Recipient female genotype	Days after first mating[a]	Weight of recipient (g)	Kits born	Live kits after 10 days
Breeding season 2002							
1	7	5	honorik	6	835	1	0
2,3	7[b],8[b]	3+5	honorik	7[b]	930	2	0
4,5	8	6+1	honorik	7	735	4	2
6,7	7,8	4+1	honorik	7	830	4	4
8	8	6	nohorik	6	642	5	3
9	7[b]	1	honorik	6[b]	835	0	0
Total		32				16	9
Average		5.3±1.0			801±41	2.7±0.8	1.5±0.7
Breeding season 2004							
10,11	7,8	3+4	honorik	7	894	6	5
12,13	8,9	5+3	nohorik	8	584	3	3
14	11	2	nohorik	10	656	0	0
15,16[c]	7,8	5+5	honorik	7	764	5[d]	3[e]
17,18	8,10	5+4	honorik	8	804	5	3
19	10	4	honorik	10	720	1	0
Total		40				20	14
Average		6.7±1.2			737±45	3.3±1.0	2.3±1.0
Breeding seasons 2002 and 2004							
Total		72				36	23
Average		6.0±0.8			769±30	3.0±0.6	1.9±0.5

[a] Day 0 = first day of mating of the donor females and recipients
[b] Donor females and recipients were mated once daily on two consecutive days
[c] European polecat donor female
[d] One of the kits born was a European polecat, all other kits were European mink
[e] One of the surviving kits was a European polecat, two other kits were European mink

Figure 16. Litter born in 2004 as a result of transfer of European mink embryos into a honorik recipient female. Kits are in the hands of Dasha Volkova. (Courtesy of Valery Cindrenko, photo taken during weaning period).

Figure 17. European polecat (lightest coloured and biggest, in the upper part of the figure) and two European mink kits born as a result of a mixed pregnancy: transfer of five European polecat and five European mink embryos into a hybrid (honorik) recipient female. Kits are in the hands of Yulia Ternovskaya. (Courtesy of Valery Cindrenko, photo taken during weaning period).

Table 9. Breeding results of the control European mink females during the breeding seasons of 2002 and 2004. Values are means ± S.E.M.

No of female	Weight of female	Duration of pregnancy	No of kits born	No of kits at Day 10
Breeding season 2002				
1	790	42	6	6
2	555	43	2	2
3	735	42	4	4
4	749	42	4	4
5	765	44	3	3
6	890	43	4	4
7	740	43	6	5
8	736	42	1	1
9	717	41	4	1
10	800	41	4	4
Total			38	34
Average	748±27	42.3±0.3	3.8±0.5	3.4±0.5
Breeding season 2004				
11	702	41	5	5
12	607	42	7	7
13	739	41	5	5
14	745	42	5	5
15	673	42	7	7
16	691	43	3	3
17	674	42	6	6
18	693	42	3	3
19	620	42	4	4
20	760	43	3	0
Total			48	45
Average	690±16	42.0±0.2	4.8±0.5	4.5±0.7
Breeding seasons 2002 and 2004				
Total			86	79
Average	719±16	42.0±0.2	4.3±0.4	4.0±0.4

5.4.2 Monitoring kit survival and development

In the 10 whelping hybrid recipients, the average litter size was reduced from 3.6±0.6 to 2.3±0.6 kits (range of reduction 0 - 2 kits per litter) after the first 10 days. Thus, in the embryo transfer group, 13 of the 36 term European mink kits were lost (36 % postnatal mortality). In the naturally bred group of European mink, death was observed only in 3 of 10 litters with a loss of 1 kit, 3 kits and 3 kits respectively (Table 9), i.e. 7 of 86 kits

were lost (8 % postnatal mortality). After postnatal day 10, no loss of kits was registered in either ET or Control groups (Table 10). During both breeding seasons, 2002 and 2004, mortality rate during first ten days of life of ET-born kits was higher as compared to naturally born kits. However, in 2004, it was less pronounced in both groups (30.0 % and 6.3 % in 2004 vs 43.8 % and 10.5 % in 2002, respectively). Chi-square test confirmed that mortality rate during first ten days of postnatal life was significantly higher in the ET group as compared to the naturally bred group (P=0.05).

Table 10. Survival rates of European mink kits born after embryo transfer and natural mating during the breeding seasons of 2002 and 2004.

Offspring	No of kits born	No of kits at day 10	Kits surviving at day 90	
			Number	% of kits born
ET male kits	20[a]	13[a]	13[a]	65
Control male kits	38	36	36	95
ET female kits	16	10	10	63
Control female kits	48	43	43	90
Total ET kits	36	23	23	64
Total control kits	86	79	79	92

[a] The European polecat kit born as a result of embryo transfer is included

In 2002, the average birth weight of the kits in the embryo transfer group was significantly lower than that of the kits born after natural breeding of European mink (Figure 18A). This difference (P<0.001) was more pronounced for female than for male offspring (P<0.01). The average birth weight of newborn females was below the lower border of the birth weight range for European mink. The weight differences between males and females observed in the embryo transfer group were already apparent at birth (P<0.05), although there were no such sex differences in birth weight in naturally born kits. At 10 days of age, the female offspring from the embryo transfer group still weighed less than the controls (P<0.05), but there were no significant differences in the weights of the male offspring. Both groups were sexually dimorphic at this age, with females weighing less than males (Figure 18C). At 90 days of age, no weight differences between the embryo transfer and control groups were detectable, although there was pronounced sexual dimorphism within both the ET and control groups (Figure 18E).

