The Wild Male Mink as a Sentinel for Endocrine-Disrupting Chemicals and Reproductive Toxicity

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Abstract

Increasing evidence indicates that pollutants may affect the hormone system of humans and wildlife. These endocrine-disrupting chemicals are related to an increased risk of a variety of diseases and disorders, including adverse effects on the reproductive system. By using a sentinel wildlife species, an early warning of adverse health effects due to pollutants in the environment may be provided. This is also relevant for humans, as wildlife and humans can be exposed to similar mixtures of pollutants.

With this in mind, the aim of this thesis is to investigate the possibility of establishing the wild mink (*Neovison vison*) as a sentinel species in Sweden, for both exposure to pollutants and effects of pollutants on the reproductive system. Mink were collected from local hunters and necropsies were performed focusing on the male reproductive system. The collected mink provided an insight in how to handle the variation in data due to sample season, age and nutritional status. These factors significantly influenced many, but not all, of the concentrations of chlorinated, brominated and perfluorinated compounds and also some reproductive organ variables. In addition, the results offer information on how to optimize the design of future studies, and some baseline data for reproductive organ measurements were compiled. Considerable concentrations of PCBs were found in some areas and the concentrations of PFOS were among the highest ever recorded in mink. Associations were found between measurements on the reproductive organs and pollutant concentrations. The anogenital distance was inversely associated with concentrations of some perfluoroalkyl acids and DDE. Several associations were also found between some PCB congeners and measurements on the penis and baculum.

In conclusion, the wild mink males may serve as an indicator for environmental exposure to pollutants in Sweden. In addition, the wild mink seems to be a suitable sentinel species that may provide an early warning of alterations in the male reproductive organs related to environmental pollution.

Keywords: Endocrine disrupting chemicals, mink, male reproduction, PCB, PBDE, perfluoroalkyl acids, PFOS, DDE, anogenital distance, penis, baculum

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The choice, after all, is ours to make

Rachel Carson, *Silent spring (1963)*

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Persson, S., Bäcklin, B.-M., Kindahl, H., Brunström, B. & Magnusson, U. (2011). Influence of age, nutritional status and season on the reproductive system in wild male mink (*Neovison vison*). *European Journal of Wildlife Research* 57(5), 1057-1063.

II Persson, S., Rotander, A., van Bavel, B., Brunström, B., Bäcklin, B.-M. & Magnusson, U. (2013). Influence of age, season, body condition and geographical area on concentrations of chlorinated and brominated contaminants in wild mink (*Neovison vison*) in Sweden. *Chemosphere* 90(5), 1664-1671.

III Persson, S., Rotander, A., Kärrman, A., van Bavel, B. & Magnusson, U. (2013). Perfluoroalkyl acids in subarctic wild male mink (*Neovison vison*) in relation to age, season and geographical area. *Environment International* 59, 425-430.

IV Persson, S., & Magnusson, U. Environmental pollutants and alterations in the reproductive system in wild male mink (*Neovison vison*) from Sweden (manuscript).

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Abbreviations

DDE	Dichloro-diphenyl-dichloroethylene
DDT	Dichloro-diphenyl-trichloroethane
EDC	Endocrine disrupting chemical
HCB	Hexachlorobenzene
MeO-PBDE	Methoxylated PBDE
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PFAA	Perfluoroalkyl acid
PFBS	Perfluorobutane sulfonate
PFDA	Perfluorodecanoic acid
PFHxS	Perfluorohexane sulfonate
PFNA	Perfluorononaoic acid
PFOS	Perfluorooctane sulfonate
PFUnDA	Perfluoroundecanoic acid

1 Introduction

The number of new chemicals that are synthesized and marketed has increased exponentially since the 1960's (Binetti *et al.*, 2008). Chemicals can be found in the environment all over the world, in wildlife and in humans. Some of these chemicals are toxic and may interfere with the hormonal system of the body, so called endocrine-disrupting chemicals (EDCs). There is increasing evidence that EDCs play an important role in the adverse effects on for example reproduction, thyroid function and the immune system that have been observed in wildlife and/or in humans (Diamanti-Kandarakis *et al.*, 2009; WHO/UNEP, 2013).

Effects of endocrine disruptors on the reproductive system of wildlife were recognized first during the 1950s and 60s (Markey *et al.*, 2002). In Sweden, the reproductive success of the grey seal, the otter and the white-tailed sea eagle declined from the 1950s, and was associated with high levels of PCB and DDT (Roos *et al.*, 2012). After these chemicals were banned, the levels have slowly decreased and the populations have recovered due to improved reproduction.

Wildlife and humans are not exposed to one individual chemical at a time, but to mixtures of chemicals, prenatally and throughout life. Chemicals may act together, often in a dose-additive way (Kortenkamp, 2007). In experiments on rats, it has been shown that mixtures of chemicals can induce male reproductive tract malformations even if the individual chemical does not lead to observable effects at that dose (Rider *et al.*, 2009). To further complicate matters, hormones and EDCs can act at very low concentrations, and the responses can be non-monotonic (Vandenberg *et al.*, 2012). In other words, the effect of an EDC may be observed at both low and high concentrations, but not at intermediate concentrations (U-shaped dose-response curve) or vice versa.

Studies on laboratory animals can provide mechanistic information that help understand the effects of EDCs on wildlife (Gray *et al.*, 1998) and also test the hypotheses generated in the field (Colborn, 1994). However, in order to understand the effects of mixtures of EDCs, studying laboratory animals on a chemical-by-chemical basis will not suffice (Colborn, 1994). In this case, studying wildlife is advantageous as a mixture of relevant chemicals and concentrations is already present. In a recent report from the World Health Organization it was stated that

> "wildlife are important sentinels for human male reproductive health as they are more easily sampled and live in direct contact with similar or the same complex mixtures of anthropogenic environmental contaminants to which humans are exposed" (WHO/UNEP, 2013).

1.1 Endocrine disruption of the male reproductive system in mammals

The complex physiology of the reproductive system is regulated by a number of hormones. In the male, androgens are crucial for the development of the male reproductive organs and for supporting spermatogenesis (Bhasin, 1999). Leydig cells in the testicles produce mainly testosterone, while the cortical cells of the adrenals produce androstenedione and other androgens (Robaire, 1999). Testosterone can be converted into a more potent form, dihydrotestosterone, in several different locations in the body (reviewed by Marchetti & Barth, 2013).Testosterone can also be aromatized into estrogen, another important steroid for spermatogenesis and male fertility (reviewed by Carreau *et al.,* 2003).

The production, receptor binding or metabolism of hormones may be disrupted by exposure to endocrine-disrupting chemicals (Diamanti-Kandarakis *et al.*, 2009). Exposure to EDCs during the fetal or neonatal period may alter the functions of the reproductive organs in the adult (McLachlan *et al.*, 1975; Norgil Damgaard *et al.*, 2002; Newbold *et al.*, 2006) or could possibly influence the onset of puberty (reviewed by Magnusson & Ljungvall, 2013). In the rat, there is a fixed masculinization programming window during gestational day 15.5 to 19.5, during which exposure to anti-androgens reduces the anogenital distance and induces hypospadias, cryptorchidism and testicular malformations (Carruthers & Foster, 2005; Welsh *et al.*, 2008). There are similarities between the effects seen in prenatally exposed rats and the disorders associated with the testicular dysgenesis syndrome (TDS) in humans (Fisher, 2004). The TDS hypothesis was formulated as declining sperm counts and increased incidence of testicular germ cell cancer, cryptorchidism and hypospadias in boys and men seemed to have common risk factors. They were therefore proposed to all be symptoms of the same condition (Skakkebæk *et al.*, 2001), and exposure to EDCs may be one of many causes (Sharpe & Skakkebaek, 2008).

