Male reproductive health in environmental research

Aspects of histological evaluation of testicular tissue

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Abstract

There is increasing concern that chemicals in the environment are causing male reproductive disorders in animals and humans. This thesis investigated different aspects of histological evaluation of testes, as a tool to measure effects of environmental chemicals on male reproductive health in wild and laboratory animals. When collecting samples from wild animals, unpredictable conditions can result in poor fixation of tissues and robust endpoints are therefore important. In mink, testis length and weight, area and diameter of seminiferous tubules and acrosome marked with Gata-4 were found to be the most reliable measurements after delayed pre-fixation time. Knowledge of the normal morphology and seasonality of testes is crucial when evaluating male reproduction. Staging of the seminiferous tubules in mink and polar bear is described in this thesis. A computerised image analysis method, including semi-automatic delineation of seminiferous tubules, was developed to objectify and improve measurements of testicular tissue. The method was used to measure the area and diameter of tubules in polar bear, rat and mink. Gata-4 was used as the key component in developing a novel morphology-based approach for evaluating testicular development in pubertal laboratory rats. In controlled settings, effects of a single chemical, bisphenol A (BPA), was investigated in Fisher rats. No major effects on male reproduction in midpubertal or adult rats were seen, apart from higher prevalence of mild inflammatory cell infiltrate in cauda epididymis in adult rats exposed to 50 µg BPA/kg bw/day *in utero* and during lactation. In polar bears, testes histology and organ measurements enabled identification of reproductive status, independent of season and age. Reproductive organ measurements were found to differ with reproductive status, stressing the importance of considering this factor when evaluating effects of chemicals in polar bear. Inverse correlations between testes and baculum measurements and oxychlordane concentrations indicated that this chemical may cause reproductive disruption in East Greenland polar bears. Another sign of disturbed reproduction was disorganisation of germ cells in testis, seen in half of all polar bears with active sperm production, but no correlations to target chemicals were detected. In conclusion, the factors season, reproductive status and handling of tissues prior to fixation must be taken into consideration when evaluating testis histology and organ weight, in order to obtain accurate results and draw correct conclusions. Computerised image analysis proved to be a useful objective tool when evaluating testis histology in polar bear, mink and rat.

Keywords: Testes histology, mink, polar bear, Fisher rat, bisphenol-A, persistent organic pollutants, wild animals, image analysis, morphometry.

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Dedication

To my family

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 Spörndly-Nees, E., Ekstedt, E., Magnusson, U., Fakhrzadeh, A., Luengo Hendriks, C.L., Holm, L. (2015). Effect of pre-fixation delay and freezing on mink testicular endpoints for environmental research *PLoS ONE*, 10 (5), pp. 1-17.

II †Fakhrzadeh, A., †Spörndly-Nees, E., Ekstedt, E., Holm, L., Luengo Hendriks, C.L. (2016). New computerized staging method to analyse mink testicular tissue in environmental research. *Environmental Toxicology and Chemistry*, 36 (1), pp. 156-164.

III Spörndly-Nees, E., Boberg, J., Ekstedt, E. Holm, L., Fakhrzadeh, A. Dunder, L., Kushnir, M.M., †Lejonklou, M. H., †Lind, P.M. (2018). Low-dose exposure to Bisphenol A during development has limited effects on male reproduction in midpubertal and aging Fischer 344 rats. *Reproductive Toxicology*, 81, pp. 196-206.

IV Spörndly-Nees, E., Holm, L., van Beest, F.M., Fakhrzadeh, A., Ekstedt, E., Letcher, R., Magnusson, U., Desforges, J.P., Dietz, R., Sonne, C. Age and seasonal variation in testes and baculum morphology in East Greenland polar bears (*Ursus maritimus*) in relation to high concentrations of persistent organic pollutants. (*manuscript*).

Papers I-III are reproduced with the permission of the publishers.
† Authors contributed equally

Abbreviations

BPA	Bisphenol A
EFSA	European Food Safety Authority
DDT	Dichlorodiphenyl-trichloroethanes
GD	Gestation day
HCH	Hexacyclohexanes
HCB	Hexachlorobenzene
HE	Haematoxylin eosin
OCS	Octaclorostyrenes
PAS	Periodic acid shiffs
PCB	Polychlorinated biphenyls
PND	Post-natal day
POP	Persistent organic pollutants

1 Introduction

During recent decades, there has been growing concern about the increased frequency of reproductive disturbances seen in both animals and humans. In humans, lowered semen quality, increased incidence of testicular cancer, cryptorchidism and hypospadias have been reported (Bergman *et al.*, 2013; Toppari *et al.*, 1996). Defects in sex organ development and function in alligators, reduced reproductive organ weight and disrupted histopathology in polar bears, and eggshell thinning in birds are just some of many reproductive disturbances reported in wildlife. A number of studies have linked adverse effects on male reproduction in mammals, birds and fish to environmental pollutants (Bellingham *et al.*, 2012; Sonne *et al.*, 2006; Fox, 2001; Vos *et al.*, 2000). There has been a marked increase in new chemicals marketed since the 1960s (Binetti *et al.*, 2008). Close to 800 chemicals are known or suspected to interfere with hormone receptors, hormone synthesis or hormone conversion. These chemicals are often referred to as endocrine disrupting chemicals (Bergman *et al.*, 2013). The mechanism of action varies and has been described as *e.g.* mimicking oestrogens, having masculinising effects or acting as an anti-androgen. Some chemicals may act as both oestrogenic agonists and androgen antagonists (Tyler *et al.*, 1998). On combining wildlife and human evidence in a major scientific report, Bergman *et al.* (2013) concluded that exposure to endocrine disrupting chemicals during foetal life and puberty could possibly be the cause of male reproductive health problems in humans.

Different approaches can be used when investigating the relationship between chemicals in the environment and their potential harmful effects on humans and wildlife. Depending on the aim, the effect of a single chemical on male reproduction can be studied in a controlled laboratory environment, or real-life exposure to chemical cocktails can be monitored in wild animals. In the laboratory, it is possible to control most confounders. However, examining one chemical at a time does not address the effect of the chemical cocktail in the modern environment. Exposure studies on chemical cocktails in a laboratory

environment are a way of addressing this. Combinations of endocrine disruptors are reported to produce significant effects even when the dose is so low that no observed effect is seen after exposure to the individual chemicals (Kortenkamp, 2007). However, one obvious difficulty is deciding which chemicals to include in the experimental cocktail. Studying real-life exposure in wildlife addresses this problem. Animals at the top of the food chain are especially interesting for studies of the cumulative effect of chemicals in the environment (Basu *et al.*, 2007; Oskam *et al.*, 2003). However, using wild animals is most likely associated with a number of difficulties, as discussed later in this thesis.

1.1 Male reproduction

The male reproductive organs consist of testes, epididymis, ductus deferens, accessory sex glands (*e.g.* prostate) and penis (Knobil & Neill, 1994) . In this thesis, the focus is on the testes. To detect adverse effects on male reproduction, histopathology of testicular tissue is considered the most sensitive tool (Creasy, 2003).

1.1.1 Testis histology and spermatogenesis

The testis consists of seminiferous tubules surrounded by interstitium with blood vessels, nerves, connective tissue and testosterone-producing Leydig cells. Seminiferous tubules are composed of a high epithelium with spermatogonia, developing germ cells and Sertoli cells. Spermatogenesis is the process whereby diploid stem cells give rise to haploid spermatozoa (Knobil & Neill, 1994). Spermatogenesis can be divided into three phases. First, the spermatogonia undergo a series of mitotic divisions, resulting in spermatocytes and at the same time maintaining their number by renewal. The second phase involves primary and secondary spermatocytes that undergo meiotic division, resulting in haploid spermatids. In the third phase, called spemiogenesis, the round spermatids go through a series of complex cytological transformations, followed by condensation of the nucleus leading to the production of the mature, elongated spermatozoon (Figure 1, steps 1-19). The first step in spermiogenesis is called the Golgi phase, where the newly formed round spermatids are characterised by formation of an acrosomic granule, visible next to the nucleus, from the Golgi complex. This is followed by the cap phase, where a head cap expands around the acrosomic granule and grows to cover the surface of the nucleus. At the same time, a flagellum sprouts from the other side of the nucleus. In the acrosome phase, the nucleus rotates and the acrosome becomes orientated towards the basement membrane. The nucleus is displaced to the periphery of the cytoplasm,

followed by condensation of the nuclear chromatin. In the final step, the maturation phase, the nucleus completes its condensation and the acrosome covers two-thirds of the nucleus. Excess cytoplasm forms a residual body and is finally absorbed by the Sertoli cell at spermiation, when the spermatozoon is released from the seminiferous epithelium into the lumen (Creasy, 1997; Clermont, 1972).

Cycle of seminiferous epithelium

Apart from the Sertoli cells, the seminiferous epithelium consists of two generations of spermatogonia seen along the basement membrane, one or two generations of spermatocytes and one or two generations of spermatids at the border of the lumen of the tubule. A group of cells that are in the same step of development are defined as a generation of cells (Clermont, 1972). The generations of cells are combined to form cellular associations in a fixed manner. For example, a spermatid at a certain step is always associated with the same types of spermatogonia and spermatocytes. The cells can only be associated in a limited number in the various cross-sections of seminiferous tubules, called stages of the cycle of the seminiferous epithelium (see Figure 1 for stages in mink). Because the process is continuous, the demarcation between the end of one stage and the beginning of the next is imprecise. The duration of one cycle of the seminiferous epithelium is constant for a given species, but the individual stages differ in duration from each other (Creasy, 1997; Russel *et al.*, 1990). The frequency with which the different stages are seen reflects their duration. The most frequent stage in rat (stage VII) lasts for 58 hours, while stage IX only lasts for seven hours (Creasy, 1997; Russel *et al.*, 1990).

Different animal species have varying number of stages, depending on the criteria defining them (Russel *et al.*, 1990). Over the years, many classifications have been proposed, but the most commonly used staging method is that proposed by Leblond and Clermont (1952), who described stages I-XIV in the rat. They used acrosome development and the position of the spermatid in the seminiferous epithelium to define the stages (Leblond & Clermont, 1952). Spermatogenesis in mink was first described by Tiba *et al.* (1968), who defined eight stages. Pelletier (1986) described 12 stages in mink, based on similar criteria used by Leblond and Clermont (1952).