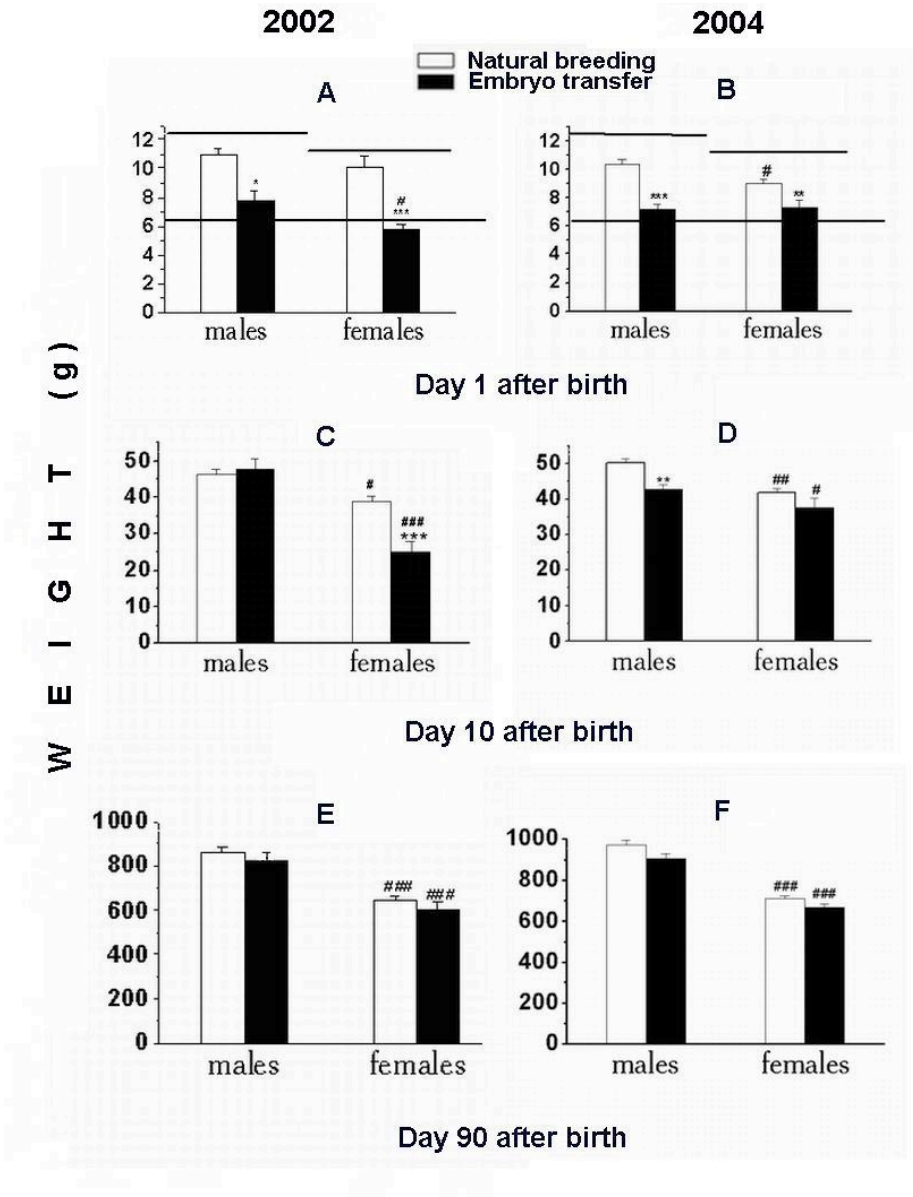

Figure 18. Body weights (±S.E.M.) of European mink term offspring produced after embryo transfer and after natural mating during the breeding seasons of 2002 and 2004. *-P<0.05; **- P<0.01; ***-P<0.001 as compared to the weight of naturally born kits within the same category. #-P<0.05; ##-P<0.01; ###-P<0.001 as compared to the males within the same category. Lines in A and B show the upper and the lower borders of the birth weight for European mink according to more than 20 years of observation at the *Mustelidae* Research Station in Novosibirsk (from Ternovsky, Ternovskaya, 1994). The European polecat kit born as a result of embryo transfer is not included.

In 2004, the average birth weight of the kits from the embryo transfer group was significantly lower than that of the kits born after natural breeding (Figure 18B), this

difference being more pronounced for male offspring ($P<0.001$), though there was also a difference in the female offspring ($P<0.01$). It is noteworthy that the weight differences between males and females observed in the control group were already apparent at birth ($P<0.05$), which was not the case in the 2002 experiment. In contrast, there was no weight difference between males and females at birth in the embryo transfer group, which was also opposite to the result of the year 2002. At 10 days of age, the male offspring from the embryo transfer group still weighed less than the controls ($P<0.01$), but there were no longer any significant differences in the weights of the female offspring in the two groups. Both ET and naturally-born kits were sexually dimorphic at this age, with male heavier than females in both groups (Figure 18D). At 90 days of age, no weight differences between the embryo transfer and control groups were detectable: the only difference registered at this age was that males weighed more than females (Figure 18F).

6. DISCUSSION

6.1 Oestrus and early pregnancy in the stoat

Investigation of early pregnancy events with special emphasis on preimplantation embryo development in the stoat, a diapausing mustelid species, was one of the aims of this study and was addressed in article I. Principal results were the confirmation of oestrus and successful mating in juvenile stoat female and, for the first time, documentation of ovulation, preimplantation embryo development, embryonic diapause and implantation in these stoat females impregnated as juveniles.

6.1.1 Oestrus in juvenile stoat females

One of the important findings of this study is that during the period between May and August 1991 all the juvenile stoat females from the captive population at the *Mustelidae* Research Station in Novosibirsk demonstrated oestrus and all the females used for natural mating with adult males were already in oestrus at the age of 20 days (I).

This is in good agreement with the results of Ternovsky and Ternovskaya (1994) who produced litters from 58 young stoat females mated during May - August over a number of consecutive years starting in 1971. At the time of mating these females were aged 17 to 88 days (with two exceptions: one female was aged 133 days and another was aged 134 days).

Taken together, observations reported in this Thesis and by Ternovsky and Ternovskaya (1994) allow us to conclude that, in the northern hemisphere, female stoats enter oestrus during the first month of their life, i.e. during May - June, and may stay in oestrus the whole summer until impregnated by an adult male. Similar precocious sexual maturation and the possibility of females entering oestrus during their first month of life have been reported for the long-tailed weasel (*Mustela frenata*) (Wright 1964 cited in King 1989), an American close relative of the stoat (Koepfli et al. 2008).

It is not known what triggers the follicular development in the juvenile females of the stoat and long-tailed weasel. Milk of the mother might be considered as such a trigger if the dams themselves were to be in oestrus during this *post-partum* period. However, dams are not normally in oestrus while nursing their kits; normally they enter oestrus after weaning, when they have finished lactating (Ternovsky and Ternovskaya 1994). *Post-partum* oestrus might occur in adult stoat (but not in long-tailed weasel) females (King 1989), but this has never been observed in the captive-bred stoat population in Novosibirsk (Ternovsky and Ternovskaya 1994; personal communication from Ternovskaya 2009) although, in Novosibirsk, 39 litters were born from the adult females and 58 litters were born from females that conceived while still kits. Therefore, the stoat represents one fascinating enigma of reproduction, when kits reach oestrus and start to reproduce before their own mothers reach their oestrus after weaning.

Precocious sexual maturation of females sometimes takes place in other species, including mice (Vandenberg 1967, 1969) pigs (Brooks and Cole 1970) and humans (Dacou-Voutetakis 1997), but it is associated with either the effects of pheromones from the adult male (Vandenberg 1967, 1969, Brooks and Cole 1970) or disorders of puberty (Dacou-Voutetakis 1997, Blondell et al. 1999): the role of environmental oestrogens in particular has been discussed (Hermann-Giddens et al. 1997). In the *Mustelidae* species studied so far, the sexual maturity of newborn females has been observed exclusively in the stoat (*Mustela erminea*) and in its American counterpart the long-tailed weasel (*Mustela frenata*) (Wright 1964, Ternovsky 1983, King 1989, Ternovsky and Ternovskaya 1994). In these two mustelid species precocious sexual maturation of females is not pathological but, rather, a reproductive peculiarity associated with obligate diapause.

The stoat is not the only mustelid species to display continuous oestrus for several months; ferrets/polecats, species of the same genus (*Mustela*) (Koepfli et al. 2008), demonstrate prolonged periods of oestrus as well. Once a female domestic ferret (*Mustela putorius furo*) enters oestrus, it normally continues for more than 3 months until it is terminated by mating or hormonal injection (Hammond and Marshall 1930, Williams et al. 1992). Other polecats/ferrets (e.g. steppe polecat, black-footed ferret,

European polecat) demonstrate a similar prolonged oestrous period confirmed by vaginal smear cytology (Mead et al. 1990, Williams et al.1992).

6.1.2 Preimplantation embryo development in stoat

Preimplantation embryo development in stoat was thoroughly investigated as the first aim of this Ph.D. study. From a formal point of view preimplantation embryo development in the stoat may be considered to consist of three phases:
oviductal passage/cleavage/compaction,
uterine migration/diapause,
and activation/implantation.

Ovulation in the stoat and European mink is discussed below in 6.3.1 in a comparative context. Our results demonstrate that the rate of embryonic development during oviductal passage is slower in stoats than in most mammalian species (I). Only on day 9 pc did stoat embryos reach the morula stage, although they were still in the oviducts (I). These findings are in agreement with those of LaFalci and Molinia (2007), who recovered cleavage-stage stoat embryos from the oviducts 5 - 6 days after mating.