Although fetal exposure to chemicals can cause these adverse effects, androgen action after birth is also important for the development of the reproductive organs. For example, disruption of androgen action during the masculinization-programming window reduces penis size and anogenital distance in rats, but in combination with postnatal exposure to anti-androgens, these parameters are reduced even further (MacLeod *et al.*, 2010).

1.2 Environmental contaminants and their effects on the male reproductive system

This section gives a brief overview of the contaminants analyzed in this thesis; the use, the overall time trends of levels found in biota (with focus on Sweden) and their potential impact on male reproduction. Most of the chlorinated and brominated contaminants in this thesis are either banned or under restriction in Europe. For example, the PCBs, chlordane, hexachlorobenzene (HCB), perfluorooctane sulfonate (PFOS), DDT and some of the brominated flame retardants (PBDEs) are listed as persistent environmental pollutants (POPs) by the UN through the Stockholm Convention, an international environmental treaty with 172 parties (The Stockholm Convention, 2010). After legislation, most of the contaminant levels in the Swedish environment have decreased, although slowly. For example, in the Swedish contaminant monitoring programme in marine biota, the yearly decrease in PCB, DDE and HCB has been approximately 2-11% per year. The PBDEs show both decreasing and inconsistent trends and no decrease has been observed for PFOS (Bignert *et al.*, 2012).

1.2.1 Polychlorinated biphenyls (PCBs)

PCBs were used in many different industrial and commercial applications, such as electrical equipment, paints and heat- and pressure-transfer fluids (Voogt & Brinkman, 1989). In 1966, the Swedish chemist Sören Jensen found very high concentrations of an unknown contaminant in wildlife tissues and identified it as PCB (Jensen, 1972). Production and (new) use of PCB was banned in Sweden in the early seventies (Jensen, 1972), and production in other countries ceased during the 70s and 80s (Voogt & Brinkman, 1989) and came to an end in the 90s (Breivik *et al.*, 2002). There are 209 different PCB congeners, having different numbers and positions of chlorine atoms. Some congeners are dioxin-like and will bind to the aryl hydrocarbon (Ah) receptor (Van den Berg *et al.*, 1998), while others are estrogenic (Soto *et al.*, 1995), anti-androgenic or anti-estrogenic (Bonefeld-Jørgensen *et al.*, 2001). In rats, examples of adverse effects due to PCB exposure are reduced weight of reproductive organs and testosterone levels (Sager, 1983; Faqi *et al.*, 1998a; Hany *et al.*, 1999). Also, decreased sperm production and increased abnormal sperm morphology have been reported in rats (Faqi *et al.*, 1998b; Hsu *et al.*, 2007). The latter has also been observed in young men accidentally exposed to PCBs and dibenzofurans *in utero* (Guo *et al.*, 2000).

1.2.2 Polybrominated diphenyl ethers (PBDEs)

PBDEs are used as flame retardants in, for example, textiles, electronics and furniture (Alaee *et al.*, 2003). Like the PCBs, they are lipophilic and the congeners have different numbers and positions of bromine atoms (Rahman *et al.*, 2001). PBDE 47 is often the most predominant congener in fish-eating birds and mammals (de Wit, 2002). The commercial mixture penta-PBDE consists mainly of PBDE 47 and PBDE 99, whereas the predominant congeners in the octa-PBDE formulation are PBDE 183 and PBDE 209 (La Guardia *et al.*, 2006). Both of these mixtures are listed as POPs. They were banned in the European Union in 2004, and in 2005 the production in the USA was stopped – but deca-PBDE is still used in many countries (de Wit *et al.*, 2010). Deca-PBDE consists mainly of PBDE 209 (La Guardia *et al.*, 2006). The penta-PBDE mixture (DE-71) has been shown to inhibit binding of the androgen receptor (Stoker *et al.*, 2005) and delay male puberty in rats (Stoker *et al.*, 2004; Kodavanti *et al.*, 2010). In addition, PBDEs seem to affect sperm function and/or spermatogenesis in rats (Kuriyama *et al.*, 2005), mice (Tseng *et al.*, 2006) and men (Abdelouahab *et al.*, 2011).

Methoxylated PBDEs (MeO-PBDEs) have been found in marine mammals (Verreault *et al.*, 2005; Rotander *et al.*, 2012). The origin of MeO-PBDEs is not completely clarified, but there are indications that they are mainly of natural origin and probably not metabolites of PBDEs (Teuten *et al.*, 2005; Teuten & Reddy, 2007; Rotander *et al.*, 2012). It seems that biomagnification through the food chain could explain some of the presence in wildlife (Kelly *et al.*, 2008; Jaspers *et al.*, 2013). Structural analogs of PBDEs (MeO-PBDEs and OH-PBDEs) are potentially endocrine disruptors (reviewed by Wiseman *et al.,* 2011). For example, some MeO-PBDEs have been seen to increase testosterone production in a human adrenocortical carcinoma cell line (He *et al.*, 2008).

1.2.3 Dichloro-diphenyl-trichloroethane (DDT)

DDT was initially used as an insecticide in the 1940s, and it was later used in large quantities for the control of agricultural and forest pests. DDT was banned in many countries in the 70s and 80s, mainly because of the adverse effects on wildlife (Turusov *et al.*, 2002). However, it is still used to control malaria in several African and Asian countries (WHO, 2012). P'p-dichloro-diphenyl-dichloroethylene (DDE) is the major metabolite of DDT and the predominant DDT-related compound in biota. While DDT is considered to have estrogenic effects (Bitman & Cecil, 1970), DDE is considered to be an anti-androgen and in studies on rats, reductions in anogenital distance, testicle weight and number of sperm have been observed (Kelce *et al.*, 1995; Loeffler & Peterson, 1999; Wolf *et al.*, 1999) In addition, DDT exposure has been associated with impaired seminal parameters in men (Ayotte *et al.*, 2001; De Jager *et al.*, 2006; Aneck-Hahn *et al.*, 2007).

1.2.4 Hexachlorobenzene (HCB)

HCB was used as a fungicide and in different applications in industry, but was banned subsequently in different countries from the 1960s up until 2000s (Barber *et al.*, 2005). HCB binds weakly to the Ah receptor and could therefore be classified as a dioxin-like compound (Hahn *et al.*, 1989; Birgelen, 1998). Binding of dioxin to the Ah receptor causes disruption of the development of the male reproductive system (Gray *et al.*, 1995). Moreover, HCB partially agonizes androgen action in the prostate in low doses, but interferes with androgen action in high doses (Ralph *et al.*, 2003).