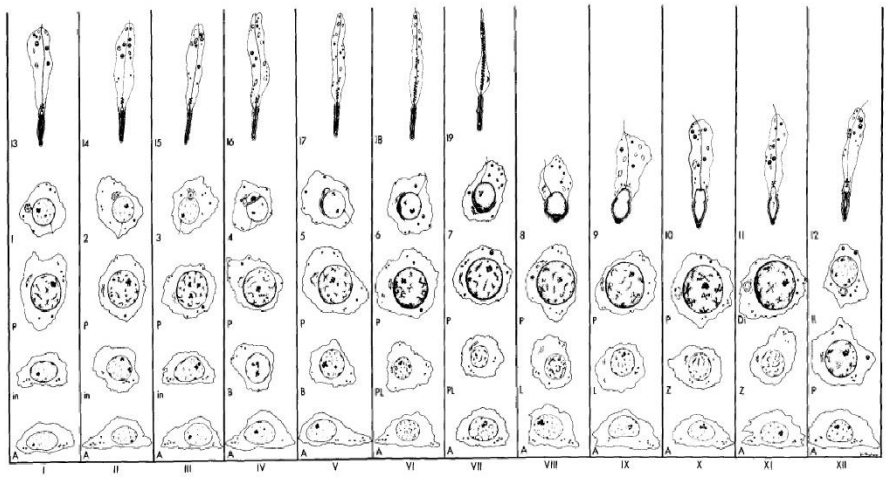

Figure 1. Spermatogenesis in mink testis (Pelletier, 1986). Reproduced with permission from the author.

1.2 Methods to measure male reproductive health

Reproductive toxicology concerns indications of xenobiotic exposure resulting in adverse effects on physiological processes and behaviours and/or anatomical structures involved in animal reproduction or development (Evans, 2018). Measuring male reproductive health and reproductive toxicology is a complex area. This thesis focuses on effects on anatomical structures, primarily organ measurements and histological evaluation of testes. There are large differences in what is considered normal male reproduction between animals and humans. As an example, men with more than 4% normal-shaped spermatozoa are still considered as having normal sperm morphology (WHO, 2010), whereas most test species, including rat, generally have to have less than 5% abnormal sperm to be considered healthy (Working, 1988). The human male is therefore at relatively greater risk of being affected by toxic agents due to the difference in gonadal function. In human ejaculate, the number of sperm is typically only two- to four-fold the number at which fertility is significantly reduced. In contrast, ejaculate from most animal species contains many times the number of sperm that produces maximum fertility (Working, 1988). Therefore, testis histology is considered a more sensitive endpoint to detect effects on sperm production than other fertility measurements (Creasy, 1997; Working, 1988).

1.2.1 Organ measurements

Reproductive organ weights are easy to obtain and provide informative data on the function of reproductive tissue. Morrissey *et al.* (1988) show that alterations in reproductive organ weights (testis and epididymis) have a high probability of being detected and conclude that this is a sensitive endpoint to detect reproductive toxicants. Disturbed fluid balance can cause an increase in testes and epididymis, while decreased spermatogenesis can cause decreased organ weight (Creasy, 2003). In accessory sex glands such as prostate, decreased organ weight is a sensitive indicator of decreased testosterone levels (Creasy, 2003).

1.2.2 Histological evaluation of testes

Histopathology

When administering a cytotoxic compound, morphological changes in germ cells are frequently seen as a primary manifestation (Vidal & Whitney, 2014). Spermatogonia are not protected by the blood-testis barrier and are therefore most vulnerable to toxic effects. However, the stem cell spermatogonia divide infrequently and are therefore less sensitive than spermatogonia that have entered the proliferative and developmental pool (Meistrich, 1986). Cytotoxic damage to testes can be reversible, depending on the cells that are affected and the extent of the damage (Meistrich, 1986). Hormonal disturbances can result in varying histopathological changes depending on animal species (Ramaswamy & Weinbauer, 2014). In a position paper for the Society of Toxicologic Pathology, Lanning *et al.* (2002) reviewed recommended approaches for evaluation of testicular toxicity and also covered recommendations for regulatory guidelines from authorities used when pharmaceuticals and new chemicals are evaluated. Endpoints to evaluate in testicular tissue are described in the paper and include germ cell degeneration, depletion or exfoliation of germ cells into tubular lumen, tubular vacuolisation, contraction or dilatation, sperm retention and effects on Leydig cells (atrophy, hypertrophy or hyperplasia). Lanning *et al.* (2002) emphasise the need for evaluating testicular histopathology with stage awareness to detect whether the combination of cells in a certain stage is abnormal. Two frequent stage-specific findings are retained spermatids and lack of round spermatids or pachytene spermatocytes (Vidal & Whitney, 2014; Creasy, 2003; Lanning *et al.*, 2002). Decreased intratesticular testosterone levels can result in retained spermatids and degeneration of spermatocytes and round spermatids in stage VII/VIII (Vidal & Whitney, 2014; Lanning *et al.*, 2002; Creasy, 1997).

Histomorphometry

The reproductive function of testis can also be evaluated by histomorphometry, which is defined broadly as measurements of form or shape in tissue. Histopathology is a subjective method and the need for objective measures has been discussed (Berndston, 1977). Different techniques have been described for quantification of testicular function, for example determination of relative spermatozoa production rate where all germ cells and Sertoli cells are counted in a certain stage (Clermont & Morgentaler, 1955). When counting cells in testicular tissue, the size of cell nuclei must be considered, as larger cells will be detected more frequently than smaller cells. This bias must be corrected to get the true cell count (Berndston, 1977). To perform this correction, the thickness of the histological section needs to be known and, even if the microtome is set at a certain thickness, there is a variation in the actual thickness (Berndston, 1977). Berndtson *et al.* (1989) describe the sampling intensity and number of animals required to detect a given alteration in seminiferous tubular diameter in rat and rabbit. They report that to detect a difference of 10% from the treatment group when using eight rats per group, it is adequate to measure 10 tubular cross-sections (Berndtson *et al.*, 1989). Another approach is to count cells in the seminiferous tubules. Leptotene/Sertoli cell ratio and number of round spermatids per tubular cross-section have been used. However, Berndtson *et al.* (1989) show that fewer animals are needed to detect changes in seminiferous tubular diameter than are needed to detect changes when counting cells.

Regardless of the chosen method to detect impaired reproduction, sensitivity (the proportion of actual positives correctly identified as such), specificity (the proportion of actual negatives correctly identified as such) and reproducibility (the closeness of agreement) are important, but not always easy to achieve. The probability of toxic damage caused by a chemical being detected or not is influenced by several factors. Magnitude of the damage in relation to the variation among animals is one important factor. Number of observations per male and number of animals per treatment group should also be considered (Berndtson *et al.*, 1989).

Computerised image analysis

In histopathological research and clinical diagnostics, digital image analysis is used frequently to facilitate and objectify qualitative measurements. Manual histopathology is time-consuming and suffers from inter- and intra-observer disagreement, which could be improved by image analysis. Computerised imaging methods are already assisting pathologists in making accurate diagnoses in diseases (Madabhushi, 2009). However, it is important to acknowledge the

difference between computerised readings and analysis by the human eye and to note that the histological slides must be optimised for the digital "eye", so that reproducible results correlate with clinical findings. Digital image analysis requires some principal steps for optimal results, including proper sampling, tissue and staining optimisation, scanning and digital storage and algorithm design (Riber-Hansen *et al.*, 2012). The algorithm design step requires collaboration between pathologists and skilled engineers for optimal results. The main steps in algorithm set-up are pre-processing the image to enhance the structures of interest and suppress noise, segmentation of the image and post-processing to refine the segmentation and generation of output (Grunkin *et al.*, 2011). Numerous image analysis tools exist on the market and are used frequently without requiring a high level of computer skills to preform easier measurements (Prasad & Prabhu, 2012). To further improve computerised image analysis, whole slide imagining can be used, but validation is needed to ensure that the results are equivalent to glass slides and light microscopy (Pantanowitz *et al.*, 2013).

1.3 Chemicals in the environment and their effect on male reproduction

In laboratory studies, the effect of a single chemical on male reproduction is commonly investigated. However, during recent years there has been increasing interest in the effect of interactions between chemicals found in the environment, which is named the 'cocktail effect'. New chemicals are continually being produced and the effect of the resulting chemical cocktail is unclear and will vary depending on the environment. A recent report from the World Health Organization (WHO) on endocrine disruption underlined this issue and urged future investigations to take it into consideration when studying reproductive disturbances in humans and wildlife (Bergman *et al.*, 2013). One way of confirming the effect is by collecting samples from wildlife and analysing them for signs of disturbed reproduction. This provides a good picture of how the complex mixture of chemicals can affect humans and wildlife. Using wild animals, in particular top predators, to study the effect of endocrine disrupting chemicals on male reproductive disorders has been suggested (Bergman *et al.*, 2013). However, a recent review by Matthiessen *et al.* (2018) concluded that there are generally greater effects on wildlife of banned chemicals with endocrine activity than the currently used chemicals. Most of the chemicals measured in polar bears in this thesis are banned or under restrictions and listed as persistent organic pollutants (POPs) through the Stockholm Convention (Convention, 2010). Below, a brief description is provided of two selected

chemicals and their potential impact on male reproduction. These are bisphenol A, the chemical to which rats were exposed in Paper III, and oxychlordane, the only chemical correlated with impaired male reproduction in polar bears in Paper IV.

1.3.1 Bisphenol A

Bisphenol A (BPA) is used as an additive in the production of different types of plastics. It is present in food containers, recycled paper and dental sealants (Liao & Kannan, 2011). Humans are exposed to the chemical via several different exposure routes, *e.g.* it can pass through the placenta to the foetus and via breast milk to neonates (Rochester, 2013; Salian *et al.*, 2011; Schonfelder *et al.*, 2002). The estimated dietary intake of BPA can be between 0.39 and 0.6 µg/kg body weight (bw) per day in breast-fed infants and women of child-bearing age (EFSA, 2015). Different modes of action have been described for BPA, *e.g.* it can act by binding to oestrogen receptors ER α and β, functioning as an ER modulator or binding to androgen receptors and inhibiting androgen action (Richter *et al.*, 2007). The increasing incidence of reproductive disorders observed over the past decade, together with the hormone-like activity of BPA, has led to health concerns regarding human exposure (Salian *et al.*, 2011). Both adverse health effects and no or limited effects of BPA on male reproduction in rats have been reported (see Table 1 in Paper III). Negative effects on reproductive parameters observed after low-dose BPA exposure during development include *e.g.* altered prostate histopathology (Prins *et al.*, 2017; Bernardo *et al.*, 2015; Timms *et al.*, 2005), reduced sperm count (Hass *et al.*, 2016; Salian *et al.*, 2009; vom Saal *et al.*, 1998) and low sperm motility (Salian *et al.*, 2009). The definition of low-dose exposure has changed over the years. In 2015, the tolerable daily intake of BPA was reduced from 50 µg/kg bw/day to 4 µg/kg bw/day by the European Food Safety Authority (EFSA, 2015). Some recently published low-dose animal studies report adverse effects of BPA at 25 µg/kg bw/day on male and female reproductive endpoints, such as decreased sperm count, increased anogenital distance in newborn females and altered male mammary gland differentiation at postnatal day 22 (Hass *et al.*, 2016; Mandrup *et al.*, 2016; Christiansen *et al.*, 2014).