In the great majority of mammalian species the oviductal journey is much shorter in duration and the rate of embryonic development in most mammals is faster than in the stoat. For example, murine embryos enter the uterus 66 - 96 hpc at the stage of 4 - 8 cells (McLaren 1982, Handyside and Hunter 1986, Hardy and Spanos 2002, Stanton et al. 2003). However, in some *Canidae* such as dogs the oviductal passage of embryos lasts longer, until day 8 - 9 after ovulation or 10 - 12 days after the preovulatory LH surge (Concannon and Lein 1989, Valtonen 1992). This prolonged period of oviductal passage in canids is associated with the ovulation of immature oocytes (Valtonen 1992); in contrast to most other mammalian species, oocytes in *Canidae* are ovulated at the Prophase I stage of meiosis (Farstad 1993, Farstad et al. 2001) and thus additional time for their maturation in oviducts is needed. In stoats, however, oocytes are ovulated at the M-II stage and we always have seen the first polar body in ovulated stoat oocytes (I). Thus the reasons for the prolonged oviductal passage in dogs and stoats may be different.

Bats are another group of mammals which are characterized by a slow rate of embryonic development until the morula stage and prolonged passage in oviducts. In some bats, cleavage embryos are unable to begin compaction for about 8 - 10 days and stay in the oviducts at the 6 - 8-cell stage (Rasweiler 1993, Pakrasi and Tiwari 2007). Although the precise mechanisms of these phenomena may differ in bats and stoats, both of these species, along with dogs, may be counted as the mammals with the slowest rate of embryonic cleavage and the longest duration of passage through the oviducts. It has been shown recently that the differential arrest of embryo cleavage and the delay in compaction are caused in some species of bats by the inability of the *corpora lutea* to provide enough progesterone (Pakrasi and Tiwari 2007).

In stoats, we observed only very small CL in the ovaries during the oviductal passage of embryos and it has been shown earlier that plasma levels of progesterone in pregnant stoat females remain very low during the whole implantation delay period, which lasts throughout autumn and winter until the following April (Gulamhusein and Thawley 1974). Thus, insufficient progesterone secretion by the CL may be considered as one of the main reasons for the prolonged oviductal passage in both stoats and bats. Another obvious reason for the late entrance of stoat embryos into the uterus is that there is a 3 - 4 day gap between copulation and ovulation (I).

Even though stoat embryos enter the uterus at 11 dpc, later than do embryos in other mustelids, they still migrate into the uterus at the morula stage. This is characteristic of mustelids, being the stage at which embryos enter the uterus for both the species under investigation in this thesis, i.e. the European mink (III) and stoat (I), and also for polecats/ferrets (Robinson 1918, Lindeberg and Järvinen 2003, Lindeberg 2003, Lindeberg 2008).

6.1.3 Delayed implantation in the stoat

It was experimentally demonstrated in this study that delayed implantation in stoats lasts 8 to 9 months (I). This is in agreement with the results of Ternovsky and Ternovskaya (1994) who, in 58 young stoat females impregnated as juveniles, found that the whole pregnancy (including diapause and the postimplantation period) lasted from 224 to 356 days (on average 317 days) i.e. about 10.5 months.

In European mink, during the first week after mating the concentration of progesterone in the faeces more than doubles, and on day 12 pc it is even four times higher than in unmated females. The doubling coincided with the embryo's advancing into the uterus (6 dpc), and the rapid expansion of the blastocyst, leading to implantation at 12 dpc (III, IV). The rapid development of CL during this period resulted in their acquiring all the signs of functioning luteocytes (IV). In ferrets, the increase in progesterone concentration in plasma, faeces and urine (Blatchley and Donovan 1976, Daniel 1976, Mead et al. 1990, Young et al. 2001) and the development of CL (Joseph and Mead 1988) during the first two weeks of pregnancy are as rapid as in European mink. In stoats, on the other hand, no visible changes occur in the CL during the first weeks after mating (I) and the concentration of progesterone remains at the level of unmated females for more than half a year (Gulamhusein and Thawley 1974). Thus, the reason for the slowing down of stoat embryo development in the uterus, and for embryos remaining in a state of dormancy for several months, may be non-functional CL during the period of diapause.

It has been shown that the passive immunization of pregnant ferret females with monoclonal anti-progesterone antibody (Rider and Heap 1986) drastically slowed down the rate of embryonic development, i.e: arrested embryonic cleavage, and in many cases also prevented their migration from the oviducts into the uterus. A similar effect on the rate of early embryonic development has been observed in ferrets and American mink after ovariectomy. If ovaries were removed (2 dpc in mink or 3 dpc in ferret), then significantly more embryos appeared to be behind in their development when they entered the uterus than in sham-operated controls in both species (McRae 1994). Exogenous progesterone administered as silastic implants to ovariectomized pregnant mink females alleviated the effect of ovariectomy, significantly decreased the percentage of degenerated embryos, and also decreased the percentage of embryos which were behind in their development (McRae 1994).

The sable (*Martes zibellina*) may be used as a reference for the stoat because it has similar obligate implantation delay. In sables, the overall length of gestation is 250 - 300 days (Ternovsky and Ternovskaya 1994, Bates 2002) and the duration of the implantation delay is about eight months, with postimplantation embryonic

development taking only 25 - 30 days (Baevsky 1955, 1960, Bates 2002). The duration of the implantation delay in the badger (*Meles meles*) is even longer, about 10 months (Canivenc and Bonnin 1979, Yamaguchi et al. 2006).

Some North American mustelids, e.g. the western spotted skunk (*Spilogale putorius*) and marten (*Martes Americana*), have a long obligate period of delayed implantation which normally lasts approximately 6.5 - 7 months in skunks (Enders et al. 1986) and 7 - 8.5 months in martens (Jonkel and Werkwerth 1963). These species may also be used as references for stoats, especially if we take into account the fact that in the western spotted skunk the morphological changes in the blastocyst during delayed implantation (Enders et al. 1986) as well as the mechanisms of implantation (Enders and Mead 1996) have been comprehensively studied.

In mustelids and marsupials, the size of blastocysts normally increases during the period of implantation delay (Renfree and Shaw 2000). About a five-fold increase in the diameter of stoat blastocysts was observed during the period of delayed implantation although, after 3 months, the stoat embryos were more ellipsoidal than spherical and these changes from round to ovoid form were more pronounced towards the end of the delay (I). The gradual increase in the diameter of the diapausing blastocyst in *Mustelidae* is due to fluid accumulation within the blastocoele and increased cell numbers in the trophoblast, as has been confirmed for the sable (Baevsky 1955, 1960), western spotted skunk (Enders et al. 1986) and European badger (Yamaguchi et al. 2006). Blastocysts of the western spotted skunk increase in size as well, reaching more than 1 mm in diameter toward the end of the implantation delay period (Enders et al. 1986).

6.2 Reproductive biology and early development in European mink

Two other aims of the study were (1) to investigate the *in vivo* embryo development in European mink from ovulation/fertilization until implantation and (2) to investigate oestrous cyclicity and early pregnancy events in European mink, in particular the development of *corpora lutea* in ovaries, and to confirm the time of implantation. These questions were addressed in articles III and IV. Thorough investigation of luteogenesis in ovaries on the one hand and preimplantation embryos on the other

hand proved that implantation delay does not occur in European mink and provided basic biological knowledge necessary for subsequent use of conservation programmes that have reproductive technologies as their core element.

6.2.1 Oestrous cyclicity in European mink

It has been demonstrated that in the conditions current in Novosibirsk, Russia, the majority of European mink females enter oestrus during April, with entry dates ranging from late March to early May (IV). This is in a good agreement with earlier reports on captive bred populations of this species observed at the same Research Station (Ternovsky and Ternovskaya 1994) and elsewhere (Moshonkin 1981, Maran and Robinson 1996). Data from wild animals are scarce and not as reliable as for captive bred populations, but Ceña et al. (2006) have reported that wild females come into heat between mid March and late April. One may therefore conclude that the onset of the breeding season is similar in wild and captive populations. Also, the geographic position does not appear to be a determining factor since the initiation of the breeding season occurs at a similar date in Novosibirsk and in southern (Moshonkin 1981) and northern (Maran and Robinson 1996) regions of the species' historical range.