1.2.5 Chlordane

Technical chlordane is an organochlorine pesticide mixture with up to 120 components, for example cis-chlordane, trans-chlordane, heptachlor and trans-nonachlordane (Dearth & Hites, 1991). Metabolism of technical chlordane results in oxychlordane and heptachlorepoxide (Tashiro & Matsumura, 1978; Brimfield & Street, 1979). In Sweden, the use of technical chlordane was banned in 1971 (KIFS, 2008:3), but it was not extensively used prior to the ban (Cato, 2008). Chlordane has been found to be a weak anti-androgen *in vitro* (Lemaire *et al.*, 2004) and has been shown to induce testicular lesions in mice (Balash *et al.*, 1987). In addition, it has been suggested that chlordane is associated with testicular germ cell tumors in men (Hardell *et al.*, 2003; Purdue *et al.*, 2009).

1.2.6 Perfluoroalkyl acids (PFAAs)

Poly- and perfluoroalkyl acids have been widely used in products such as textiles, paper, fire-fighting foams and paints but also a variety of other applications – and many are still in use (Lindstrom *et al.*, 2011). Perfluorinated means that all hydrogens on all carbon atoms in an alkyl chain have been replaced with fluorine, for example perfluorooctane sulfonate (PFOS), that consists of a chain of eight carbons (Buck *et al.*, 2011). PFOS is the predominant PFAA in wildlife and seems to be the most bioaccumulative (Houde *et al.*, 2011), although PFAAs with longer chains also seem to have a high bioaccumulation potential (Conder *et al.*, 2008). Consequently, the production has shifted from PFOS towards short chain PFAAs such as perfluorobutane sulfonate, PFBS (3M, 2000), which seems to be less toxic than PFOS (Lieder *et al.*, 2009). However, PFOS is still produced in China (Zhang *et al.*, 2012). PFAAs appear to have the ability to affect both Leydig cells (Cook *et al.*, 1992; Biegel *et al.*, 1995) and Sertoli cells (Qiu *et al.*, 2013) *in vivo*. They also affect the estrogen and androgen receptors *in vitro* (Kjeldsen & Bonefeld-Jørgensen, 2013) and may reduce the expression of growth hormone receptors in the testis (Yeung *et al.*, 2011). Feeding PFOS to adult mice resulted in lower serum concentrations of testosterone and epididymal sperm counts (Wan *et al.*, 2011).

1.3 Reproduction and reproductive toxicity in the mink

The mink is a seasonal breeder with mating occurring in late February and March (Enders, 1952), or, for wild mink in southern Sweden, March or occasionally early April (Gerell, 1971). Female mink are induced ovulators and shortly after fertilization, the development of the embryo is arrested (Hansson, 1947). The female may then mate with another male and give birth to kits from two ovulations (Enders, 1952). After a period of delayed development, the increasing day length during spring stimulates the production of prolactin which in turn initiates implantation (Sundqvist *et al.*, 1989) in the beginning of April (Enders, 1952). The kits are then born in the beginning of May (Hansson, 1947). Litter size in wild mink has been observed to be on average 3-4 (Mitchell, 1961; Gerell, 1971; Sidorovich, 1993). The kits are looked after by the female up until late summer or early autumn when juvenile dispersal starts (Gerell, 1970; Bonesi & Macdonald, 2004). However, the female can occasionally allow the juveniles to linger in her home range for some time (Mitchell, 1961).

Mink reproduction follows an annual cycle. During autumn, the decreasing day length stimulates the secretion of follicle stimulating hormone and luteinizing hormone (Martinet *et al.*, 1992). This, in turn, stimulates the production of androgens and estrogen by the gonads and an increased weight of the ovary and testis during autumn and winter can be observed (Pilbeam *et al.*, 1979). In males, testosterone peaks in January and then falls abruptly in early February (Sundqvist *et al.*, 1984), just before the breeding season. Spermatozoa are generally present in the epididymis by the end of February

(Onstad, 1967). After the breeding season, testosterone concentrations continue to decrease (Nieschlag & Bieniek, 1975) and the testicles regress and remain very small and inactive until autumn (Onstad, 1967).

In the 1960s, domesticated mink in the USA experienced poor reproductive performance and it was discovered that it was caused by feeding the mink PCB-contaminated fish (Aulerich *et al.*, 1971; Aulerich *et al.*, 1973). Mink were found to be very sensitive to PCBs (Bleavins *et al.*, 1980). Feeding female mink PCB-contaminated fish (that could also contain other chemicals) or commercial mixtures of PCB results in reduced kit survivability, reduced kit body weight, lower whelping rates and decreased litter size (Aulerich *et al.*, 1971; Aulerich *et al.*, 1973; Bleavins *et al.*, 1980; Hornshaw *et al.*, 1983; Kihlström *et al.*, 1992; Heaton *et al.*, 1995; Restum *et al.*, 1998; Brunström *et al.*, 2001; Bursian *et al.*, 2006; Beckett *et al.*, 2008) . The reduced whelping rate is probably attributed to early and/or late fetal death due to lesions in the maternal vessels in the placenta (Bäcklin & Bergman, 1992; Bäcklin *et al.*, 1998)

Similar adverse effects have been observed in mink fed heptachlor (Crum *et al.*, 1993), HCB (Rush *et al.*, 1983; Bleavins *et al.*, 1984), polybrominated biphenyls (Aulerich & Ringer, 1979), PBDEs (Zhang *et al.*, 2009) and also in mink fed thyroid glands and diethylstilbestrol (reviewed by Aulerich & Bursian, 1996). In contrast, these parameters were not affected when exposing mink to DDT and its metabolites (Aulerich & Ringer, 1970; Jensen *et al.*, 1977).

1.4 The wild mink as a sentinel species

A sentinel species, sometimes referred to as a surrogate or an indicator species, is an organism that can be studied in place of other organisms for various reasons, or used to gather information about the environment it lives in, for example level of pollution. Sentinels may provide an early warning of potential health risks before human populations are affected (Van der Schalie *et al.*, 1999).

O'Brien et al (1993) listed a number of criteria for choosing a good sentinel species. For example, it should be able to accumulate contaminants to levels that reflect the local contamination in space and time, i.e. not be a migratory species. It should also be omnivorous and of a high trophic level, and the route of exposure should be similar to that of humans. Ideally it is sensitive to pollutants and similar enough to humans to be able to make comparisons of health effects. The possibility of studying it in captivity is advantageous, as this could confirm findings from the field. In addition, the time from exposure to appearance of subsequent effects should be short, so that an early warning may be identified.

Knowledge about wild mink biology is quite extensive (Gerell, 1972; Dunstone, 1993). Since it has been farmed for its fur for many years, its reproduction has been the subject of many studies (Hansson, 1947; Enders, 1952; Sundqvist *et al.*, 1988; Sundqvist *et al.*, 1989). Information is also available on mink disease and toxicology (Hunter & Lemieux, 1996), basic physiology (Aulerich *et al.*, 1999) and anatomy (Klingener, 1979). Toxicological data from mink studies have been suggested to be a useful complement in risk-assessment for human exposures (Calabrese *et al.*, 1992). The mink is a top predator that feeds mainly on fish, but also crayfish, frogs, birds, rodents and insects (Gerell, 1967b). This means that the mink is presumably highly exposed to contaminants that biomagnify in the food chain, as contaminants often end up in the aquatic environment. Due to the choice of prey and the fact that the home range of the mink is always close to some sort of body of water, the mink is often said to be semi-aquatic (which in other words means that it may be considered semi-terrestrial). The home range of the mink is relatively small, on average 2.5 km along a shore-line in a Swedish habitat (Gerell, 1970). An unequal amount of time is spent in the different parts of the home range, and mink tend to spend most time in core areas that are richer in dens and prey (Dunstone, 1993; Halliwell & Macdonald, 1996). The limited area in which a mink resides means that concentrations of contaminants found in mink reflect local pollution (Wren *et al.*, 1986; Foley *et al.*, 1988).