1.3.2 Oxychlordane

Oxychlordane is a chlordane metabolite previously used as an insecticide but still found in the environment, in animals and in humans (González-Alzaga *et al.*, 2018; Navarrete *et al.*, 2018; Zhang *et al.*, 2016; Persson *et al.*, 2013b; Dearth

& Hites, 1991). In surveys in 2005-2008, combined chlordanes were the most prevalent measured organochlorine pesticide with relative uniform concentrations in polar bears from Alaska, Canada, East Greenland and Svalbard (McKinney *et al.*, 2011). A decline in oxychlordane during the past three decades (1982-2010) has been observed in East Greenland polar bears (Dietz *et al.*, 2012). In humans, oxychlordane is reported to affect male reproduction by increased risk of testicular germ cell tumours (McGlynn *et al.*, 2008).

1.4 The laboratory approach

1.4.1 Rat

Rats are one of the most common laboratory animals and millions of rats are used every year in pharmaceutical and chemical testing. Laboratory rats are bred for the purpose and are genetically very similar. They are kept in a strict laboratory environment and controlled for confounding exposure, *e.g*. by using glass bottles for drinking water and evaluating the feed for oestrogenic content. There are numerous different rat strains. In this thesis, Fischer 344 rats were used to study the effects of BPA on male reproduction. Previous studies have reported differing sensitivity towards BPA in different rat strains. For example, Hossaini *et al.* (2003) found a greater effect of BPA on male reproductive endpoints in Fischer rats compared with Wistar rats. The Sprague Dawley rat has been commonly used in BPA studies, but is also reported to be less sensitive to BPA than Fischer rats (Long *et al.*, 2000; Steinmetz *et al.*, 1998; Steinmetz *et al.*, 1997). Fischer rats were therefore chosen to be the model animal in Paper III. Gavage and injection are commonly used administration routes when studying the effects of BPA on male reproduction. However, this results in bolus exposure, whereas BPA exposure through drinking water gives a more even distribution of the chemical throughout the day, due to the water consumption habits of the rat (Spiteri, 1982; Stephan & Zucker, 1972). The effects of BPA on various periods of the reproductive cycle have been investigated previously, but there are few animal studies investigating the effects on male pubertal development and reproduction in ageing rats.

1.5 The wild animal approach

Thousands of chemicals surround modern humans in their everyday life and it is not possible to include all these chemicals in a pre-fabricated mixture to which laboratory animals can be exposed. The wild animal approach aims to use the

real-life exposure that occurs in nature. Wildt *et al.* (2010) encourage the use of non-traditional species in reproductive science and point out that cow, rat and mouse were the study object in 44% of reproduction-oriented articles published between 1999 and 2009. Wild animals can be used as a sentinel species or for determining the risk to the animal itself. A sentinel species is an organism that can be studied instead of other organisms or used to gather information about the environment they live in, *e.g.* level of contamination. It can provide an early warning of the potential risk to human health. However, using wild animals in research adds complexity when designing the study. For example, tissues for histopathological studies should ideally be placed in fixative directly post mortem. When using wild animals this is almost impossible to achieve, as capture is usually both time-consuming and difficult, and transport to the laboratory may take several hours or days post mortem. For practical reasons, the animals are sometimes even frozen (Persson *et al.*, 2012). This results in a variety of post mortem changes before the tissues can be investigated. Problems with delayed fixation have been discussed in previous environmental studies (Sonne *et al.*, 2006; Bubenik & Jacobson, 2002). Both polar bear and mink are seasonal breeders and only produce sperm in the spring, which further complicates evaluation of effects on male reproductive health in these species.

1.5.1 Polar bear

Wild polar bears are found in Greenland, Svalbard, Alaska and North Canada. They are at the top of the food chain, preying mainly on Arctic ringed seals (McKinney *et al.*, 2013). Male polar bears do not become sexually mature until around 3-6 years of age and are only reproductively active during the spring (Sonne *et al.*, 2007; Rosing-Asvid *et al.*, 2002; Lønø, 1970). Based on breeding behaviour, testis measurements and presence of spermatozoa in testes, the breeding season for polar bears is between February and June (Lentfer *et al.*, 1980; Lønø, 1970; Erickson, 1962). They do not produce spermatozoa during the rest of the year (Lønø, 1970). Detailed histological descriptions of polar bear testes in the reproductive season and in the non-reproductive period, *i.e.* before sexual maturation and during the non-reproductive seasons of the year, are scarce in the literature and, to my knowledge, have only been presented by Lønø (1970) and Rosing-Asvid *et al.* (2002). Polar bears are especially vulnerable to population effects from contaminant-induced reproductive disruption due to their slow reproduction rates (Lentfer *et al.*, 1980). Cubs stay with their mother for about two years and female bears are therefore only available for mating every three years (Lentfer *et al.*, 1980). Previous studies indicate that persistent organic pollutants have negative effects on male polar bear reproduction, with

reduced organ weight and decreased testosterone levels (Sonne *et al.*, 2006; Oskam *et al.*, 2003).

1.5.2 Mink

Mink has been suggested as a suitable sentinel species in environmental monitoring (Persson *et al.*, 2012; Basu *et al.*, 2007). Knowledge about wild mink biology is extensive. It is a seasonal breeder that only produces sperm in the spring (Pelletier *et al.*, 2015). Mink is a semi-aquatic top predator that can accumulate certain chemicals and is sensitive to their toxic effects (Basu *et al.*, 2007; Kihlström *et al.*, 1992). Another advantage of mink is its relatively narrow home range (Gerell, 1970). The limited area in which mink spend their life means that concentrations of pollutants found in their fat are representative of the area where they were caught (Foley *et al.*, 1988). The animals can easily be housed in the laboratory if a controlled experimental setting is needed. In Sweden, mink is generally hunted in order to protect other wildlife, as it can do substantial damage to the population of birds and is considered an invasive species (Jaktförordningen, 1987:905).

1.6 Rational for this thesis

Most studies that set out to examine whether chemicals in the environment affect male reproductive health are conducted in laboratory animals and most endpoints described in the literature are developed for these types of studies, *i.e.* short-term exposure studies on adult rat or mice kept in strict laboratory conditions. However, some questions cannot be answered with this type of study. Effects of chemicals on other ages of animal after chronic exposure with long recovery time and real-life chemical cocktails must be investigated. In this thesis, different aspects of histological examination of the testes in wild and laboratory animals were considered. The issues examined were whether histological examination of testes using commonly described endpoints is applicable in studies on wild animals and laboratory animals in different set-ups, whether chemically induced changes can be revealed by such examinations, and the factors that should be considered when seeking to draw accurate conclusions from the results.

2 Aims

The main aim of this thesis was to investigate different aspects of histological evaluation of testes as a tool to measure the effect of environmental chemicals on male reproductive health in wild and laboratory animals.

Specific objectives were:

- To identify and validate objective methods and endpoints, including image analysis, morphometry and histopathology, and to apply some of these methods in wild animals to investigate the impact of prolonged pre-fixation time and seasonality on important endpoints.
- To establish the effect of prolonged pre-fixation delay and freezing on mink testicular endpoints such as gross morphology and histopathology, in order to determine robust endpoints in suboptimally fixed testicular tissue.
- To describe a staging method in mink and polar bear, using Gata-4 immunohistochemistry to visualise development of the acrosome, and to test the reproducibility of this method in mink.
- To design and validate a computerised staging method that automates staging in mink testicular tissue.
- To investigate long-term and short-term effects on reproductive health in midpubertal and adult male Fischer 344 rats following exposure, via drinking water, to low doses of bisphenol A *in utero* and during lactation.
- To describe the normal histology of polar bear testes during the reproductive season and to assess possible effects of environmental chemicals on male polar bear reproduction.

3 Comments on material and methods

This chapter comprises three parts. Section 3.1 provides an overview of the animals and study design. Section 3.2 presents some comments on the endpoints used to evaluate male reproductive health, while section 3.3 provides a description of the statistical analyses used in Papers I-IV. For a complete description of materials and methods, see the individual papers.

3.1 Animals and study designs

Papers I-IV in this thesis present findings from studies using the animal species rat, mink and polar bear. Mink were collected from a fur farm and used in Papers I and II to develop methods and gain knowledge for future studies on wild mink. Rats were kept in laboratory settings and used as a model animal for human exposure to study the effect of BPA on male reproduction in Paper III. Wild polar bears caught by local hunters in East Greenland were used in Paper IV to study the biology of male reproduction and the impact of persistent organic pollutants on male reproduction in polar bear.

3.1.1 Mink, method development (Papers I and II)

For Papers I and II, 30 10-month-old, sexually mature mink (*Neovison vison*) were obtained from a fur farm at culling in March, their reproductively active season. In Paper I, these animals were used to investigate how prolonged pre-fixation time and freezing of testicular tissue affect endpoints frequently used to measure male reproductive health. In Paper II, perfectly fixed tissue samples from five mink in Paper I were used in the development of methods to stage mink testes, both manually and automatically by computerised image analysis.

In Paper I, the 30 mink were divided post mortem into six treatment groups, depending on time and treatment between euthanisation and fixation/ measurements:

Treatment group 0 h (n=5): Immediately after euthanisation, body weight, left testis weight and length were measured, followed by fixation of left testis. These animals were also used in Paper II.

Treatment groups 6, 18, 30, 42 h, and 6 h + frozen (n=5 per group): After euthanisation, the animals were transported intact to the laboratory and left at room temperature (21 °C) for 6, 18, 30 or 42 h, after which measurements of body weight, left testis weight and length and fixation of left testis tissue were performed. The five animals in the last group (6 h + frozen) were frozen (-20 °C) 6 h post mortem and then thawed at room temperature (21 °C) for 30 h prior to measurements of body weight, left testis weight and length and fixation of left testis tissue.

3.1.2 Rat, laboratory bisphenol A exposure (Paper III)

The rat material for Paper III was obtained from a large experiment designed by Assoc. prof. Monica Lind and Dr. Margareta Halin Lejonklou, Department of Medical Sciences, Occupational and Environmental Medicine, Uppsala University. Fischer 344 rats were kept under laboratory conditions and exposed to BPA *in utero* and during lactation. A number of endpoints for male reproductive health were investigated in pups, either at midpuberty on post-natal day (PND) 35 or in adult, 12-month-old rats.

Forty-five mated female Fischer 344 (F344/DuCrl) rats (9 weeks old) were randomly distributed into three dosing groups. To mimic the most likely route of human exposure, dams were exposed to BPA via their drinking water *ad libitum* from gestation day (GD) 3.5 until PND 22. Control females received water containing 1% ethanol (vehicle). Based on water consumption by the dams in a pilot study, the average intended doses were 0.5 µg BPA/kg bw/day and 50 µg BPA/kg bw/day. The main routes of exposure of the pups were assumed to be via the placenta *in utero* and via lactation. On PND 22, dams were sacrificed and one male and one female from each litter was selected at random and moved to a new cage. In the present study, only the males were used. The animals were randomly divided into two groups: midpubertal males, euthanised at PND 35 (control n=13, 0.5 µg BPA/kg bw/day n=11, 50 µg BPA/kg bw/day n=9) and adult males, euthanised at 12 months of age (control n=12, 0.5 µg BPA/kg bw/day n=8, 50 µg BPA/kg bw/day n=8). Male reproductive organs were fixed within a few minutes post mortem.