The present study shows that the duration of oestrus in European mink is highly variable and can range from 1 to 12 days, as was suggested by Moshonkin (1981) who, with a captive-bred population in the south of the European part of Russia (Astrakhan), arrived at an estimate of 1 to 10 days. Moreover, we found that, if not mated, most European mink females enter a second and even a third oestrus. It has been also reported earlier by Moshonkin (1981), as well as by Maran and Robinson (1996) that the period between the first and second oestrus is about one month. Taken together, all these data indicate that the European mink may be described as a seasonally polyoestrous species.

Recent molecular phylogenetic analysis has confirmed that European mink are most closely related to the ferret/polecat species (Davison et al. 2000, Sato et al. 2003, Koelpfi et al. 2008), although in its cyclicity it resembles more the phylogenetically remote sable (*Martes zibellina*). It is known that after a short period of prominent

oestrus and receptivity, sable females demonstrate a period of non-receptivity with corresponding changes in their vaginal smear cytology. After 7 to 10 days (sometimes even 20 days) they demonstrate all the signs of oestrus again (Kler 1941).

6.2.2 Preimplantation embryo development in European mink

The results presented in this thesis (III) demonstrate that preimplantation embryo development in European mink resembles that of polecats/ferrets for key events including the rate of embryo development, the time of entering uterus, and the timing of implantation.

The first ovulated eggs were observed in European mink oviducts on day 2 pc, as stated above, and by day 4 pc 6- to 13-cell embryos had been flushed from the oviducts (III). Similar cleavage stages of preimplantation embryos were flushed from the domestic ferret (Daniel 1970) and Finnish farmed European polecat (Lindeberg and Järvinen 2003, Lindeberg 2003) at 3 - 4 dpc.

On day 5 pc, mostly morula stage embryos were flushed from the oviducts of the European mink (III). It has also been reported (Daniel 1970 on domestic ferrets and Lindeberg 2003 on Finnish farmed European polecats) that the first morulae in polecats/ferrets can be observed on day 5 of pregnancy. These observations confirm that the timing of embryo development during the first five days of pregnancy is similar in European mink and polecats/ferrets. The number of cells counted on day 5 in ferret morulae has been reported to range from 8 to 60 (Daniel 1970). The number of cells in 5 dpc European mink embryos in the present study (III) ranged from 19 to 43, i.e. in the same range as in the domestic ferret. The diameter of 5 dpc European mink embryos was in the range 178 - 190 micrometers, which is not very different from the figures for domestic ferrets (Daniel 1970) and farmed European polecat (Lindeberg and Järvinen 2003).

Day 6 was clearly the day of migration of the European mink embryos from the oviducts into the uterus, some embryos being still at the morula stage and some being early blastocysts (III). This was also the case in polecats/ferrets. Since the publication

of the classic work of Robinson (1918) it has been known that ferret embryos enter the uterus on the sixth day of pregnancy. It has been shown that in Finnish farmed European polecats all the embryos are still in the oviducts on day 5 after the first mating, and they move into the uterus 6 - 7 dpc; on day 8 nearly all embryos are in the uterus (Lindeberg and Järvinen 2003, Lindeberg 2003). Recently Li et al. (2006a) reported that by day 7 most ferret embryos are in the uterus, already at the blastocyst stage, and our observations in European mink are consistent with this.

On day 8 pc, European mink embryos start to expand and we found that the average diameter of blastocysts was around 500 micrometres (438 - 600) (III). Ferret embryos are about the same size on this day of development (460 - 500 micrometres according to Daniel 1970 and an average of 685 micrometers according to McRae 1992). Kidder et al. (1999a) counted cells in ferret blastocysts on day 8 and reported an average of 285 cells per blastocyst, which is similar to our observation of around 300 cells for European mink on this day (III). On 9 - 10 dpc the embryos reach 1 mm in diameter and more both in European mink (III) and in ferrets (Daniel 1970).

On day 11 pc, the last day before implantation, the size of European mink blastocysts slightly exceeded 1.5 mm (III). This is well within the range for ferret blastocysts which, according to McRae (1992) is 1.4 to 2.1 mm, and according to Mead et al. (1988a) is 1.5 to 2.2 mm (average 1.82 mm). In both species the embryos were still not implanted on day 11 pc (III, Daniel 1976, Mead et al. 1988a, Kidder et al. 1999a).

6.3 Characteristic features of ovulation and implantation in mustelids

6.3.1 Ovulation in stoat and European mink

It is well known that induced ovulation is characteristic of mustelids (Carroll et al. 1985, Murphy 1989), so it was not surprising that ovulation was observed exclusively after copulation in both the stoat and the European mink. Although the number of animals studied around ovulation was relatively small for both species (only 7 stoat females studied 1 - 4 dpc and only two European mink females studied on the first and second days after copulation, one female on each of these days), all these cases are consistent with the concept that in both species ovulation is triggered by coital

stimuli. It has been demonstrated that the ferret needs full spectrum copulation to induce ovulation – gripping or any other elements of mating do not induce ovulation in the absence of intromission (Carroll et al. 1985), although it is not known whether or not elements of mating behaviour (e.g. mounting without intromission) may induce ovulation in stoats or European polecats.

Our results clearly demonstrate (I), that stoat spermatozoa retain their viability and fertilizing capacity in female reproductive ducts for as long as 3 - 4 days after copulation (i.e. 84 - 104 hpc). Very recently this observation was indirectly confirmed in New Zealand. LaFalci and Molinia (2007) inseminated oestrous stoat females with motile frozen-thawed stoat semen, and found motile spermatozoa in these females' reproductive tracts three days after insemination. It is not surprising that the survival time of spermatozoa in the female stoat reproductive tract is much longer than in most other mammalian species, with notable exception of horses and some bat species (Johnson and Everitt 2000, Senger 2003). The relatively long survival time for stoat sperm in female reproductive ducts may be evolutionarily important in the context of induced ovulation, especially if we take into account the fact that ovulation in stoats occurs 3 - 4 days after copulation as described in this study (I).

The time elapsing between coital stimuli and ovulation in European mink was 24 - 48 hours, as no ovulated eggs were observed on the first day pc, but on the second day ovulated eggs and two-cell embryos were flushed from the oviducts (III). Similarly, Daniel (1970) did not find ovulated eggs on day 1 pc in ferrets. The timing of ovulation in ferrets, 24 - 36 hours after copulation (Hammond and Walton 1934), is similar to that observed in this study for European mink. Although the stoat is a member of the same genus, *Mustela*, as the European mink and European polecat (Ternovsky and Ternovskaya 1994, Koepfli et al. 2008), and phylogenetically these species are members of the same clade (Koepfli et al. 2008), we found that the duration between coital stimuli and ovulation is much longer in the stoat, i.e. 72 - 96 hours. The comparison of ovulation-related events of the stoat with those of European mink and ferrets/polecats indicates that even species belonging to the same genus can demonstrate pronounced species-specificity. Although in all of these species ovulation is mating-induced, the lag period between copulation and ovulation, as well as time of sperm survival in female reproductive tract, is very different. This is not surprising

considering that extensive ecomorphological diversity is a characteristic feature of extant mustelids, and they exhibit within-family differences more than do most other mammalian families (Koepfli et al. 2008).

6.3.2 Implantation in the stoat and European mink, extra-embryonic coats

The mechanisms regulating the onset of diapause are still poorly understood (Renfree and Shaw 2000). In contrast, the activation of blastocysts at the end of diapause (Enders et al. 1986) and implantation itself (Enders and Mead 1996) have been thoroughly studied in the western spotted skunk which, like the stoat, has a long period of obligate diapause. Within the last 24 - 48 hours before implatation, blastocysts of the western spotted skunk expand rapidly and reach a size of 1.7 - 2 mm at implantation (Enders et al. 1986, Enders and Mead 1996). During this activation period the visceral endodermal layer starts to differentiate on the internal surface of the ICM (Enders et al. 1986). The beginning of the differentiating process in the ICM was considered as the marker of the activation in the spotted skunk and indicated the very beginning of gastrulation (Enders et al. 1986). A similar expansion in the size of blastocysts has been reported in American mink after the prolactin-induced escape of blastocysts from diapause (Desmarais et al. 2004). In their experiments, Desmarais et al. (2004) showed that the activation of American mink embryos has a similar sequence of events to that seen in skunks: at first, slow growth and then rapid enlargement 2 - 3 days before implantation.