Mink originate from North America but have been introduced in many other parts of the world. There are many established populations in different countries in Europe (Bonesi & Palazon, 2007), but also in east Russia (Dunstone, 1993), Japan (Ooi *et al.*, 1992), and south Chile (Ibarra *et al.*, 2009). In Sweden, the mink was introduced during the 1930s and soon spread all over the country (Gerell, 1967a). An estimated amount of 11 500 mink were hunted in 2007/2008 (Viltforum, 2009); thus, collecting sufficient amounts of samples is realistic. In Sweden, the mink is mostly hunted for protective measures, rather than trapped for its fur, as it can do substantial damage to populations of birds (Roos & Amcoff, 2010). As the mink is considered to be an invasive species, legislation permits hunting all-year round in unlimited numbers (Ministry for Rural Affairs, (1987:905)), which facilitates sampling for monitoring purposes. Moreover, in other European countries mink carcasses should be relatively easy to collect as it is often hunted, for example to protect populations of the European mink (Bonesi & Palazon, 2007) or for the protection of its prey (Bonesi *et al.*, 2007). In Northern America, where the mink originates, it is often trapped for its fur (Basu *et al.*, 2007).

In summary, the mink fulfills many of the basic criteria for a good sentinel species. The wild mink has previously been studied for assessment of wildlife exposure to contaminants (Martin et al., 2004; Kannan et al., 2005; reviewed by Basu *et al.,* 2007) and domesticated mink have been used for toxicological studies (Aulerich & Bursian, 1996; Fox, 2001). However, the use of wild mink as a sentinel for health effects has been rare (Harding *et al.*, 1999; Basu *et al.*, 2005), but the mink has been singled out as a good species for this purpose (Basu *et al.*, 2007). In general, when using a wild sentinel species for studies on the health effects of contaminants, ecological and biological factors need to be separated out so that the risk associated with EDCs may be identified (Lister & Van Der Kraak, 2001).

2 Aims

The overall objective of this thesis is to investigate the possibly of establishing the wild mink as a sentinel for environmental pollution and the effects thereof on the reproductive system. Monitoring possible effects rather than exposure has the advantage that it enables surveillance of additive and synergistic effects attributed to the complex mixture of chemicals found in the environment. This thesis also addresses the importance of handling the variation found in field data, as this may facilitate future sampling and study design for the assessment of contaminant-related health effects. The specific aims were:

- To study parts of the reproductive system in wild male mink that may be suitable for screening and/or monitoring in the field
- To describe the variation in the reproductive system and study the influence of age, sample season and nutritional status on the concentrations
- To describe the variation in concentrations of chlorinated, brominated and fluorinated contaminants analyzed and study the influence of age, sample season and nutritional status
- Characterize the differences in exposure between and within the four sample areas
- Investigate possible associations between tissue concentrations of contaminants and a range of variables of the reproductive system in wild male mink

3 Methodological considerations

3.1 Collection of mink

Collection of mink carcasses started in autumn 2006, when mink from all over Sweden were gathered for the initial study on reproduction (paper I). Local hunters were asked to send in mink that they caught during their regular hunting. Frozen carcasses were used, as the aim was to collect many mink from distant locations in a time-efficient manner. In order to collect fresh carcasses, hunting with dogs, live traps or trap guards are needed, which is not always possible or demands a lot more work in the field in order to cover a wide geographical area. Using frozen carcasses is a practical procedure for the hunter (and the person doing the autopsies), as the hunter could freeze trapped mink sequentially and then send in all the carcasses at the same time.

The majority of the collected mink were male, so therefore we chose to focus on male reproduction. A skewed sex ratio is expected when using traps, probably a result of the more limited home ranges of the females (Buskirk & Lindstedt, 1989).

3.2 Selection of sample areas

In order to sample mink with presumed differences in exposure, the sampling focused on four areas (papers II-IV). One area is located on the coast of the Baltic Sea, which is relatively heavily polluted by industry (the Gävle area). Another coastal area in the west of Sweden was also chosen as the exposure was expected to differ from that in the Baltic Sea (the Koster Islands area, 2 km off the mainland). In an attempt to find mink from rural areas, samples from the northern part of Sweden were collected. In addition, an area just outside Stockholm was chosen, as it could reflect both the urban and agricultural surroundings (the Märsta area). A map and more detailed information of the areas are found in paper III.

There are no mink farms situated in these four areas. Proximity to mink farms should be considered when choosing the sample area (Basu *et al.*, 2009), since escaped domestic mink may bias the sample (Bowman & Schulte-Hostedde, 2009). In Sweden, all mink farms must be properly fenced and maintained in order to prevent escapees (NSF, (2009:10)). However, there could be occasional escapees, and mink are sometimes released by so-called animal rights activists. Kidd et al (2009) did not identify any domesticated mink in two presumably wild populations in Canada (40 km from the nearest mink farm), but some hybrids could be found (6/50 in total). Tentatively, this could serve as an indication of how far away the sample area has to be in order to avoid domesticated mink during sampling. The closest mink farm to the Koster area was 66 km away (and the next 120 km away). The farmer states that a maximum of ten mink managed to escape when the farm was active (from the 80's to 2010) (S-O Bandgren, personal communication). Thus it is not likely that escapees are common in the Koster population. The Gävle area lies approximately 110-120 km from the nearest farm, although this farm was closed down in 2008. The closest farms to the Märsta area and sampling sites in the northern parts of Sweden are at least 160-170 km away.

3.3 Necropsies

Necropsies were performed on newly thawed carcasses. As an indicator of long term nutritional status, the subcutaneous fat on the ventral part of the abdomen between the hind legs was weighed and divided by body weight. This fat pad is actually often the only subcutaneous fat present and it is quickly and easily removed. The epididymis was separated from the testis. The penis was cut off where it enters the pelvis and the length and weight were measured as well as the length of the baculum. In this way, the somewhat time-consuming work of opening the pelvis was avoided, which may be desirable when handling large numbers of carcasses. In a pilot study, the mean coefficient of variation was calculated for length measurements, and the variation was found to be low (1.0-3.1%) (Persson, 2007).

The large variation in prostate size was somewhat challenging, as the outline of this accessory gland is almost undetectable in juveniles caught in early autumn. The difficulties in finding a standardized way of measuring the size made us discard it from the protocol. However, the prostate is an interesting organ as it is a target for EDCs (see review by Prins, 2008) and should not be overlooked when assessing male reproduction.

During necropsy, great care was taken to ensure that free-ranging farmed mink were avoided by examining fur color (and quality) and body size. Male mink in farms mostly weigh over 2 kg, some even exceeding 3 kg (Korhonen & Niemelä, 1998; Socha *et al.*, 2010), which is considerably larger than wild mink (approximately 1 kg, paper II). The larger body size of farmed mink can be partly explained by a larger fat deposit, but also because farmed mink have been selectively bred for body size (Lagerkvist *et al.*, 1993).