3.1.3 Polar bear, normal biology and real-life exposure (Paper IV)

The polar bear study was performed in collaboration with Prof. Christian Sonne, Bioscience Department, University of Aarhus, who designed the sampling methods and collection routines for all material from the bears. Samples from 214 male polar bears in the Scoresby Sound area (69°00-74°00'N) in East Greenland were collected from subsistence hunting regulated by the Home Rule of Greenland (Nuuk) during the period 1997-2016. All data and tissue samples were collected from bears during hunting under harsh conditions in a very cold climate, which resulted in missing values and most likely affected handling of tissues prior to fixation. Body length and girth were recorded on 29 of the polar bears. Testes (n=214) and baculum (n=73) were collected and fixed, then stored in fixative at -20 °C during transport to Roskilde, Denmark, for further storage. From there, they were collected and processed for histology at the Swedish University of Agricultural Sciences, Uppsala, Sweden. Sternal subcutaneous adipose tissue was also sampled from 69 of the bears and kept at -20 °C until analysis for dieldrin, mirex, hepoxide, octaclorostyrenes (OCSs), polychlorinated biphenyls (PCBs), dichlorodiphenyl-trichloroethanes (DDTs), oxychlordane, hexachlorobenzene (HCB) and hexacyclohexanes (HCHs). The age of 76 of the bears was determined by counting the cementum growth layer groups of the lower left incisor (I_3) (Hensel & Sorensen, 1980).

Fixative

In Papers I, II and III, modified Davidson's fluid was used as the fixative for testes and epididymis. It consists of 30% 37-40% formaldehyde, 15% ethanol, 5% glacial acid and 50% distilled water, and is suggested to be a superior substitute to Bouin's (Latendresse *et al.*, 2002). It is recommended for use by the Society of Toxicologic Pathology (Lanning *et al.*, 2002). Dorsolateral prostate samples in Paper III were fixed in 4% formalin for 24 hours at 4 °C. In Paper IV, a formaldehyde/alcohol solution (3.5% formaldehyde, 86% ethanol and 10.5% phosphate buffer) was used, to prevent freezing due to the harsh conditions during collection in East Greenland.

3.2 Endpoints used to measure male reproduction

An evaluation was performed on how a number of endpoints measuring male reproductive health were affected by prolonged pre-fixation time and freezing in Paper I, by chemicals in Papers III and IV and reproductive group in Paper IV.

3.2.1 Organ measurements

Testes weight, length, height, epididymal weight, anogential distance (AGD), baculum weight, height and length were measured in the four studies as described in Table 1.

Table 1. *Organ measurements in Papers I (mink), III (rat) and IV (polar bear)*

Measured endpoints	Paper I	Paper III	Paper IV
Testes weight (g)	x	x	x
height (mm)			x
length (mm)	x		x
Accessory sex gland (g)		x*	
Epididymis weight (g)		x	
Baculum weight (g)		-	x
length (mm)		-	x
density		-	x
AGD (mm)		x	

AGD= anogenital distance. *Only 52-week-old rat. – Not applicable, rats do not have baculum.

3.2.2 Testes histology and stains

In Paper I, the histology of testes was used to evaluate the effect of prolonged pre-fixation time and freezing on commonly used endpoints for detecting damage in testes. Haematoxylin-eosin (HE) was used for staining in Papers I and IV and periodic acid shiffs (PAS) in Paper III. PAS is recommended in rodents, because the staining of the acrosome enables staging (Creasy, 2003). However, PAS did not stain the acrosome as distinctly in mink and polar bear testes, and therefore the Gata-4 antibody (a zinc finger transcription factor) was used to mark acrosome development in these species, as described below. In Paper II, testicular histology of testes was used to validate manual and computerised staging methods. In Paper III, histology of testes was used to evaluate the effect of BPA exposure on male reproduction, with Gata-4 used to evaluate testes maturation in rats euthanised at PND 35. In Paper IV, testicular histology was used both to describe the reproductive biology of polar bears and to evaluate whether reproduction was impaired and possible connections to levels of some measured POPs.

3.2.3 Semi-automatic tool to measure seminiferous tubules

A semi-automatic image analysis technique was developed on mink testicular tissue in Paper I to measure area, epithelial height and diameter of the seminiferous epithelium. This method was then used for segmenting seminiferous tubules in mink, a first step for the computerised staging method developed in Paper II. The same method was used to measure area, diameter and epithelial height of seminiferous tubules in rat in Paper III. In Paper IV, only area and diameter were calculated, based on results in Paper I showing that these measurements were the most stable when fixation time is delayed, which could be expected in the polar bears due to the conditions during sampling.

To develop the method in Paper I, a variant of PAS was used to obtain maximum colour differences between the seminiferous epithelium and the interstitial tissue. This stain was used in an attempt to develop an automatic, computerised delineation of basement membrane surrounding the seminiferous tubules, which did not succeed. Instead, a semi-automatic method with some user interaction was developed. This semi-automatic method was less sensitive to choice of stain and therefore regular PAS and HE were used in Papers III and IV, respectively.

The semi-automated algorithm includes user input to produce a correct delineation to find the boundaries of the seminiferous tubules. The user adds a seed point on the boundary of a tubule and the algorithm calculates the cost of the optimal boundary between this seed point and all other pixels in the image. By moving the mouse over the image, the user can then instantly see the optimal boundary between that seed point and the mouse position. By clicking, that portion of the boundary becomes fixed, and the newly selected pixel becomes the seed point, repeating the whole process. In this manner, the user can interactively, and with only a few clicks, very precisely delineate the whole tubule boundary (Figure 2). After a tubule is delineated, its area is given by the number of pixels inside the drawn boundary, and its radius is computed as the average distance from all pixels on the drawn boundary to the tubule's centre of mass. For tubules with visible lumen, the lumen is segmented using the same procedure as described above. The epithelial height is then computed as the average distance between the border of the lumen and the border of the tubule. More details can be found in Paper I.

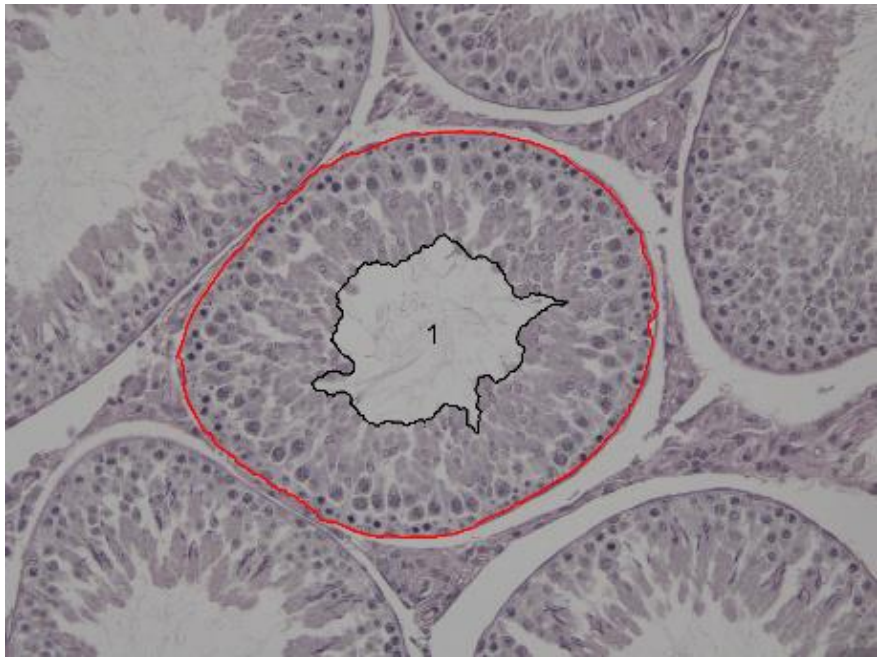

Figure 2. Semi-automatic image analysis for detecting area, diameter and epithelial height in mink seminiferous epithelium.

3.2.4 Immunohistochemistry Gata-4

Transcription factor Gata-4 is crucial for Sertoli cell function and is expressed in Sertoli cells and Leydig cells in post-natal and adult mice testis (Kyrönlahti *et al.*, 2011; Ketola *et al.*, 1999). Immunohistochemical localisation of Gata-4 can be used to visualise Sertoli cells. McClusky *et al.* (2009) found that the polyclonal Gata-4 antibody also distinctly marks the complete development of the acrosome in rat testis and described how it can be used for staging of the seminiferous epithelium in rats. In this thesis, the antibody was tested on both mink and polar bear testis tissue, where it distinctly marked acrosome development. Paper I investigated how stable the acrosome marker is after prolonged pre-fixation time and freezing. In Paper II, the stain was used to develop computerised image analysis based on applying pattern recognition to stage seminiferous epithelium. This thesis presents additional novel results, not presented in Paper IV, on testes morphology and acrosome development after Gata-4 immunohistochemistry on polar bear testes. With polar bear samples, problems arose with tissue falling off the slides during the staining procedure, and therefore the antigen retrieval step with pressure-heater described in Papers I-III was excluded. In rat, Gata-4 was used to evaluate how far midpubertal rats

had progressed in their sexual development, by marking acrosome development and facilitating identification of spermatid steps

3.2.5 Manual staging of seminiferous epithelium in mink

In Paper II, 12 stages in mink testicular tissue, stained with Gata-4, were described and the method was tested by intra- and inter-observer agreement. A total of 545 tubules were staged manually, using the criteria described in Paper II, by two investigators (E. Ekstedt and E. Spörndly-Nees), with each investigator staging all tubules twice. Next, the two investigators examined all cases and obtained a consensus staging for each tubule. From the four independent stagings, two from each investigator, intra- and inter-observer agreement was determined. A confusion matrix for intra-observer agreement was computed by averaging the confusion matrices for the two investigators.

The 12 stages described were pooled into five groups according to morphological criteria, based on the formation of the acrosome in the round spermatid and whether the seminiferous epithelium consisted of one or two layers of spermatids. Stages important in toxicological evaluation were also considered when grouping the 12 stages into five groups.

3.2.6 Computerised staging of seminiferous epithelium in mink

The computerised staging tool describes the Golgi/acrosome shapes visible within each tubule (for further details, see Paper II). The tool was created using 12 stages and then tested on the pooled five groups.