The embryo flushed from one stoat female at the beginning of April showed signs of the start of differentiation in the ICM, i.e. was at the stage of activation. In this embryo, a distinct layer of visceral endoderm on the internal surface of the ICM was visible. The first black spots appeared on the winter fur of this female 5 days after operation. These first black spots indicated the start of moulting and, according to Ternovsky, are indirect indicators of implantation in this species as described in the Chapter 2 of this thesis. This female, in which the second uterine horn had been left intact, gave birth to a young kit 27 days after the operation day. This case suggests that in stoats the postimplantation period of pregnancy is not longer than 27 days, in agreement with earlier suggestions that the actual (postimplantation) pregnancy lasts about four weeks in stoats (Rowlands 1972, cited in McDonald and Larivière 2002).

In marten (*Martes americana*), a mustelid with a similar length of delayed implantation as in the stoat, the interval between implantation and parturition has also been estimated to be about 27 days (Jonkel and Werkwerth 1963). In sable, another mustelid with a similar implantation delay, postimplantation embryonic development also takes only 25 - 30 days (Bates 2002).

Implantation occurs on day 12 pc in European mink (III, IV) and in ferrets (Daniel 1970, Enders and Schlafke 1972, Gulamhusein and Beck 1973, Mead et al. 1988a). Enders and Schlafke (1972) suggest that implantation in ferrets occurs between 12 and 13 dpc. It was also confirmed recently in experiments with Finnish farmed European polecats that all the females studied, with one exception, had embryos implanted about 13 days after the first mating (Lindeberg and Järvinen 2003, Lindeberg 2003).

Generally, the timing of embryo development, migration into the uterus and implantation in European mink and the closely related polecats/ferrets follow the same course. However, some difference in the functional activity of CL and other regulating mechanisms may occur even in these closely related species.

Several studies of ferrets/polecats address progesterone concentrations in plasma, urine, or faeces (Blatchley and Donovan 1976, Daniel 1976, Mead et al. 1990, Young et al. 2001). Due to the huge methodological differences it is not possible to compare the studies where progesterone was measured in plasma (Blatchley and Donovan 1976, Daniel 1976) with those where progesterone was measured in faeces (Young et al. 2001, IV) or in urine (Mead et al. 1990). However, for endangered and wild mammalian species, it is obvious that monitoring ovarian steroids in faeces or urine, rather than in plasma, is preferable (Young et al. 2001, Brown 2006).

The peak concentration of progesterone in faeces at the time of implantation was five-fold lower in European mink (IV) than in polecats/ferrets (Young et al. 2001). In European mink the progesterone concentration was less than 200 ng/g faeces on 12 dpc (IV). However, progesterone concentrations in European polecats on day 12 pc were five times higher and reached a 1000 ng/g in faeces (IV^{u}). Young et al. (2001) found a similar high level of progesterone at implantation time in the closely related

black-footed ferret (about 900 ng/g feces). These data permit the conclusion that even though the timing of preimplantation embryo development and implantation, as well as the rate of embryonic development during the first twelve days of pregnancy, are similar in the European mink and European polecat, the former species is characterized by lower levels of progesterone, most probably due to less active CL. Recently progesterone and its metabolites were monitored in the stoat with the use of non-invasive faecal measurements. It has been shown that an increase of progesterone was observed prior to implantation and parturition in pregnant females but that in those females which did not deliver kits progesterone level remained low throughout the whole year (Molinia et al. 2007). These recent observations are in good agreement with early results of Gulamhusein and Thawley (1974) indicating that there is clear peak of plasma progesterone concentration in pregnant stoats, and that this peak coincides with implantation as both occur in April.

There is great species and family variation with regard to extra-embryonic coats in mammals. The coats of uterine origin like gloiolemma are associated with normal development of rabbit embryos. However, visible extra coating of stoat eggs (I) and of some cleavage-stage European mink embryos (Amstislavsky, unpublished results) indicated the opposite in these species – the extra layer was a clear indicator that the eggs were dead. This observation is in agreement with that of Adams (1973) who flushed degenerated eggs from American mink females that had failed to mate. These degenerated unfertilised eggs were covered with a similar extra layer outside the zp, sometimes of regular, but sometimes of irregular shape. These extra coats looked like "insulating coats" which embraced dead eggs. It is possible to conclude that in some species like the stoat and European mink extra-embryonic coats might be an indicator of degeneration and that such "insulated" embryos should not be chosen for embryo transfer or *in vitro* culture. These "insulating coats" are most probably specific for mustelids.

6.4 Embryo transfer as an approach for endangered mustelids conservation

An important aim of this study was to explore the applicability of a surgical embryo recovery technique for European mink donor females and the possibility of transferring European mink embryos into hybrid recipient females (hybrids between

the European polecat and European mink, i.e. honoriks and nohoriks). Another aim related to embryo transfer was to study the rate of postnatal survival of kits born after transfer of more advanced stage European mink embryos into honoriks/nohoriks. European mink embryos were transferred into honorik/nohorik recipient pseudopregnant females in two separate experiments (II, III) and 50 % of transferred embryos developed to term in both series. Kit survival rate was as low as 56.26 % in one series (II) but improved to 70 % in a later experiment (III). These main results are discussed in more detail below.

6.4.1 Embryo transfer in European mink

The ferret and American mink were the first *Carnivora* species in which embryo transfer was applied (Chang 1966, 1968, 1969). Thus, embryo transfer was started in mustelids at least ten years earlier than in other *Carnivora* families, including felids (Kraemer et al. 1979), canids (Kraemer et al. 1979, Kinney et al. 1979) and ursids (Boone et al. 1999); see also Betteridge (1981), Adams (1982) and Kraemer (1983) for historical reviews. Despite this long history, relatively few embryo transfer experiments have been conducted in mustelids. Besides our work with European mink (II, III), transfer results have been published only for ferrets/polecats (Chang 1966, 1968, 1969, McRae 1994, Kidder et al. 1999b, Li et al. 2001, 2006a, b, Lindeberg et al. 2002, Lindeberg et al. 2003, Piltti et al. 2004, Sun et al. 2008) and American mink (Chang 1968, Zhelezova and Golubitsa 1978, Adams 1982).

6.4.2 Choice of embryo transfer method for the European mink

Different methods can be used for mammalian embryo transfer. One well-known system relies on the transfer of embryos during the luteal phase of the oestrous cycle into a synchronised recipient female. This is widely used in spontaneously ovulating animal species such as sheep or cattle, in which the *corpora lutea* of the oestrous cycle are functional and produce relatively large quantities of progesterone (Gordon 2005). Another embryo transfer system exploits a pseudopregnant recipient; pseudopregnancy induced either by hormonal treatment (Chang 1968, Mead et al.

1988b) or by mating with a sterile male (Hogan et al. 1986, Lindeberg 2003). This is widely used in various species, including mice (Hogan et al. 1986) and ferrets/polecats (Lindeberg et al. 2002, 2003, Li et al. 2001, 2006a, Piltti et al. 2004). Sometimes, although not very often, a third approach is undertaken: embryo transfer into a pregnant female. In this case alien embryos and the recipient's own embryos develop concurrently, and a bispecific pregnancy is established. This approach has been used in cattle (Summers et al. 1983), rats (Mayer and Fritz 1974, Stein et al. 1993, Amstislavsky et al. 1996) and even bears (Boone et al. 1999). This model is also known as "mixed pregnancy" and fur colour or other markers, e.g. molecular, are used to identify term offspring developed from the transferred embryos.