Age was determined by tooth cementum analysis at Matson's laboratory (Milltown, Montana, USA). This laboratory is experienced in ageing mink, and they state that most species (including mink) should be aged with an accuracy of 70% and 90% within 1 year of the correct age. The reliability of the age determination on our mink was rated as high for 92.6% of the samples, meaning that the teeth sections nearly match those of the standardized cementum aging model. Ageing is performed by counting the darker annular rings formed during winter.

3.4 Analysis of contaminants

Analyses of contaminants were performed by MTM Research Centre at Örebro University, Sweden (papers II-IV). Brominated and chlorinated compounds were analyzed in subcutaneous fat, as these lipophilic compounds accumulate in adipose tissue. Gas chromatography and low-resolution mass spectrometry were used, after elution of lipids with hexane/dichloromethane (1:1, v/v) and sample clean-up using a multi-layer silica column (for details see paper II).
The PCBs were of interest as the mink is known to be sensitive to them. Of the dioxin-like PCBs, several mono-*ortho*-substituted PCBs were analyzed (PCB 114, 105, 118, 156, 157 and 189). The three coplanar dioxin-like PCB congeners (PCB 77, 126 and 169) were not analyzed. The brominated flame retardants (PBDEs) were also of great interest due to their potential effects. In addition, the deca-PBDEs are still in use in many parts of the world and are debrominated in the environment into penta-PBDEs (Söderström *et al.*, 2004; Stapleton *et al.*, 2006). Perfluorinated alkyl acids were considered as especially interesting to study, since high concentrations had been noticed in otter from Sweden (Järnberg U, 2008) and the general knowledge of levels in wildlife is limited.

For the PFAAs, liver tissue was used for analysis, as high levels often are found in this organ (Ishibashi *et al.*, 2008; Ahrens *et al.*, 2009). Unlike the organochlorine and organobromine compounds, PFAAs are not lipophilic but tend to bind to proteins, for example liver fatty acid binding protein and serum albumin (Luebker *et al.*, 2002; Jones *et al.*, 2003), which are situated or produced in the liver. PFAAs were extracted with acetonitrile, and the solution was put through a WAX solid phase cartridge. The analysis was then performed using a UPLC coupled to a MS/MS mass spectrometer (for details see paper III). As the samples were transported and analyzed in two batches with approximately one year in between; differences between the batches were investigated, but no differences were found.

3.5 Statistical analysis

Multiple linear regression was used as it is an established statistical method for modelling the relationship between one single outcome variable of interest that may be associated with, or depend on, more than one predictor variable. All models were built manually. Least square means (LSmeans) were used when pairwise comparisons between groups were made. LSmeans (or marginal means), are within-group means adjusted for the independent variables in the model, and are often used in unbalanced designs (SAS, 2003). Controlling the pairwise comparisons of LSmeans for Type I errors ("false positive") with for example Bonferroni or Tukey adjustment usually increases the risk for Type II errors ("false negative") (Rothman, 1990). Therefore, it was decided to keep the results unadjusted as the objective of these studies was to unravel all possible influences of environmental and biological factors (papers I-III). The results should be interpreted with this in mind.

To summarize and visualize the variation of chemical concentrations between the four sample areas (papers II-III), multivariate analysis using principal component analysis (PCA) was performed. PCA is suitable for data with many variables, even if they are highly collinear, and it describes the correlation structure so that related observations or variables can be indentified (Eriksson *et al.*, 2006).

4 Main results and discussion

4.1 Measurements of the reproductive system of wild male mink

The reproductive system of wild male mink was studied in order to find robust measurements that could possibly be used for assessments in environmental monitoring and to generate some baseline data (paper I). Frozen carcasses were used, but some baseline data are probably applicable for fresh carcasses as well. Baculum length and the anogenital distance will most likely be relatively unaffected by either freezing or some decomposition.

A low frequency of miscellaneous lesions of the reproductive tracts was found during the necropsies (paper IV), and some of these have been described in studies on domestic mink (Onstad, 1967; Sundqvist, 1987). However, three out of 35 mink from the same area (the Koster area, on the west coast) were cryptorchid. This percentage was somewhat higher than expected when compared to studies on domestic mink. Cryptorchidism is a relatively rare condition, so a larger sample of mink would be needed in order to make a justifiable assessment.

Histological examination of the testes was challenging to perform due to decomposition and freeze fractures. Even so, the structure of the testes was often sufficiently intact to enable recording of the seminiferous tubular diameter (paper I). This diameter was found to follow the same variation as testis weight, a finding that has been observed in farmed mink as well. Seminiferous tubular diameter could be an interesting variable to study, as it may be affected by EDCs. A reduction in tubular diameter has been observed in mice exposed to chlordane (Balash *et al.*, 1987) and DDT (Krause *et al.*, 1975). However, as the variation in tubular diameter within the testis of the mink was sometimes quite large, further studies on how the freezing affects tubular diameter and how to handle intra-assay variation are recommended and fresh carcasses may be preferable. The histological evaluations also revealed that the adult mink seemed to produce spermatozoa earlier in the season than the juveniles, as spermatozoa were found in only 64% of the juveniles and 94% of the adults during spring (paper I). This is in line with findings in a study by (Gerell, 1971), where juvenile females and males seemed to breed later than the adult mink.

As sperm morphology may be targeted by EDCs (Faqi *et al.*, 1998b; Kuriyama & Chahoud, 2004), spermatozoa were collected from the cauda epididymis by needle aspiration for analysis (paper I). Occasionally this procedure failed to collect sufficient spermatozoa for the sperm morphology procedure, or alternatively, the epididymal sperm count was low. Cutting the cauda epididymis into small pieces with scissors in formol-saline could possibly yield better results. The sperm morphology indicated that most mink would probably be fertile (assuming normal sperm count and motility), as the percent defective spermatozoa was low. However, comparing with the results from a study regarding the effect of freezing on cat spermatozoa (Larsson, 2012), the observed low frequency of coiled tails, as well as the increased frequency of abnormal acrosomes, might be an effect of freezing. On the other hand, pathological heads, mid-piece defects and detached sperm heads are most likely unaffected by freezing. So naturally, in order to get all possible information from a sperm morphology evaluation, freezing should be avoided. Still, the results from using frozen samples suggest that the epithelium in the seminiferous tubules is intact and functioning in most mink, as the average percentages of pathological heads, mid-piece defects and detached heads were low (paper I).

4.2 Contaminant levels in wild male mink

Very high levels of PFOS (mean 1250 ng/g wet weight, range is shown in Table 1) and the detection of PFBS and long chain PFAAs in many of the mink indicate that mink readily accumulate several fluorinated organic contaminants (paper III). The concentrations of ∑PCBs varied considerably (Table 1) and the mean concentration of the PCB congeners (n=24 congeners) analyzed was 7320 ng/g lipid weight (lw). The mean concentration of ΣPBDE (n=10 congeners) was 42.5 ng/g lw. The predominant pesticides found in mink were oxychlordane, HCB and p,p'-DDE (mean 21.5, 22.0 and 476 ng/g lw, respectively). The most commonly detected methoxylated PBDEs were 6MeO-PBDE 47 and 2MeO-PBDE 68, although the concentrations were generally low (mean 2.1 and 1.6 ng/g lw, respectively) (paper II).
Table 1 shows the range of contaminant concentrations analyzed in some top predators in Sweden. Note that the sample areas, sample years, and method for

Table 1. *Organohalogen compounds in some mammalian top predators in Sweden (ng/g)*[a]