3.2.7 Morphology in polar bear testes

Testicular histology was used in Paper IV to describe polar bear male reproductive biology. Polar bears are seasonal breeders (Rosing-Asvid *et al.*, 2002; Lønø, 1970). Hunters collected animals of various ages during the whole year. The testes were evaluated for fixation damage, to select the most suitable samples for histological evaluation (n=90). All sections were blind-evaluated by the examiner, which was the same person throughout the study (E. Spörndly-Nees). The polar bears were then divided into four different reproductive groups, based on histological characteristics modified from categories previously described by Lønø (1970) and Rosing-Asvid *et al.* (2002). These were: i) Reproductive (REP): polar bears with spermatozoa in testis (n=36); ii) non-reproductive-degenerated (DEG): polar bears without spermatogenesis and degenerated/atrophic seminiferous tubules (n=22); iii) non-reproductive-undeveloped (UND): polar bears with small seminiferous tubules containing

only two cell types, lacking active spermatogenesis (n=24); and iv) in-transition (INT): polar bears identified as in transition between groups, with spermatogenesis but where the elongated spermatids were not mature enough to leave the epithelium (n=8). Testes from polar bears in the REP group were used to identify and describe four stages in the cycle of the seminiferous epithelium. This information was not included in Paper IV and is presented for the first time in this thesis.

3.2.8 Histopathology of male reproductive organs in rat and polar bear

Histopathological examination was performed on testes to evaluate the effect on male reproduction of exposure of rats to BPA in Paper III and exposure of polar bears to POPs in Paper IV. In polar bears, histopathological examination was performed of testes with active sperm production (REP group). In adult rats (12-month-old), histological examinations of testes, caput and cauda epididymis and ventral and dorsolateral prostate were performed. Testes were evaluated with stage awareness, as described by Lanning *et al.* (2002) and Creasy (2003). The tubular stages of the spermatogenic cycle were considered when identifying missing germ cell layers or cell types, retained spermatids, multinucleate or apoptotic germ cells and sloughing of spermatogenic cells into the lumen. Tubular dilatation/contraction was also studied.

Histopathology in rat

For adult rats, caput and cauda epididymis were evaluated and scored separately for inflammation, vacuolisation or cribriform pattern of the epithelium and presence of germ cells and cell debris in the epididymal lumen (score 1-3, where 1 is a few, 2 is ≤50% and 3 is ≥50% affected ducts). In one section each of the ventral and dorsolateral prostate from each rat, the following effects were scored: epithelial atrophy (score 0-3 according to the proportion of acini affected), interstitial inflammation (score 0-3 relative to severity of inflammation), luminal concretion, atypical hyperplasia, reactive hyperplasia, vacuolisation, and atrophic acini (score 1-3, where 1 is a few, 2 is 10-50% and 3 is ≥50% affected acini).

In the midpubertal rats, one section per left testis from all treatment groups and controls was examined by identifying the seminiferous tubule with the most developed spermatids (steps 1-19 according to Russel *et al.* (1990)). This was done to evaluate how far the rats had progressed in sexual development. Gata-4 stain was used to mark acrosome development and facilitate identification of spermatid steps, according to McClusky *et al.* (2009). The animals were then scored according to stage of development: Score 0: no spermiogenesis, *i.e.*, no

spermatids present; score 1: spermatid steps 1-9, round spermatids; and score 2: spermatid steps 10-19, elongated spermatids.

Histopathology of polar bear testes

Detailed histological examination was performed on the testis of the 90 polar bears selected as described above. Depending on group (REP, UND, DEG or INT), different endpoints were chosen. Inflammation and presence of giant cells were evaluated in testes from all 90 bears, regardless of reproductive group. In the REP group, the following endpoints were evaluated: cells in lumen of seminiferous tubules, retained spermatids (elongated spermatids that did not leave the epithelium at spermiation), absence of pachytene spermatocytes at spermiation and organisation of seminiferous tubules. The endpoints were scored on a scale of 1-3 (where 1 is less than 10%, 2 is 10-50% and 3 is ≥50% affected tissue).

3.3 Statistics

Papers I and III

The gross morphology data in Papers I and III were analysed by one-way ANOVA followed by Tukey's (Paper I) or Dunnett's (Paper III) multiple comparison. Residuals were analysed and were considered normally distributed with equal variances. The morphometric measurements [area (mm^2), diameter (µm) and epithelial height (µm) of seminiferous tubules] were based on several measurements per animal and a hierarchical ANOVA was therefore used. In Paper I, two models were used, one including the fixed effect of time (0, 6, 18, 30 and 42 h) and the other the effect of freezing (0 and 6 h, 6 h + frozen). In Paper III, hormone data were considered to be normally distributed after log-transformation and were analysed by one-way ANOVA followed by Dunnett's multiple comparison test. In Paper III, the correlations between testis weight and testis maturation score were considered to be non-normally distributed and were therefore tested with a Kruskal-Wallis test followed by Dunn's multiple comparison test. Histological scores in Paper III were analysed using Fisher's exact test without correction for multiple testing. The analyses were carried out with SAS software (SAS Institute Inc., Cary, NC, USA, version 9.3) using the MIXED procedure. All values presented in diagrams represent mean ± S.E in Paper I and mean ± S.D in Paper III and differences were considered significant at $p<0.05$.

Paper II

Intra- and inter-observer agreement is presented as confusion matrices, which give insights into stages that are most frequently confused. These matrices were constructed by taking the two stages assigned to each tubule (through two different processes, for example by two different investigators) and using them as rows and columns in a matrix. This matrix element was then increased by 1. The result was a number $\boldsymbol{C}_{i,j}$ at each matrix element (i,j) that indicates how many tubules were assigned to stage i by the first process and stage j by the second. For display purposes, each row was normalised to 100 and the numbers rounded. This normalisation converted the absolute number of tubules into a relative percentage of tubules, for ease of comparison across experiments.

The weights were also used to reduce the penalty for disagreement between consecutive stages. In this thesis, kappa values were translated to degrees of agreement, according to Viera and Garrett (2005) and Watson and Petrie (2010).

Paper IV

For statistical analyses of polar bear reproductive status, tests were performed for differences in testes and baculum measurements, bear age, length and girth, and POP concentrations between the four reproductive groups (REP, DEG, UND and INT) using multivariate ANOVA, followed by Tukey's multiple comparison. Prior to the multivariate ANOVA, the Shapiro-Wilks test was used to verify the assumption of normality and Levene's F test for equality of variances was used to verify homogeneity of variance among groups. The analyses of testes and baculum measurements also included age as a covariate in the ANOVA, to account for possible confounding effects. For the statistical analyses of reproductive toxicology, only data from male polar bears in the REP group were considered. In that case multivariate linear regression was used, with testes and baculum measurements as the response variables and the various POPs as the predictor variables. The main assumptions of linear regression analyses were verified by inspection of model residuals. If these assumptions were not met, the model was re-run using a polynomial spline function for the POPs, to assess non-linearity in the response. Age was also included as a covariate in the analyses to account for a potential age effect in the response. All analyses were conducted in R and regression coefficients and differences per group were considered statistically significant at $p<0.05$.

4 Main results

The main results are presented below in two separate sections. Section 4.1 presents the results on method development and description of normal morphology in testes. This includes results on manual and computerised staging in mink (Paper II), reproductive groups in polar bear (Paper IV), staging in polar bears (not included in any paper) and a method describing testes development during puberty in rat (Paper III). Section 4.2 describes the effect of prolonged pre-fixation time and freezing (Paper I), BPA (Paper II) and POPs (Paper IV) on frequently used endpoints to measure male reproductive health.

4.1 Method development and description of normal morphology

Mink for Papers I and II were collected in their reproductively active season. In Paper II, 12 stages previously described by Pelletier (1986) were identified in the testicular tissue and adjusted to light microscopic evaluation using immunohistochemistry for Gata-4. Polyclonal Gata-4 antibody stained the acrosome dark brown, highlighting differences in the shape of the developing acrosome. A detailed description of the 12 stages can be found in Paper II. The 12 stages were then pooled to five groups (A-E), as described below. Polar bear testes were collected during the whole year and four reproductive groups were identified; reproductive (REP), non-reproductive-degenerated (DEG), in transition (INT) and non-reproductive-undeveloped (UND). Testes from polar bears in the REP group were used to describe four stages of the seminiferous epithelium, as presented below. Morphological details in the Gata-4 stained mink testicular tissue were greater than in the polar bear testes, making it possible to identify 12 stages in mink, while only four stages were evident in polar bear (stages AB-E). Differentiation between the first two pooled groups in

mink (groups A and B) were not visible in polar bears, and these were therefore pooled into one stage (stage AB).

4.1.1 Polar bear reproductive groups based on testicular morphology

In Paper IV, four reproductive groups were identified in the 90 histological tissue samples from polar bears.

The criteria for testes in the REP group were active seminiferous tubules with elongated spermatids at spermiation. Spermatogonia, Sertoli cells, early and late spermatocytes and round and elongated spermatids were seen in different stages of the cycle of the seminiferous epithelium, see detailed description of the stages below. The average age of the bears was 9.8 years. They were captured between January and August, but most (83%) were caught in February-April. Most animals had relatively pronounced interstitial tissue with Leydig cells containing lipid droplets (Figure 1a, b in Paper IV).

The histological criterion for the INT group was spermatogenesis with elongated spermatids embedded in the epithelium (*i.e*. not at spermiation) (Figure 1c, d in Paper IV). The polar bears in the INT group were on average 9.1 years old and most were captured in January, except for three bears that were captured in February or March.

The DEG group was defined by seminiferous epithelium with no active spermatogenesis that contained few, partly degenerated, disorganised germ cells. Sertoli cells and spermatogonia were present in all tubules and occasionally early spermatocytes. Lumen was often occupied by debris or extracellular matrix (Figure 1e, f in Paper IV). The polar bears in the DEG group had an average age of 8.2 years. Almost all bears above four years of age and captured between August and January fell within this group (there were two exceptions). However, four polar bears did not follow the expected pattern and showed degeneration of testes in February to April.

The UND group was defined by testes with seminiferous tubules small in diameter without lumen and only Sertoli cells and gonocytes (Figure 1g, h in Paper IV). The average age of polar bears in the UND group was 2.5 years and they were captured throughout the year. This group displayed sexually immature testes, with undeveloped seminiferous tubules without any spermatids. The interstitial tissue was pronounced, with Leydig cells and small blood vessels.

Three groups (REP, UND and DEG) differed significantly in testes weight, length, height and width and in baculum weight, length and density (Table 1 in Paper IV). The mean age was similar in REP, DEG and INT, but these three groups differed significantly from the UND group. Three groups (REP, UND and DEG) differed significantly in area and diameter of seminiferous tubules.

The polar bears in the UND group had the smallest seminiferous tubules and the reproductive group (REP) had the largest (Table 1 in Paper IV).

4.1.2 Staging of seminiferous epithelium in mink

In Paper II, manual staging was performed and a computerised image analysis tool was developed to enable objective staging of mink testes. The distinct dark brown stain of Gata-4 antibody was the base for pattern recognition and it was used to identify the stages manually and by the computer (Figure 3). The manual and computerised staging results were evaluated by agreement, Cohen's kappa and Cohen's weighted kappa (intra and inter, and computer vs consensus staging), which were calculated for the 12 stages and the five pooled groups (Figure 4 and 5). The five groups were pooled according to morphological criteria based on the formation of the acrosome in the round spermatid and whether the seminiferous epithelium consisted of one or two layers of spermatids. Stages important in toxicological evaluation were also considered when grouping the 12 stages into five groups. The main Gata-4 characteristics of acrosome development were described in the five pooled groups.