Because polecats/ferrets and European mink are induced ovulators (Carroll et al. 1985, III, IV), only the second and third options described above can be utilized if embryo transfer has to be performed within or between *Mustelidae* species. Although in our earlier experiments with rats, the third option, i.e. embryo transfer into pregnant recipients, was successfully used (Stein et al. 1993, Amstislavsky et al. 1996), this approach proved to be non-effective in mustelids (Amstislavsky et al. 2006)

This model of "mixed pregnancy" was successful in rats, because there is apparently no migration of preimplantation embryos between the uterine horns (McLaren 1982) and as transfer was done into one of two horns before the own recipient's embryos arrive there from corresponding oviduct and ligature was put between oviduct and this uterine horn to prevent own embryo migration (Amstislavsy et al. 2006). However, it has been shown that in virtually all mustelid species studied so far, e.g. ferrets (Chang 1968, Li et al. 2006a), sables (Baevsky 1955, 1960) and stoats (King 1990), the embryos easily migrate between the horns. Recently, Li et al. (2006a) demonstrated that as many as 37 to 41 % of the embryos transferred into the left uterine horn in ferrets migrated to the contralateral uterine horn and implanted there. The high rate of embryonic migration between horns is the obstacle for successful application of "bispecific pregnancy" model to mustelids.

In our subsequent experiments only embryo transfer into pseudopregnant recipients was used, the model which has proved effective for Finnish farmed European polecats (Lindeberg et al. 2002, 2003, Piltti et al. 2004) and ferrets (Li et al. 2001, 2006a). Five intraspecies embryo transfers in polecats/ferrets were performed (Amstislavsky et al. 2006). In these experiments it was shown that transfer of 7 dpc embryos into the uterine horn of a 6 dpc recipient female was effective, and resulted in term kits if more than two embryos were transferred. It is noteworthy that the highest rates of embryo survival in experiments on embryo transfer in mustelids have been reported so far by Li et al. (2006a), who used the same approach: transfer of 7 dpc embryos into a 6 dpc recipient female (73.3 - 90 % of transferred embryos developed into live fetuses on day 21 of pregnancy).

Previous papers have reported attempts at interspecies embryo transfer between ferrets and rabbits (Chang 1966) and between ferrets and American mink (Chang 1968). No live kits were registered in either of these studies. The taxonomic distances between ferrets and rabbits are enormous: they represent different orders of mammals, thus pregnancy failure in this case is not surprising. The interspecies transfer between American mink and ferrets also failed to result in term kits (Chang 1968) despite these two species both belonging to the *Mustelidae*. When American mink embryos were transferred into ferret recipient uteri, the majority of them implanted, but deteriorated later. No implantation was recorded when ferret embryos were transferred into American mink recipients (Chang 1968). There are many other examples illustrating that when two species are used for embryo transfer, it often happens that transfers are more successful in one direction than in the other (Anderson 1988). The reason for pregnancy failure in the case of interspecies embryo transfer between ferret and American mink is, however, rather clear, as preimplantation embryo development in American mink normally includes a period of implantation delay (Hansson 1947, Enders 1952), whereas there is no such delay in ferrets (Hamilton 1934, Daniel 1970, Kidder et al. 1999a, Lindeberg 2008).

In four of the five experiments on interspecies embryo transfer between the European mink and European polecat, mink embryos were transferred into polecats, and in one

case transfer was in the opposite direction (Amstislavsky et al. 2006). However, all these straightforward embryo transfers failed, even though more than two embryos were transferred in all cases but one (Amstislavsky et al. 2006). Interspecies embryo transfer in its traditional form is usually ineffective in mammals (Pukazhenthi and Wildt 2004), as shown by the poor success rate in the great majority of attempts. In those rare cases where transferred embryos developed to term, the success rate in interspecies embryo transfer is usually lower than 25 - 30 % (Kydd et al. 1985, Antczak et al. 1985, Summers et al. 1987, Allen et al. 1993).

Some special tricks have been used to increase pregnancy rates in interspecies embryo transfer. Interspecies embryo transfer has been systematically applied to the *Equidae* family in Cambridge (Kydd et al. 1985, Antczak et al. 1985, Summers et al. 1987, Allen et al. 1993; also reviewed recently by Allen 2005), and it has been found that the transfer of donkey embryos into mares is much less successful than *vice versa*, so a donkey-into-horse model has been comprehensively studied with the goal of improving the success rates of interspecies embryo transfer (Allen et al. 1993, Allen 2005). Attempts have been made to administer donkey serum to recipient mares when embryos were transferred from donkeys to horses (Allen et al. 1993) and the success rate did improve. It has also been shown in experiments with mice that an injection of 50 µl of allogenic murine blood to pregnant females led to increased plasma progesterone levels in these females, larger fetuses, and more live pups at the end of the weaning period (Gerlinskaya et al. 2000). In contrast to its usefulness in horses (Allen et al. 1993, Allen 2005) and mice (Gerlinskaya et al. 2000), this approach has not yet been properly addressed for mustelids.

However, in earlier studies by the Cambridge group with *Equidae*, donkey-horse hybrids (mules) were effectively used as recipients for the transfer of horse or donkey embryos (see Allen et al. 1993, Allen 2005 for review), and a success rate of 40 % was achieved (Antczak et al. 1985, Allen et al. 1993, Allen 2005). This successful use of interspecies hybrids as recipients in *Equidae* encouraged us to try a similar approach with *Mustelidae*.

6.4.3 Use of hybrids as recipients to overcome pregnancy failure

The use of hybrids (honoriks and nohoriks) in the framework of European mink conservation is beneficial not only because female nohoriks and honoriks are fertile (Ternovsky and Ternovskaya 1994, Ternovskaya et al. 2006), but also because the great majority of honorik males are sterile (Ternovsky and Ternovskaya 1994, Ternovskaya et al. 2006) and thus may be used to induce pseudopregnancy in female recipients. The possibility of using genetically sterile honorik males to induce pseudopregnancy in recipient females has been demonstrated (Amstislavsky et al. 2006). Some other types of male hybrids (e.g. offspring of a European polecat male and a nohorik female) can be selected as "sterile and eager to copulate" and might be used for the same purposes (III).

Although honoriks have been produced on a regular basis for several years on the *Mustelidae* Research Station in Novosibirsk, producing *vice versa* hybrids is much more laborious, and experiments on the hybridization of European mink males to European polecat females very rarely ended up with term offspring due to ethological, behavioural and other barriers (Ternovskaya et al. 2006). One possible solution may be to develop a technique of *in vitro* fertilization to get a developing nohorik embryo and then to transfer it into either a European mink or a European polecat recipient. Interspecies and even intergeneric "hybrid embryos" have been obtained from domestic puma (*Felis concolor*) or leopard (*Panthera pardus*) oocytes, fertilised *in vitro* by frozen-thawed semen of the domestic cat *(Felis catus*) (Johnston et al. 1991). Phylogenetic distance, body size and ethological/behaviour differences between these three felid species are much more pronounced than between the European mink and European polecat. The latter two belong to the same genus and to the same phylogenetic clade (Bininda-Edmonds et al. 1999, Koepfli et al. 2008). Thus, applying the *in vitro* hybridization technique to these mustelid species sounds promising.

In our studies, a success rate of 50 % was achieved after the transfer of European mink embryos into either nohoriks or honoriks. Thus, the rate of success was higher than that recorded for embryo transfer in ferrets by Chang (1968) and Kidder (1999b), and was about at the same level as reported for ferrets by McRae (1994) (51.85 %) and for

Finnish farmed European polecats (42 %); the latter study done by the same method but on a larger scale (Lindeberg et al. 2002). However, the embryo survival rate in our embryo transfer experiments was lower than recently obtained with ferrets in North America (Li et al. 2001, 2006a): Li et al. (2001) recorded a 68.5 % success rate, and recently a 73.3 - 90 % level of success has been achieved by the same research group (Li et al. 2006a). In the latter case no full-term kits were counted, but the live fetuses on day 21 of pregnancy.