Species	Sample years	*N*	∑PCB	∑PBDE	DDE	HCB	PFOS	References
American mink (*Neovison vison*)	2004-2009 [b]	101	108–124 000	2.0-390	8.8–9708	2.7–439	<0.8–21 800	(Papers II-III)
Lynx (*Lynx lynx*)	1999-2002 [c]	25	46-1590	n.d.[e]	<LOQ[f]-210	n.d.	2.8-202	(Lind, 2012)
Pine marten (*Martes martes*)	1989-1992 [c]	79	190-11 500		0.47-350	9.5-180		(Bremle *et al.*, 1997)
Otter (*Lutra lutra*)	1990-1994 [c]	43	600-860 000					(Roos *et al.*, 2001)
	2000-2004 [c]	26	1200-78 000					(Bisther & Roos, 2006)
	1970-2011	140					19-16 000	(Roos, 2013)
	2005-2011 [c]	49	-72 000	- 649			32-7352	(Roos, 2012)
Grey seal (*Halichoerus grypus*)	2005-2008	15					156-1444	(Kratzer *et al.*, 2011)
	2000-2001 [d]	10			2300-15 000			(Larsson *et al.*, 2004)

[a] ng/g lipid weight for ∑PCB, ∑PBDE, DDE, HCB and liver wet weight for PFOS, other contaminants were analyzed in: [b] adipose tissue, [c] muscle, [d] liver
[e] not detected
[f] limit of quantification

analysis vary between these studies. The mink, which is relatively short-lived, contains relatively high concentrations of PCB and DDE. This might reflect the semi-aquatic diet of the mink. Mink and otter both seem to have extremely high levels of PFOS. As the otter is a vulnerable species protected from hunting in Sweden, mink could serve as an indicator of the otter to PFOS (and other PFAAs) exposure to the otter in a certain areas of interest.

4.3 Influence of age, season and nutritional status on the reproductive system and contaminant concentrations

4.3.1 Age variability

There was a clear influence of age on the reproductive organs (paper I). The weights of the testes, epididymides and penis were lower in juvenile mink compared to older mink. After the first year of life there was no further increase in weight noted in these organs, nor in the development of the anogenital distance, which indicates that most of the development of the reproductive system is completed after one year. However, baculum and penis length (including the baculum) increased continuously with age, a phenomenom seen in other studies where the baculum has been evaluated as a tool for ageing mink (Elder, 1951; Lechleitner, 1954) .

The concentrations of some PCBs, as well as the concentrations of PBDE 153/154, increased with age, which is in line with the high bioaccumulation potential of these congeners (paper II). However, the concentrations did not differ significantly between juveniles and one-year-olds, possibly due to a combination of growth dilution and high exposure via milk and subsequent changes in diet. All other PBDE congeners (including 6MeO-PBDE 47), DDE, HCB, oxychlordane, all PFAAs and the lower chlorinated PCB congeners were not influenced by age (papers II-III). In general, the results regarding the influence of age on concentrations of contaminants in mammalian wildlife are often quite contradictory, sometimes even in the same species (see discussions in papers II-III). The influence of age could perhaps be less in relation to other factors, such as choice of prey, hotspot exposures or annual changes in lipid metabolism, that make age dependency per se difficult to identify. In mink, the relatively short life span may complicate the identification of an age influence even more, and might be one of the reasons for the absence of an age influence for some contaminants in our studies.

Mink in Sweden seem to be generally quite short-lived. The average age for all our collected males and females was 0.8 years (*n*=283), and only 7% were 3 years or older (unpublished data). The age ranged from juvenile to 5 years old. It is known that juveniles often dominate the trapping result, especially during early autumn (Bonesi *et al.*, 2006). In our experience, the juveniles are easily recognized upon necropsy up until approximately 7 months of age, based on body size and the length and development of the baculum. Depending on the specific research question, this may be sufficient information and the extra work and cost of teeth extraction and age determination by cementum analysis could be avoided. For example, if only assessing the exposure of PBDEs, organochlorine pesticides and PFAAs, a sample containing many juveniles will probably not underestimate the exposure to any great extent as these contaminants did not vary significantly with age. However, if PCBs are of interest, it is more important to include older animals, as the levels of PCB increased with age. Avoiding sampling during the juvenile dispersal in autumn could help to increase the proportion of older animals caught.

4.3.2 Seasonal variability

The mink is known to be a seasonal breeder, and this was clearly shown by the results in paper I. The seasonal change in the measurements on the reproductive organs was similar to observations in domestic mink. The most pronounced seasonal changes were those seen in the weights of the testes and epididymides. Naturally, in order to identify mink with abnormal weights of these organs, the sample size must be large enough to reach satisfactory statistical power. Alternatively, the sampling could be restricted to certain times of year, but one should take into account that the average contaminant concentrations may be over- or underestimated as PCB, PBDE and some PFAA concentrations also vary with season (papers II-III).

PCB concentrations, and also concentrations of PBDE 153/154 and PBDE 47, were generally at their highest in spring. Possibly, this seasonal variation is not attributed only to general weight loss during late winter and spring and subsequent increased concentration in adipose tissue. Perfluorodecanoic acid (PFDA) and perfluoroundecanoic acid (PFUnDA), chemicals that do not accumulate in fat to any larger extent, showed similar seasonal changes in concentration, which suggests influence of other factors, such as seasonal changes in home range size (Zschille *et al.*, 2012), seasonal changes in concentrations in prey or seasonal shifts in food preferences. As the mink generally feeds on any available prey, the composition of the diet is variable, and it has been found to be dominated by mammals (Hammershoj *et al.*, 2004; Ibarra *et al.*, 2009), fish (Gerell, 1967b; Salo *et al.*, 2010), birds (Hammershoj *et al.*, 2004), amphibians (Jedrzejewska *et al.*, 2001), or crayfish (Melero *et al.*, 2008). In a study from Sweden, mink preyed more on fish during winter and spring, but during other seasons preys such as mammals, birds, crayfish, frogs and insects were more common (Gerell, 1967b).

4.3.3 Variability due to changes in nutritional status

Notably, nutritional status did not influence the reproductive traits, except for the anogenital distance (paper I). Nutrition is a major environmental cue for reproduction in domestic animals (Robinson, 1996). For example, ram lambs offered improved pasture and grain supplementation had an earlier pubertal development, smaller winter regression in testicular size and earlier testicular recrudescence in spring than lambs on poorer pasture (Bielli Pallela, 1999). The lack of influence of nutritional status in our study could be interpreted as the mink eating enough for reproduction to rarely be affected by nutritional status as we have measured it.

Mink with sparse amounts of adipose tissue had higher concentrations of ∑PCB and ∑PBDEs than mink with larger fat depots, which reflects the lipophilic nature of these chemicals and the increased concentration in adipose tissue in case of an increased fat mobilization. In contrast, the analyzed pesticides and the PFAAs were not influenced by nutritional status (papers II-III). For the lipophilic pesticides this is difficult to explain, but the PFAAs are not expected to accumulate specifically in adipose tissue as they tend to bind to proteins (Luebker *et al.*, 2002; Jones *et al.*, 2003).

Nutritional status did not vary with season in paper I, but in paper II the nutritional status of mink caught in winter was significantly higher than that of mink caught in autumn or spring. Possibly, factors such as prey availability, population density, local climate and disease (for example Aleutian disease) could be the underlying reasons for the inconsistent results.