Group A: Includes stages I, II and III. The round spermatids were in Golgi phase, with the acrosome seen as a brown dot (Figure 3a).

Group B: Includes stages IV and V. The round spermatids had entered cap phase and the acrosome was seen as a triangular, U-shaped brown structure covering up to one-third of the nuclear pole (Figure 3b).

Group C: Includes stages VI and VII. The brown-stained acrosome continued to spread over the nucleus of the round spermatids and covered approximately half of the nuclear envelope (Figure 3c).

Group D: Includes stages VIII and IX. The pear-shaped spermatids were oriented towards the basement membrane and had entered the acrosome phase, with brown-stained acrosome covering over half of the nuclear membrane (Figure 3d).

Group E: Includes stages X, XI and XII. The elongated spermatids became more tapered, narrow and changed from conical to more elongated and slender (Figure 3e).

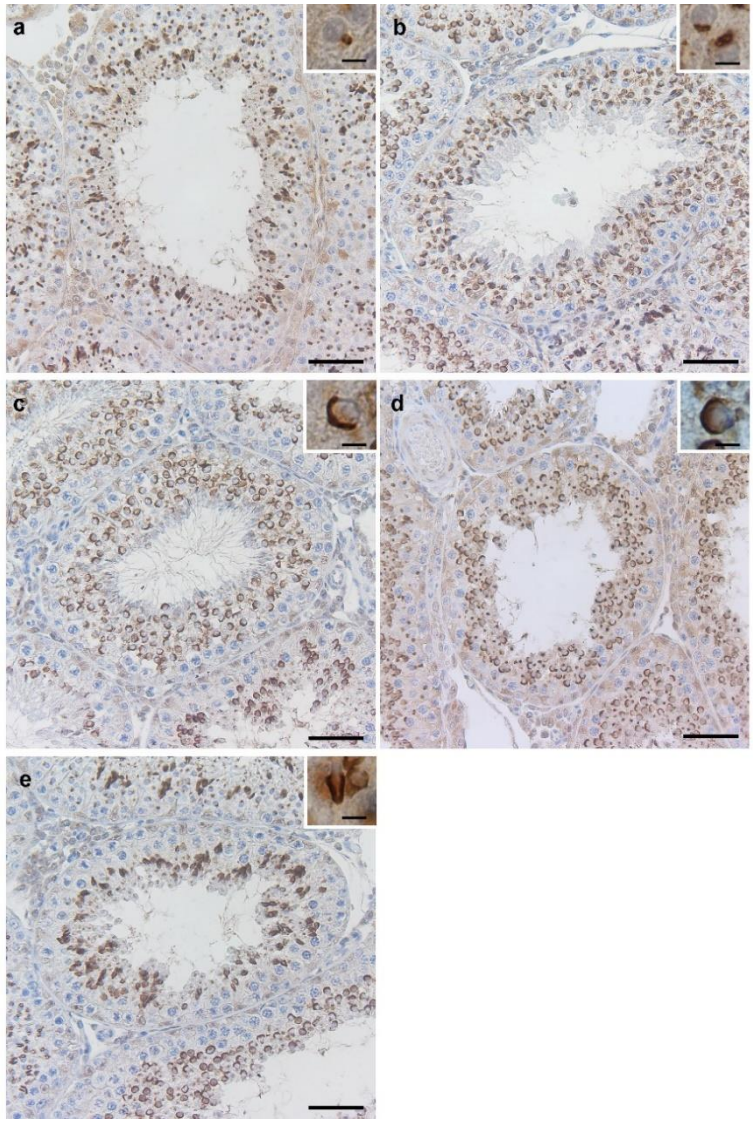

Figure 3. Five pooled groups in seminiferous epithelium showing Gata-4 immunolabelled acrosome development as brown staining in mink testes (group A-E). **Group A.** Round spermatids are seen in Golgi phase (Figure 3a) and the acrosome vesicles are seen as a brown dot. **Group B**. The round spermatids then enter the cap phase and the acrosome is seen as a U-shaped brown structure, while elongated spermatids are seen embedded in the epithelium (Figure 3b). **Group C**. The round spermatids are in cap phase, but the elongated spermatids are now lining the lumen (Figure 3c). **Group D.** The elongated spermatids are released and the lumen is empty, a new generation of elongated spermatids can be seen in acrosome phase and the acrosome is seen as a brown-stained pear-shaped structure (Figure 3d). **Group E**. The elongated spermatids are more tapered, narrow and changed from conical to more elongated and slender (Figure 3e). Weak haematoxylin counterstain, bar = 50 µm. Inserts show spermatids, bar = 5 µm.

Pooling the 12 stages into five groups clearly improved agreement and kappa statistics for both the manual and computerised staging (Table 2).

Table 2. *Summary statistics for manual and computerised staging of mink testes into 12 stages and five pooled groups*

	12 stages			5 groups		
	Intra-observer	Inter-observer	Computer	Intra-observer	Inter-observer	Computer
Agreement (%)	83.6	70.5	52.8	93.2	89.4	76.7
Kappa	0.81	0.67	0.47	0.91	0.86	0.69
Weighted kappa	0.89	0.80	0.61	0.94	0.91	0.77

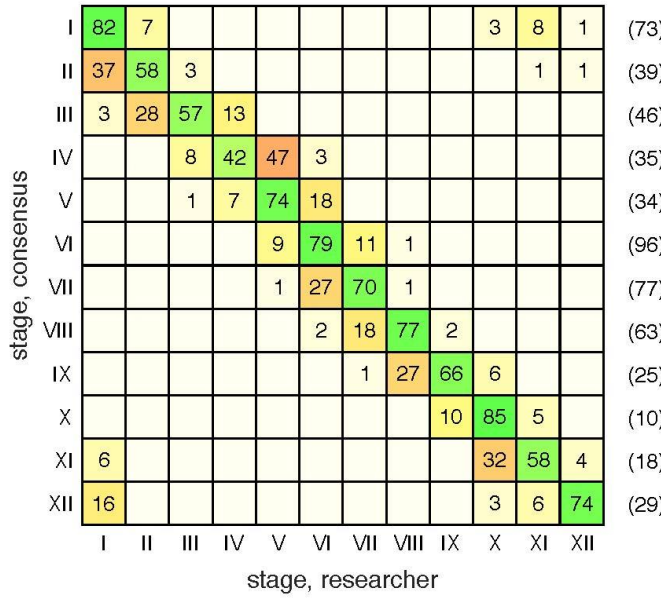

Figure 4. Confusion matrix for inter-observer agreement manual staging. Numbers indicate how many tubules were assigned to stage Y (row number) in the consensus staging (when both investigators staged the tubules together) and to stage X (the investigators' staging independently) (column number), as a percentage of all tubules assigned to stage Y. Thus, each row adds up to 100%. Along the diagonal, coded yellow (0%) to green (100%), tubules were staged identically by both investigator and consensus. Other boxes, coded yellow (0%) to red (100%), represent tubules where the investigators disagreed. Empty boxes indicate 0%. Percentages are averaged over four stagings (two stagings per investigator, two investigators). To the right of the matrix is the number of tubules assigned to stage Y (consensus), which can be used to convert percentages into number of tubules. Note that stages I and XII should be considered consecutive. In stages I-VII two layers of spermatids were seen, while after spermiation in stage VII, only one layer of elongated spermatids was present in stages VIII-XII

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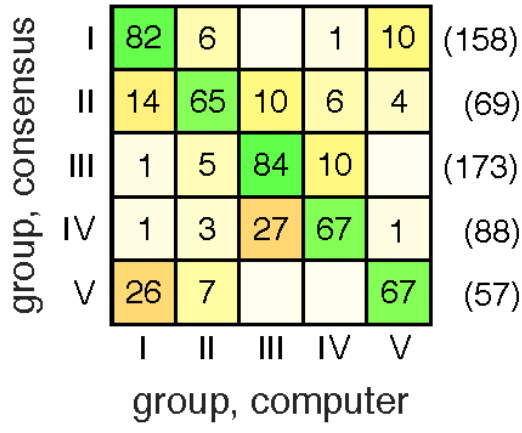

Figure 5. Confusion matrix for the computerised staging method with five groups (A-E). Numbers indicate how many tubules were assigned to group Y (row number) in the consensus staging (when both investigators staged the tubules together) and to stage X (column number) by the computerised staging tool, as a percentage of all tubules manually assigned to group Y. Thus, each row adds up to 100%. Along the diagonal, coded yellow (0%) to green (100%), tubules were staged identically in the consensus and by the computer program. Other boxes, coded yellow (0%) to red (100%), represent tubules where the program did not agree with the consensus. Empty boxes indicate 0%. To the right of the matrix is the number of tubules assigned to stage Y (consensus), which can be used to convert percentages into number of tubules. Group A corresponds to stages I-III, group B to stages IV-V, group C to stages VI-VII, group D to stages VIII-IX and group E to stages X-XII. Note that stages A and E should be considered consecutive.

Effect of prolonged pre-fixation time and freezing on the ability to stage the seminiferous epithelium in mink (Paper I)

In Paper I, it was found that staging was possible at 6 h prolonged pre-fixation time. After 18 h, staging of the seminiferous epithelium was possible, but stages 1-4 and stage 5-7s were difficult to separate. This means that the five pooled stages described in Paper II can probably be used when collecting wild mink, where fixation is likely to be delayed due to practical reasons. However, staging was difficult and only possible in larger stage categories at 30 h prolonged pre-fixation time, which indicates an upper limit on storage of animals before fixation. Staging was not possible after 42 h prolonged pre-fixation time or after 6h + freezing.

4.1.3 Manual staging polar bear

Testes morphology of the 36 reproductively active polar bears (REP) was evaluated using both HE and Gata-4 stain. Nuclear morphology and position of the germ cells in the epithelium (primary spermatocytes, round and elongated spermatids) were used to identify the stages. Stage AB corresponds to stages I-V in mink described by Pelletier (1986), stage C to stages VI- VII, stage D to stage VIII-IX and stage E to stage X-XII. In all the stages, spermatogonia and Sertoli cells were seen close to the basement membrane (SG and SC in Figure 6a, c, e, g).

Stage AB in polar bear testis (corresponds to stages I-V in mink)

Seminiferous tubules were characterised by two layers of spermatids (round and elongated) and one layer of spermatocytes (pachytene). The border of the seminiferous tubular lumen was lined by cytoplasm from elongated spermatids. Round spermatids were seen within the seminiferous epithelium, characterised by smaller nuclei with heterochromatin (RS in Figure 6a). Golgi complex-forming vesicles were seen as a dark-brown dot in proximity to the nucleus of the round spermatid (RS and inserts Figure 6b). In the luminal part, but deeply embedded in the seminiferous epithelium, elongated spermatids with ellipsoid shape were seen (ES in Figure 6a). Gata-4 stained acrosome of elongated spermatids was seen marked with dark brown (ES in Figure 6b). Spermatocytes in pachytene (characterised by larger nuclei with condensed chromatin clustered in dots) were seen in the outer part of the seminiferous tubule (SP in Figure 6a).