In our experiments, 8 (out of 9) honorik recipient females received a sufficient number of embryos (4 to 10 per female) and gave birth to live kits. One honorik recipient, however, received two embryos only and did not whelp. Two (out of 3) nohorik females which received an appropriate number of embryos (6 and 8) successfully conceived and whelped. The third nohorik female received only one embryo and this did not develop to term. Thus, the results of these experiments (II, III) are in agreement with those obtained in experiments with intraspecies embryo transfer in polecats/ferrets (Amstislavsky et al. 2006) and, taken together, suggest that if only one or two embryos are transferred into a ferret/polecat or honorik/nohorik recipient, it is not enough to establish pregnancy and produce term kits. It has been shown in *Felidae* (Pope et al. 1993) that the success rate of embryo transfer depends partly on the number of embryos transferred. Our results indicate that this is also true for *Mustelidae*, so there is no reason in transferring one or two embryos in such a polytocous species as European mink or polecats/ferrets. However, a transfer of three or more embryos was successful in the majority of cases, which is in agreement with the findings of McRae (1994) and Li et al. (2001) that the transfer of three or more embryos per recipient in mustelids resulted in term kits.

In all the cases, when embryo transfer from European mink donors to honorik/nohorik recipients resulted in the term kits, the donor females were either synchronous with, or 1 - 2 days ahead of, recipients in terms of pregnancy/pseudopregnancy (II, III). These results are in good agreement with previous findings in ferrets (Chang 1968) and farmed Finnish polecats (Lindeberg et al. 2002, Lindeberg 2003) that a one-day asynchrony of the donor female with the recipient animal does not adversely affect the results. It has also been shown, in the classic work of Chang (1968, 1969), that if

ferret 8 dpc embryos are transferred into the uterus of a pseudopregnant female on day 6 of its pseudopregnancy, they develop into healthy fetuses, but this does not occur when 6 dpc embryos are transferred into the uterus of a pseudopregnant recipient on day 8 of its pseudopregnancy.

It has been shown earlier in experiments with Finnish farmed European polecats that a higher success rate may be achieved if embryos from multiple donors are transferred into a single recipient (Lindeberg et al. 2002). It has been assumed that this is because pooling embryos of different ages provides better chances for synchronization with the recipient female. With this point in mind, embryos from two different donor females were combined and transferred into one recipient female in six cases. Although the numbers in the single donor - single recipient and multiple donors - single recipient groups were not sufficient to allow statistical comparisons of effectiveness, it is evident that the success rate in the latter group was at least as high as in the former.

6.4.4 The problem of a high postnatal mortality rate after transfer of European mink embryos to hybrid recipients

Even though the transfer of European mink embryos into honorik/nohorik recipients was successful, with 50 % of embryos developing to term, both experiments (in 2002 and in 2004) revealed that there was a high mortality rate in the resulting offspring (II, III). The low weight and decreased viability of some term kits indicates that prenatal development was altered, and this problem was accentuated in the experiments of 2002, when only nine out of sixteen kits (56.25 %) survived (II). The average birth weight of a male offspring in the embryo transfer group was within the normal range, but lower than the average birth weight of naturally born male kits. In contrast, the average weight of a newborn female offspring born as a result of ET during the 2002 breeding season was lower than the minimal weight of female ever born on this farm.

A high rate of postnatal mortality has been recorded in some experiments on intraspecies embryo transfer in ferrets. For example, only 20 out of 35 domestic ferret

kits born as a result of ET survived until week 8 *post partum* (survival rate 57.1 %; Li et al. 2001). One factor associated with the high postnatal mortality in this case may be litter size, as suggested by Li et al. (2001): they noticed that bigger litters apparently contributed more than smaller ones to the high mortality rate. Interspecies embryo transfer is often associated with high postnatal mortality rates (Anderson 1988, Solti et al. 2000, Hammer et al. 2001) because of immunological incompatibility, failure of proper placenta formation, or specific endocrine problems caused by differences in immunology, physiology and endocrinology of the donor and recipient species (Anderson 1988, Allen et al. 1993, Bainbridge and Jabbour 1998).

However, during the 2004 breeding season the rate of postnatal survival in the embryo transfer group was improved, and 14 kits out of 20 born (70 %) survived (III). The experiment of 2004 confirmed that ET kits weigh less at birth than control kits but the average weights of the kits of both sexes in the embryo transfer group was within the normal range estimated for naturally produced European mink (III).

The method of embryo transfer was not exactly the same in these two studies: during the 2002 breeding season, only 7 - 8 dpc embryos were transferred to day 6 - 7 recipients, whereas during the 2004 breeding season embryos from later stages were transferred in many of the cases. This may somehow be related to the improved postnatal survival of the kits in 2004, although there were no differences between embryo transfer success rates in these two studies (II vs III). However, it appears that late embryos of European mink (9 - 11 dpc) are too large to handle conveniently and especially to transfer.

A more plausible explanation for the improved postnatal survival of term kits in the ET group is that the animals (e.g. recipient females) were in better physiological condition during the spring of 2004 because their diet had improved. This explanation is supported by the fact that the fecundity of breeding females was higher in 2004 than in 2002: from the same number of breeding females (10), ten more kits were born in 2004 than in 2002 (an average of one kit more per litter). On day 10 of postnatal development, the difference was even more pronounced: an average 1.1 more kits in 2004 than in 2002.

6.4.5 The case of bispecific pregnancy

One honorik recipient received embryos from two different species: five European polecat blastocysts and five European mink blastocysts into the same uterine horn. The resultant litter consisted of four mink kits and one polecat kit. A similar model was used earlier in an interspecies transfer in *Felidae* (Pope et al. 1993), when a two-species mixed pregnancy was achieved in a domestic cat recipient after the transfer of a mixture of Indian desert cat (*Felis silvestris ornata*) and domestic cat (*Felis catus*) embryos. This approach resulted in the birth of Indian desert cat kittens in the domestic cat recipient, and was considered as an option to overcome the interspecies barrier.

Our approach, however, was not exactly the same as that used by Pope et al. (1993). There were embryos from two different species in both studies, but in our case (III) the recipient female was an interspecies hybrid. This technique permitted a high pregnancy rate, which was the same (50 %) as the average pregnancy rate in the whole trial. However, one technical point must be emphasized: the observed dominance of European mink embryos over European polecat embryos was to be anticipated, because the European mink blastocysts were more advanced in their development on the day of transfer (day 8 vs. day 7). This resulted in more mink kits than polecat kits despite the equal number of mink and polecat embryos that were transferred. Although there was only one case of a successful transfer from two different species with a mixed pregnancy in a honorik recipient (III), there might be good chances of overcoming the interspecies barrier with this version of "mixed pregnancy" model also by the use of ferrets/polecats as recipients if mink embryos are at a more advanced stage of development.

6.5 Relevance of the results of this investigation to conservation and immunocontraception programmes

6.5.1 Stoats

Although the stoat has a prolonged period of oestrus (Ternovsky 1983, I) there are only a few records of successful breeding of this mustelid in captivity for a number of

generations (Müller 1970, cited in McDonald and Larivière 2002, Ternovsky and Ternovskaya 1994, Doncarlos et al. 1986, McDonald and Larivière 2002). Because stoat females come into oestrus at an early age, they are normally impregnated when young and still in their nests (Ternovsky 1983), so it is not surprising that virtually all females caught in the wild have been carrying blastocysts (O'Connor et al. 2006, La Falci and Molinia 2007). There are anecdotal reports of such females being caught in the wild, brought into captivity, then giving birth to litters (East and Lockie 1965). However, a larger-scale experiment in Landcare (New Zealand) where females caught in wild were brought into captivity found that in the great majority of cases they failed to deliver term kits. It was thought that stress (either emotional or nutritional) was the main cause of this failure (McDonald and Larivière 2002, O'Connor et al. 2006).