4.4 Variability in contaminant concentrations between and within sampling areas

Mink from the Baltic coast area had the highest average concentrations of ∑PCBs, ∑PBDEs, perfluorononaoic acid (PFNA), HCB, oxychlordane and DDE (papers II-III). This may be a reflection of the fairly large industries in that area (historically), and that the large rivers Dalälven and Ljusnan may carry some pollution. Mink from the Koster area on the west coast of Sweden generally had concentration levels that were intermediate compared to the other areas.

The differences in contamination levels between the sampling areas were more pronounced for the PFAAs than for the brominated and chlorinated contaminants (as seen in the scores plots in paper II and III). The lower chlorinated PCB congeners seemed to be more abundant in the urban/agricultural area (Märsta) and the average concentrations of PFOS and perfluorohexane sulfonate (PFHxS) were high in this area. In contrast, mink from the north of Sweden contained generally low concentrations of PFOS and PFHxS. Also, many mink from the northern parts of Sweden showed similar concentrations of the chlorinated and brominated contaminants as mink from the other areas, demonstrating the ubiquity of these contaminants.

There was considerable variation in contaminant concentrations within some areas. This was clearly shown by the principal component analyses (papers II-III), where the urban/agricultural area (Märsta) had the largest within-area variations. For example, several mink showed high ∑PCB concentrations, but there were also mink with very low concentrations. In addition, PFOS concentrations were extremely high in some mink. These large variations are most likely due to point sources, such as the large international airport and the Swedish Rescue Services former training camp, within the Märsta area. The small home range of the mink possibly reflects local contamination. In addition, population dynamics such as juvenile dispersal and transient males from adjacent areas during breeding season may perhaps result in some variation in contaminant concentrations.

4.5 Associations between contaminant concentrations and the reproductive system in the mink

Using multiple regression models (with covariates), several associations between the contaminant concentrations and the reproductive organ measurements were found. In a mixture of brominated, chlorinated and perfluorinated contaminants (and other contaminants that were not analyzed), significant negative associations between DDE, PFOS, PFDA, PFUnDA, ∑PFAA concentrations and the anogenital distance were found ($p<0.05$). In addition, penis length tended to be negatively associated with DDE ($p<0.1$). These pollutants showed anti-androgenic effects in studies on laboratory animals (Kelce *et al.*, 1995; Shi *et al.*, 2007) and similar associations in humans and wildlife have been observed (Guillette Jr *et al.*, 1999; Torres-Sanchez *et al.*, 2008). These results should be further investigated, as high levels of PFAAs are found in some wildlife.

Baculum length, penis length and penis weight were positively associated with PCB 52, PCB 47/48 and PCB 110. Likewise, there were positive associations between penis length and PCB 28, and between penis weight and PCB 52. These results are difficult to interpret. As these PCB congeners are expected to have estrogenic properties, the results might be interpreted as a stimulating or protective effect on the baculum, but this is speculative. Negative associations between pollutants and baculum length were not found, but have been reported in other wildlife.

In an additional statistical analysis of sperm morphology from 22 mink, no influence of contaminant concentrations on the percentage of pathological heads or abnormal sperm was found (in formol-saline; mid-piece defects, detached sperm heads, proximal cytoplasmatic droplets and acrosome defects).

As only male mink were used for exposure analysis, comparison with laboratory studies on mink reproduction with endpoints such as litter size and kit survival should be interpreted with great caution, but could serve as a useful indication. After the first breeding season, females will probably have lower concentrations of most contaminants than males, due to transfer to the offspring during gestation and lactation (Bleavins *et al.*, 1981). Assuming that juvenile males and females contain approximately the same concentrations of contaminants prior to the first breeding season, a comparison was made between juvenile concentrations (n=51) and laboratory studies performed on mink. The levels of HCB in this study were not as high as those associated with increased kit mortality (Rush *et al.*, 1983), nor were the PBDE levels as high as the concentrations resulting in complete reproductive failure (Zhang *et al.*, 2009). Nevertheless, 12% of the juveniles had ∑PCB concentrations above the lowest observed adverse effect level (LOAEL) reported by Brunström et al (2001). At this level, reduced kit production and kit growth were observed in litters after females were fed a mixture of PCB congeners (Clophen A50) that resulted in an average concentration of 12 000 ng/g lw in muscle. However, a comparison with that study should be made carefully, as it seems that the effect was mediated mostly by congeners that are Ah receptor agonists (dioxin-like). In our study, only mono-*ortho*-substituted dioxin-like PCBs were analyzed and these PCBs have lower Ah-receptor-mediated toxicity than the co-planar PCBs. However, this analysis suggests that mink can accumulate concentrations above this LOAEL during their first year of life. As the juvenile mink with the highest concentrations were from the urban/agricultural area (Märsta) and the Baltic coast area (Gävle), further investigation of the concentrations in the female mink in these areas is justified.

Although there are considerable numbers of studies on mink fed different doses of contaminants, inconsistencies are often found in tissue selection and reporting on a wet weight or lipid weight basis when analyzing body concentrations (if reported at all). Moreover, there is generally a level of uncertainty when comparing the sum of PCBs in different studies. The number of analyzed congeners may differ between studies, which of course influences the calculated levels. Also, the congener patterns may differ; it could even vary geographically in wild mink (as seen in paper II). Therefore, some information is lost when reporting PCBs or PBDEs only as a sum of the congeners.

5 Conclusions

Wild male mink in Sweden readily accumulate different kinds of contaminants and may serve as an indicator of environmental contamination. More importantly, the wild mink seems to be a suitable sentinel species that may provide an early warning of alterations in male reproductive organs related to environmental pollution. The specific conclusions drawn from the studies in this thesis were:

- Large numbers of mink were successfully collected by working with local hunters and handling frozen carcasses. This is a good and practical approach, as mink carcasses are small enough to be easily transported and stored, but it hampers histology and the evaluation of sperm morphology. Baseline data for several reproductive organ measurements were provided, that could be used for comparisons in screening and/or monitoring programmes

- The variation in the reproductive organs in male mink was connected to both age and season, but nutritional status did not seem to have any influence. The large variation in some reproductive organs (e.g. testis weight) points to the need for carefully designed sampling strategies

- When recording concentrations of PCBs and PBDEs in wild male mink, it is advisable to take age, season and nutritional status into account. Higher concentrations were generally found in mink that were two years or older, caught in spring or with poor nutritional status. The influence of these factors on concentrations of HCB, chlordane, DDE and most PFAAs was not significant, although some PFAAs showed seasonal variation

- Mink from the Baltic coast area around Gävle and Söderhamn had generally the highest concentrations of many of the contaminants compared to the other sampling areas. However, there were mink with very high concentrations of PFOS from the urban/agricultural area north of Stockholm (Märsta), and there was more variation in contaminant concentrations within this area than in the other areas

- Several associations between tissue concentrations of contaminants and variables of the reproductive system were found. For example, the anogenital distance was inversely associated with DDE, PFOS, PFDA, PFUnDA and ∑PFAA concentrations. Also, penis length tended to be negatively associated with DDE. These results warrant further investigation

6 Future perspectives

EDCs have been a growing research area for some decades and cause-effect linkages are becoming more evident. However, there are many challenging tasks ahead, considering the concepts of mixtures and non-linear dose-responses, as well as the increasing number of chemicals. Research on wild animals will most likely continue to provide valuable information that will increase our understanding of the effects of endocrine-disrupting chemicals.