Stage C in polar bear testis (corresponds to stages VI-VII in mink)

Two layers of spermatids (round and elongated) and one layer of spermatocytes (pachytene) characterised seminiferous tubules in stage C. Round spermatids (RS in Figure 6c) had now entered the cap phase. In Gata-4 stained round spermatids, the acrosome stretched out and covered one-third to more than one-half of nuclei (RS and insert, Figure 6d). Elongated spermatids lined the lumen (ES in Figure 6c, d). Spermatocytes in pachytene were seen in the seminiferous epithelium (SP in Figure 6c).

Stage D in polar bear testis (correspond to stages VIII-IX in mink)

In stage D, seminiferous tubules were characterised by one layer of spermatids (elongated) and two layers of spermatocytes (both pachytene and leptotene or zygotene). The elongated spermatids had left the epithelium, leaving the lumen empty (Figure 6e). The new generation of elongated spermatids had now entered

the acrosome phase and started to spire, the nuclei condensed to a pear-ellipsoid shape (ES and insert, Figure 6e and 6f). The spermatid nucleus re-oriented toward the basement membrane and cytoplasm lined the border of the lumen. Two types of early spermatocytes were seen (leptotene or zygotene), both characterised by smaller nuclei than pachytene. Their chromatin was condensed in thick threads or blotches and they were difficult to distinguish between (SZ/L in Figure 6e). Pachytene spermatoyctes were identified, with large cell nuclei and chromatin condensed and clustered in dots (SP in Figure 6e).

Stage E in polar bear testis (corresponds to stages X-XII in mink)

Stage E tubules were characterised by two layers of spermatocytes and one layer of spermatids (elongated). Spermatids entered maturation phase with pointed nuclei with progressive chromatin condensation at the rostral pole and along the nuclear membrane (ES in Figure 6g, insert and red arrow, Figure 6h). Elongated spermatocytes were directed towards the basement membrane with the acrosome and tails towards the lumen. Spermatocytes were seen in both pachytene and leptotene or zygotene (SP and SZ/L, Figure 6g).

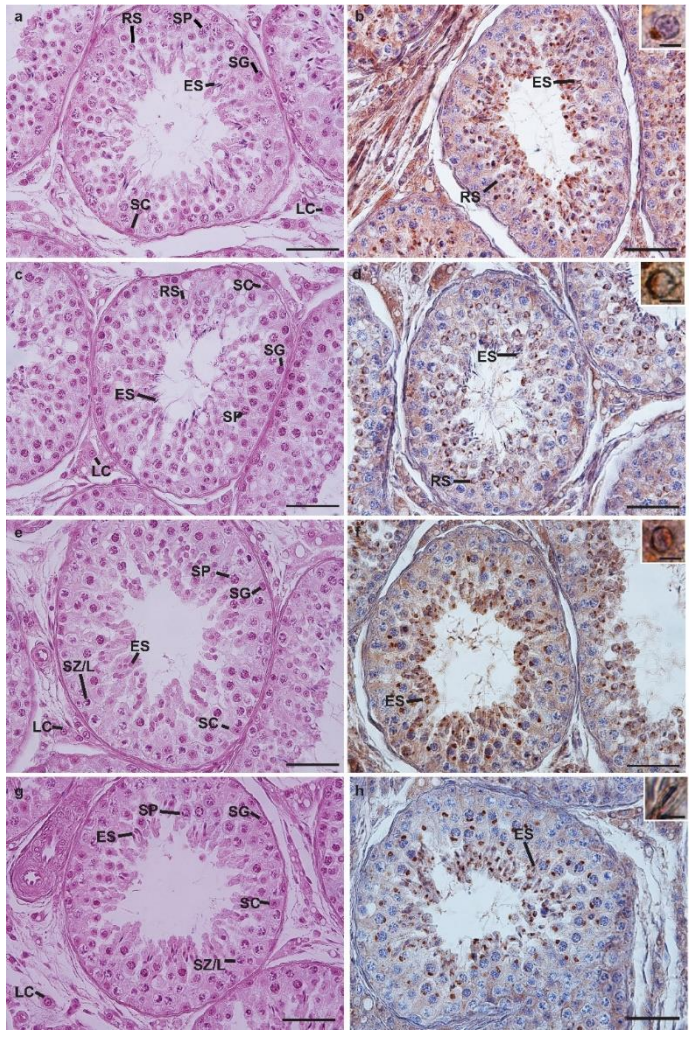

Figure 6. Four stages of spermatogenesis in seminiferous tubules of polar bear testis. **Stage AB**. Round spermatids (RS) are seen in Golgi phase (Figure 6a) and acrosome vesicles are seen as a brown dot (Figure 6b and insert). **State C**. Round spermatids (RS) enter cap phase (Figure 6c) with U-shaped acrosome in dark brown (Figure 6d and insert). Elongated spermatids (ES) line the lumen. **Stage D.** After spermiation, a new generation of elongated spermatids (ES) is in acrosome phase (Figure 6e), seen as pear-shaped structures (Figure 6f and insert). **Stage E.** The elongated spermatids (ES) (Figure 6g) are more tapered, narrow and change from conical to more elongated and slender (Figure 6h and insert). SC: Sertoli cell, SG: spermatogonia, SP: spermatocyte pachytene, SZ/L: early spermatocyte in zygotene or leptotene, RS: round spermatid, ES: elongated spermatid, LC: Leydig cell. Images to the left are stained with haematoxylin-eosin (HE). Images to the right show Gata-4 immunolabelled acrosome development as brown stain with weak haematoxylin counterstain, bar = 50 µm. Inserts shows spermatids, bar = 5 µm.

4.1.4 Gata-4 as an acrosome marker in rat pubertal development

The testicular morphology of midpubertal rats showed great variation in how far the rats had progressed towards puberty. Three distinctive morphological stages of development were identified based on spermatid development (Figure 7). Most animals (46% of rats, regardless of treatment) had round spermatids in steps 1-9 as the most developed steps (Figure 7b). Other rats (18%) had no spermiogenesis (no spermatids) and only spermatogonia, Sertoli cells and spermatocytes were present (Figure 7a). The last group of rats (36%) had elongated spermatids in steps 10-16 of spermiogenesis as the most developed step (Figure 7c). Comparing the three scores for degree of testis development at PND 35 showed increasing testis weight with increasing score (Figure 7d). Testis weight was significantly lower in rats scored to have no spermiogenesis compared with rats with step 1-9 elongated spermatids ($p<0.05$) and rats with step 10-16 spermatids ($p<0.001$). None of the rats had fully mature elongated spermatids. The rats with the most developed testes were judged to have elongated spermatids in step 16, based on the localisation of the spermatids (close to the basement membrane).

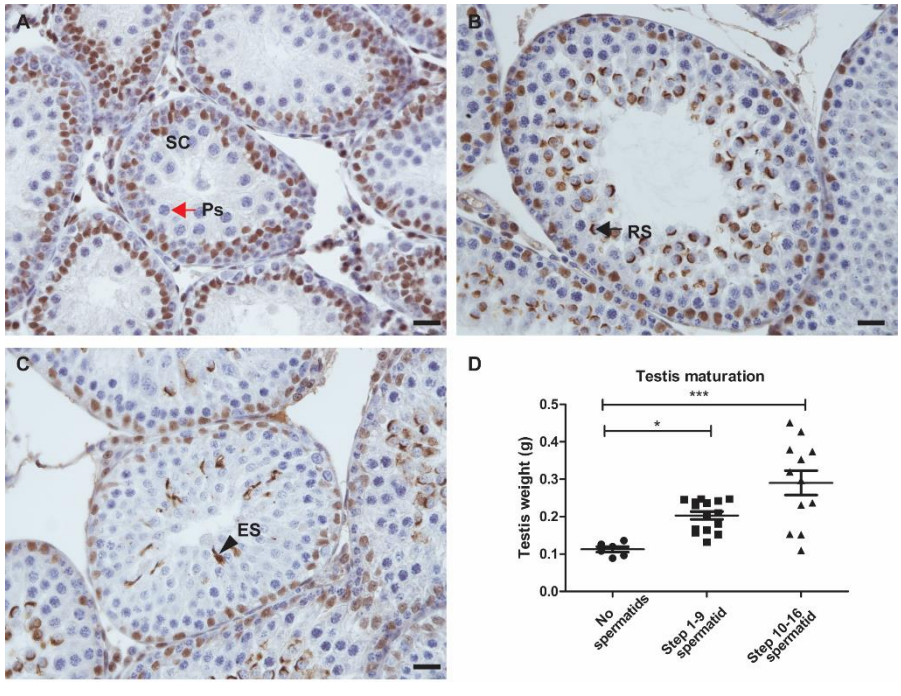

Figure 7. Histological evaluation of testis maturation in testis sections of Fischer 344 rats at postnatal day (PND) 35 after exposure to bisphenol A (BPA) *in utero* and during lactation. A-C) Sections stained immunohistochemically with Gata-4 to facilitate evaluation by marking acrosome development with brown stain. Sertoli cells (SC) also stained brown. Bar = 20 µm. Counterstained with haematoxylin. A) No spermiogenesis, i.e. no spermatids with acrosome development, pachytene spermatocytes (PS) as furthest developed stage (red arrow). B) Spermiogenesis started, score 1, step 1-9 round spermatids (RS) in cap phase (black arrow). C) Spermiogenesis proceeded further, score 2, elongated spermatids (ES) in steps 10-16 (arrowhead). D) Differences in testis weight between testis maturation scores (no spermatids, steps 1-9 spermatids, or steps 10-16 spermatids) in rat testis at PND 35 stained with Gata-4, shown as scatter plot (median testis weight 25-75% percentile, whiskers mean with SEM). Significant differences between the testis maturation scores are indicated (*$p < 0.05$, ***$p < 0.001$).

4.2 Endpoints to measure male reproductive health

4.2.1 Organ measurements

Effects of pre-fixation delay and freezing in mink (Paper I) and exposure to BPA in rat (Paper III) on organ measurements

Body weight and testicular length were not affected by prolonged storage time before fixation (6, 18, 30 or 42 h) or by freezing prior to measurements (6 h + frozen group). Testicular weight was reduced by freezing compared with the samples measured immediately after euthanisation (0 h) ($p=0.049$) and 6 h post mortem ($p=0.029$). Testicular weight was not affected by delayed fixation.

Exposure to BPA *in utero* and during lactation did not result in any significant differences in organ weight, AGD or anogenital index (AGI) in midpubertal or adult rats. However, there was a tendency for increased AGI ($p=0.069$) in adult rats exposed to 0.5 µg BPA/kg bw/day compared with the control. In midpubertal rats (PND 35), testis and epididymis weight varied among individuals, but there were no statistically significant differences between BPA-treated rats and controls.