One option for breeding stoats in captivity is to mate young suckling females to adult males (Ternovsky and Ternovskaya 1994). In this system, the males demonstrate a high reproductive capacity and polygamy. Moreover, the whole process is reliable and it is short in duration, copulation normally lasting only 1 - 2 minutes (Ternovsky and Ternovskaya 1994, I). Females at this age are not only unable to reject adult males but in most cases they demonstrate a willingness to copulate, crawling after the male after copulation (Ternovsky and Ternovskaya 1994). It is not surprising that males that were unsuccessful in copulating with adult females had greater success in copulating with juvenile females (McDonald and Larivière 2002).

In our experience, female stoats mated in their nests with adult males are a very convenient source of embryos. The females mated during the spring and summer of 1991 provided embryos until the next spring. We consider this species to be a reliable source of embryos almost year-round, which can be especially valuable in experiments requiring diapausing *Mustelidae* embryos.

Unlike the European mink, the stoat is not an endangered species and in fact is a dangerous pest in New Zealand, causing devastating harm to the local fauna, including the iconic kiwi (King et al. 2001). Recently, a number of scientific projects have been developed in New Zealand with the goal of finding a method of immunocontraception for this species (LaFalci and Molinia 2007, Molinia et al. 2007). Embryonic coats have been the focus of scientific interest for some groups for

decades, but recently it has been shown that some components of these extra coats may be the target for immunocontraception at least in some marsupials (Menkhorst et al. 2008). The brush-tailed possum, a common pest animal, was the first animal in which the idea that extra-embryonic coats as an immunocontraceptive target has been tested. Thus, recent findings of Menkhorst et al. (2008) may stimulate further studies of extra embryonic coats also in mustelids, in stoat in particular, as there might be important practical aspects of these studies.

Because the establishment of a captive-bred population is considered to be a key step in these projects, interest in stoat reproduction and captive breeding has increased dramatically (McDonald and Larivière 2002, O'Connor et al. 2006). Investigating preimplantation embryo development in the stoat is very important not only in order to find clues as to why females caught in the wild often fail to maintain pregnancy in captivity, but also to identify the "Achilles' heel" in the reproduction of this dangerous pest so that effective biocontrols can be developed. Article I provides the results of the first-ever study on preimplantation embryo development in the stoat, valuable background knowledge for future research on the disruption of its reproduction.

6.5.2 European mink

The European mink is considered to be a highly endangered species (Ternovsky 1977, Ternovsky and Ternovskaya 1994, Maran 2007, Amstislavsky et al. 2008) and breeding in captivity is currently one of the most useful conservation tools (Ternovsky and Ternovskaya 1994, Maran and Robinson 1996, Festl et al. 2006, Ternovskaya et al. 2006, Maran 2007). There are centres in Novosibirsk (Russia), Tallinn (Estonia) Osnabrück (Germany) and Lleida (Spain) where European mink are bred and these constitute core populations for re-introduction programmes (Ternovsky and Ternovskaya 1994, Maran and Robinson 1996, Festl et al. 2006, Mañas et al. 2006, Ternovskaya et al. 2006). The knowledge of the oestrous cycle in European mink and in poleats/ferrets makes it possible to produce hybrids between these related species (Ternovsky and Ternovskaya 1994, Ternovskaya et al. 2006).

Recently possibility of successful embryo cryopreservation has been demonstrated in polecats (Lindeberg et al. 2003, Piltti et al. 2004) and ferrets (Sun et al. 2008). These

recent results are promising, and embryo cryobanking has a chance to become an important integrative link between *in situ* and *ex situ* approaches to European mink conservation. In this sense, to develop a proper mode of subsequent embryo transfer with the use of honoriks/nohoriks may be considered as an important part of the future conservation programme for European mink.

Results of European mink re-introduction attempts revealed that single release of animals into the wild is not enough to transform captive-bred population into a fertile self-sustained wild population. The two largest attempts reported in the former Soviet Union (Ternovsky and Ternovskaya 1994, Shvarts and Vaisfeld 1995, Ternovskaya et al. 2006) and in Estonia (Maran et al. 2006), which are the only two large-scale projects aiming to establish island populations of European mink which have been published so far. The third large-scale attempt was initiated recently in Germany, but in this case European mink are released on the mainland and the results of this latest attempt are not published yet. In all these attempts animals have been released many times during a number of consequent years. Embryo and semen banks may also decrease the cost of captive breeding and re-introduction programmes. For example, the maintenance of genetic diversity in the form of cryopreserved embryos and/or the use of embryo transfer will provide more flexibility in breeding management. Investigation of the peculiarities of early pregnancy (IV) and preimplantation embryo development (III) in European mink are inevitable prerequisites for starting to use ART/ET to improve the success of re-introduction programmes, and for generating a Genome Resource Bank (GRB) to back up existing biodiversity in this species.

One of the biggest problems in applying ART and embryo technologies to companion animals, non-domestic and endangered species is that the efficiency usually remains relatively low. Moreover, if any study ever reports success in application of ART/ET to non-domestic and/or endangered mammalian species, these reports usually represent the first and often the only instance when the procedure was successful in the species in question (Paris et al. 2007). In contrast, embryo transfer from European mink to honoriks/nohoriks has been proved to be successful on a repeated basis as two separate attempts have been done and repeatable success (50 %) was confirmed in both of these attempts (II, III).

7. CONCLUSIONS

The newborn stoat females used in this study were impregnated in their nests 26 - 92 days after birth by mating with adult males. In these females, ovulation was induced 3 - 4 dpc. Migration of eggs into the uterus started on 11 dpc and was completed by 12 dpc. Implantation was delayed during the whole summer, winter and part of the next spring, an 8- to 9-month period, with unattached blastocysts, which slowly increased in size, present in the uterine horns during the whole period. No prominent luteogenesis was observed in stoat ovaries during the implantation delay period. Implantation was observed in April and coincided with the start of the spring moulting.

European mink proved to be a polyoestrous species, the first oestrus lasting 1 - 12 days. If not mated, the great majority of females entered a second oestrus after 12 - 55 days. In mated females, fertilised eggs underwent cleavage in the oviducts and migrated into the uterus 6 dpc at the morula stage. Blastocysts were recovered from the uterine horns from 7 dpc, when the migration into the uterus was completed, until implantation. Implantation occurred on 12 dpc without an implantation delay and was indicated by uterine swellings and the impossibility of flushing the uterine horns beyond this day. Advanced luteogenesis was observed with prominent *corpora lutea* in the ovaries around the time of implantation. Additional hormonal studies indicated a significant rise of progesterone concentration in faeces during the first week of pregnancy, with a peak around 12 dpc.

To overcome the interspecies reproductive barrier, embryos from European mink could be surgically transferred into hybrid females obtained after mating European polecat (*Mustela putorius*) males and European mink (*Mustela lutreola*) females (honoriks) and *vice versa* (nohoriks). The use of honoriks and nohoriks is beneficial in a framework of European mink conservation, as a reasonable survival rate (live kits/transferred embryos) of 50 % was achieved on a repeatable basis in two successive experiments on the surgical transfer of fresh European mink embryos to honorik/nohorik pseudopregnant recipients (36 kits/72 transferred embryos). The low birth weight of male and female kits born after embryo transfer resulted in low

survival rates of kits (56.3 %), although the high kit mortality was mitigated in the second trial and led to a higher survival rate (70 %).

Generally, these studies of reproductive biology and embryo technology in European mink and related mustelid species from the genera *Mustela* provide essential knowledge and a model for the use of embryo transfer in the framework of conservation programmes for European mink.

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