The information provided in this thesis can be used to improve the design of studies using fresh carcasses, which is beneficial as the latter may be more costly and labour-intensive to collect. Fresh carcasses can provide information about histological examinations, for example, or hormone analyses. Moreover, the assessment of sperm morphology will be more reliable. A combination of variables on frozen carcasses and other variables on fresh carcasses could be used in order to assess the reproductive system of male mink in an area of interest.

The seasonality of mink reproduction is challenging in terms of sampling and statistical analysis. However, the seasonality is in itself interesting, as the yearly timing of, for example, spermatogenesis prior to the breeding season is crucial. It has been shown that mink exposed to diethylstilbestrol (an estrogenic compound) prior to the breeding season had depressed testicular development and spermatogenesis was absent (Pridham, 1960). The annual development of the reproductive organs in male mink could in theory be delayed due to environmental exposures of estrogenic and anti-androgenic compounds. Assessing the development of the reproductive organs and spermatogenesis during the breeding season is an important step towards understanding possible adverse effects on male mink fertility. Testicular histology could be a useful tool, as well as testosterone concentrations (Sundqvist *et al.*, 1984). Moreover, as sperm count seems to be declining in humans (Carlsen *et al.*, 1992; Swan *et al.*, 2000), the number of homogenization resistant spermatids in the testicles could possibly be a useful endpoint to explore in mink.

As mink can breed during their first year of life, the generation time will be relatively short. This suggests that mink may be a suitable sentinel species for developmental toxicity in humans and wildlife with longer generation time. For example, the anogenital distance offers an easy, inexpensive and fast measurement that may be used for assessment of developmental exposure. Further studies are needed to investigate if anogenital distance in mink is correlated to, for example, sperm count, penis length and reproductive organ weights. To do this, a large set of mink of the same age sampled at the same time of year would be needed.

Using the anogenital distance together with other reproductive parameters may offer a way of screening for hot-spot exposure areas. To explore further the possible developmental effects of EDCs in the environment, studies on female mink exposure and the effects thereof on female reproduction are also of great importance. To assess better the associations between pollutants and developmental effects in males, juveniles caught during summer or autumn could be the best sample to focus on, as these mink presumably have a contaminant pattern resembling the one they were exposed to prenatally.

The mink is generally quite short-lived. Therefore, it can be assumed that the concentrations seen in mink reflect recent exposure and monitoring mink can reveal very recent fluctuations in the environment. This suggests that the mink may be suitable for monitoring time-trends or to monitor the level of contamination after measures have been taken to remove the source of pollution in an area. Following the PFAAs levels in the Märsta area would be of great interest, especially for the people resident in that area, particularly since concern for health effects due to PFAA exposure in the Swedish population has been identified in a recent risk assessment (Borg *et al.*, 2013).

As mink can be collected in areas quite close to human activities, they could be a useful tool for assessing environmental contamination of, for example, pharmaceuticals, chemicals used in consumer products or even new chemicals. Of course, other EDC-related health effects, for example on the immune system, thyroid or adrenals, remain to be studied in wild mink.

7 Populärvetenskaplig sammanfattning

Den här avhandlingen undersöker möjligheten att använda vild mink (*Neovison vison*) som ett miljöövervakningssystem, både för exponering och för effekter av miljögifter. På så sätt kan vild mink fungera som en ”alarmklocka” för miljögifter och deras påverkan på exempelvis fortplantningssystemet. En sådan varning skulle även kunna vara av betydelse för människor, eftersom både vilda djur och människor utsätts för snarlika kemikalieblandningar.

Under de senaste decennierna har studier påvisat kopplingar mellan hormonstörande kemikalier och djur och människors hälsa. Ett av de hormonsystem i kroppen som kan påverkas är det som har hand om fortplantningen. Kända exempel på miljögifter som kan orsaka fortplantningsstörningar är till exempel miljögiftet DDT, som orsakar äggskalsförtunning hos fåglar och miljögiftet PCB, som har satts i samband med nedsatt fortplantningsförmåga hos gråsälar. Även människors fortplantning tros kunna påverkas.

För att kunna övervaka vad som händer när miljögifter kommer ut i miljön kan man använda en indikatorart, det vill säga en art som återspeglar förhållanden i miljön. Den amerikanska minken blev introducerad i Sverige på trettiotalet, har många egenskaper som gör den lämplig som indikatorart för kemikalier och kemikalierelaterade effekter i miljön. Bland annat så är den högt upp i näringskedjan och födan består till stor del av vattenlevande djur som t ex fisk, kräftor och grodor, men även fåglar, fågelägg och gnagare. Troligtvis innebär dess födopreferenser att den är starkt exponerad för många olika kemikalier och i ganska höga halter. Dessutom är minken relativt vanligt förekommande i större delen av landet och får jagas året runt i obegränsad mängd.

Minkar samlades in från lokala jägare runt om i hela Sverige, men flest minkar togs från fyra utvalda områden: Kosteröarna på västkusten, östkusten kring Gävle och Söderhamn, Märsta strax norr om Stockholm och Norrlands inland. Minkhannarnas fortplantningsorgan studerades för att hitta robusta och lämpliga mått/parametrar att använda i miljöövervakningssyfte. Dessutom analyserades halter av klorerade, bromerade och perfluorerade ämnen i minkarnas lever och/eller fettdepåer. Sedan undersöktes hur faktorer som minkens ålder, insamlingssäsong och kroppskondition påverkar minkarnas fortplantningsorgan och halter av miljögifter. Resultaten från dessa analyser kan användas för att förbättra statistisk hantering eller effektivisera strategier för insamling av mink i framtiden.

Relativt höga koncentrationer av PCB fanns i vissa minkars fettdepåer, särskilt i de från områdena kring Gävle/Söderhamn och Märsta. Några minkar från Märsta hade bland de högsta koncentrationerna av perfluoroktansulfonat (PFOS) som någonsin uppmätts i mink. De höga koncentrationerna kan bero på att Räddningsverkets f.d. övningsplats och Arlanda flygplats ligger i området.

Statistiska analyser påvisade flera samband mellan halter av kemikalier och förändringar i fortplantningssystemet. Minkar med höga halter av DDE (en nedbrytningsprodukt av DDT) och vissa perfluorerade ämnen (t ex PFOS), hade ett relativt kortare anogenitalt avstånd. Det anogenitala avståndet är ett mått som ofta används i exempelvis experimentella studier på råtta för att utvärdera effekter av hormonstörande ämnen under fostertiden. Ett minskat anogenitalt avstånd hos råtthannar tyder på att könshormonerna har påverkats, det vill säga en möjlig feminisering och/eller anti-androgen effekt.

Sammanfattningsvis visar studierna i den här avhandlingen att vilda minkar fungerar utmärkt som indikatorer/alarmklocka för miljögiftsföroreningar i Sverige. I framtida studier kan minken med fördel användas till att leta efter nya miljögifter eller övervaka kända miljögifter. Att studera minkens fortplantningssystem kan ge ledtrådar att följa för att upptäcka påverkan av miljögifter i naturen och för att bringa klarhet i hur blandningar av hormonstörande ämnen påverkar djurs och människors fortplantningssystem.

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