Relationship between organ measurements and reproductive status or POP concentrations in polar bear (Paper IV)

Three reproductive groups (REP, UND and DEG) differed significantly in testes (weight, length, height and width) and baculum (weight, length and density) measurements. All values were highest for the REP group and lowest for the UND group, with the DEG group intermediate (Table 1 in Paper IV).

There was a significant negative relationship between oxychlordane concentrations in adipose tissue and testes weight, width and height, as well as baculum weight, length and density ($p<0.05$ in all cases) (Figure 8). The other POPs considered (∑PCB, ∑DDT, ∑HCH, ∑mirex, dieldrin and hepoxide) did not appear to influence testes or baculum measurements ($p>0.05$ in all cases).

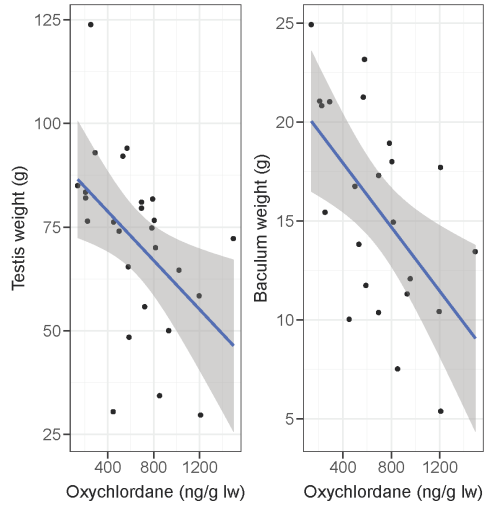

Figure 8. Effect of oxychlordane (ng/g lw) on testes weight (left) and baculum weight (right) in reproductively active (REP) male polar bears in East Greenland sampled between 1999-2016. Grey area represents 95% confidence interval around the predicted mean of a simple linear regression and black dots are data points for individual bears.

4.2.2 Morphology and histopathology

Fixation of testis tissues is crucial when studying morphology and histopathology. In Paper I, a controlled setting was created in order to study delayed pre-fixation time and freezing on mink testes. In Paper IV, pre-fixation time most likely varied between individual polar bears. In addition, some samples were clearly affected by freezing and some samples were too large for the fixative to penetrate properly. This resulted in a number of artefacts to consider during evaluation.

Effect of prolonged pre-fixation time and freezing on morphology in mink testes (Paper I)

Mink testis with a 6 h delay before fixation had a well-organised epithelium and germ cells attached to the basement membrane. Lumen of the seminiferous

tubules was filled with excess cytoplasm (Figure 9c). Overall, cells in the seminiferous epithelium and interstitium had similar morphology as at 0 h, but chromatin changes were seen in round spermatids, pachytene spermatocytes, Sertoli cells and Leydig cells (Figure 9d). With an 18 h delay before fixation, seminiferous tubular epithelium showed increased disorganisation, but the peritubular cells were still attached to the seminiferous epithelium. Lumen of the seminiferous tubules was occupied by cytoplasm, mixed with round and elongated spermatids. Chromatin changes such as pyknosis, chromatin margination and nuclear clearing were seen in round spermatids and Leydig cells. Mink testis with a 30 h and 42 h delay before fixation showed seminiferous epithelium with further disorganisation and germ cells located in abnormal positions. The peritubular cells were only partly attached to the seminiferous epithelium. Chromatin changes such as pyknosis were seen, which made identification of some cells difficult. In mink testis frozen after 6 h and thawed prior to fixation, a complete loss of organisation in the seminiferous tubular epithelium was seen. The seminiferous epithelium resembled a dense mass (Figure 9e). All cell nuclei had condensed chromatin and appeared pyknotic (Figure 9f).

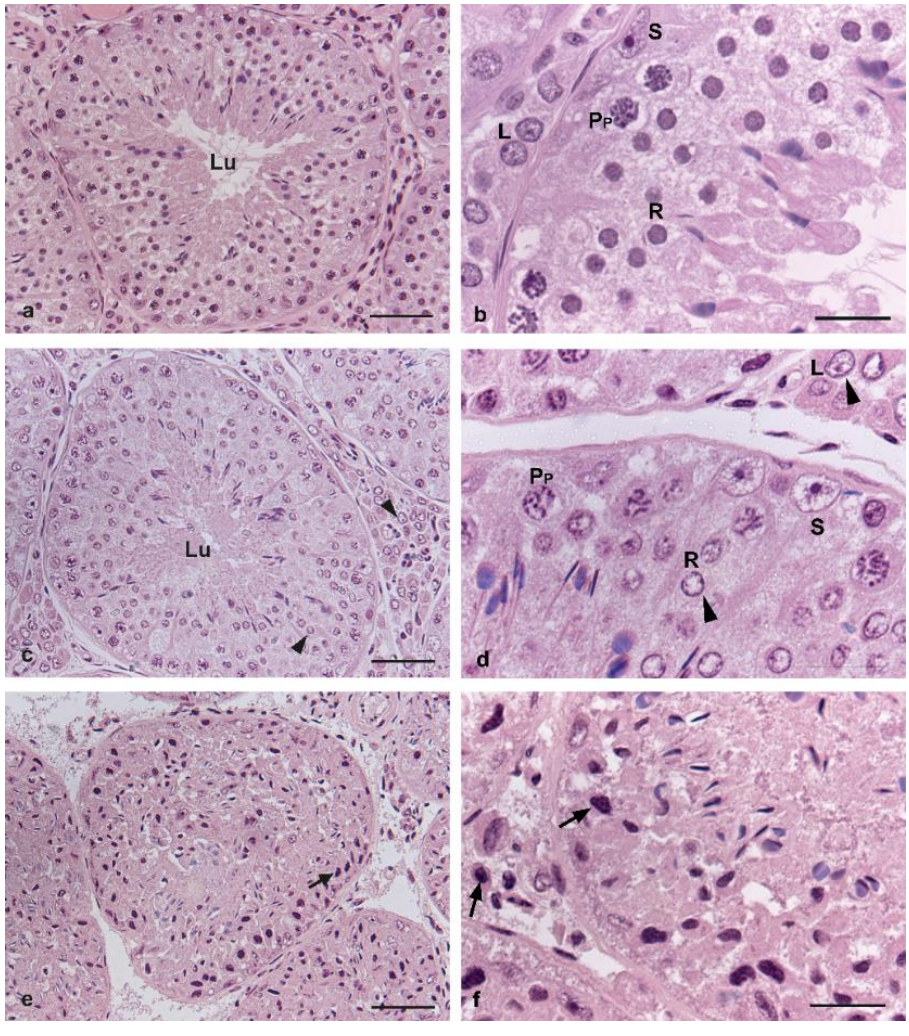
Lu
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Figure 9. Effects of pre-fixation delay and freezing on morphological organisation in mink seminiferous tubules. Cross-sections of mink testis tissue with 0 or 6 h delay before fixation, or frozen after 6 h and thawed prior to fixation. a, b) Mink testis fixed immediately (0 h) post mortem. Organised tissue with intact epithelium, a well-defined lumen in the seminiferous tubules and normal morphology with different germ cells are easy to distinguish. Germ cells are well connected and attached to the basement membrane. Sertoli cells (S) show angular nuclei with heterochromatin and a dense nucleolus, with the nucleus in close proximity to the basement membrane. The interstitial tissue shows Leydig cells (L) and capillary and lymphatic vessels are easy to recognise. c, d) Mink testis with a 6 h delay before fixation. The epithelium is well-organised and germ cells are attached to the basement membrane. Lumen (Lu) of the seminiferous tubule is filled with excess cytoplasm. Round spermatids (R) and Leydig cells (L) (arrowhead) show chromatin margination and nuclear clearing. Sertoli cells (S) show a lighter, rounder, slightly enlarged nucleus with partially condensed chromatin pattern. Pachytene spermatocytes (P_P) with chromatin clumping and slightly enlarged nuclei. e, f) Mink testis frozen after 6 h and thawed prior to fixation. A complete loss of organisation in the seminiferous tubular epithelium can be seen. The seminiferous epithelium resembles a dense mass. All cell nuclei show condensed chromatin and appear pyknotic (arrow). The exception is the elongated spermatids that can be identified. All testis samples were fixed in modified Davidson's fluid and stained with haematoxylin-eosin (HE). (Bar = 50 µm in images a, c and e; bar =20 µm in images b, d and f.).

Effect of BPA exposure in utero and during lactation on histopathology in rat testes, epididymis and prostate (Paper II)

Histological examination of the left testis of adult rats showed no differences between BPA-exposed groups and controls for any of the endpoints investigated. Interestingly, cauda epididymis had a higher prevalence of a mild degree of interstitial infiltration of mononuclear cells (inflammatory cell infiltrate score 1) in the offspring from rats exposed to 50 µg BPA/kg bw/day compared with the control ($p=0.025$). The distribution of the inflammatory cell infiltrate was mainly focal, surrounding blood vessels. Only one rat (exposed to 0.5 µg BPA/kg bw/day) had a moderate inflammatory cell infiltrate (score 2) and no rats had severe inflammation (score 3) of the epididymis. No between-group differences were observed in the caput epididymis histology or other parameters assessed in cauda epididymis.

Testicular morphology of the midpubertal rats showed great variation in how far the rats had progressed towards puberty (Figure 7), but there were no significant differences in spermatid development between the groups exposed to BPA and the control group.

Relationship between levels of POPs and histopathology in polar bears (Paper IV)

In the 90 bears evaluated histologically, two showed infiltration of mononucleated inflammatory cells in the testes, while multinucleated giant cells

were seen in 27 of the bears [REP (n=13), DEG (n=10), and INT (n=4) groups]. In the REP group, 50% of the polar bears showed disorganised seminiferous epithelium (Figure 10a) and 78% had germ cells in the lumen. There were no significant relationships between POP concentrations and any of the histopathological endpoints

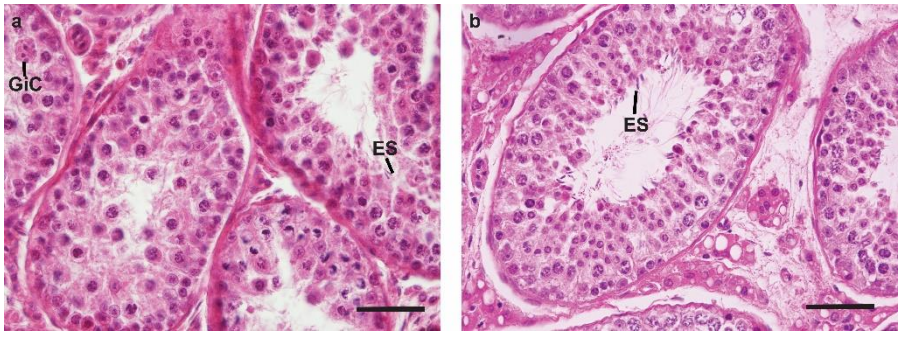

Figure 10. Histopathological evaluation of testes from polar bears captured 1999-2015 in East Greenland. The polar bears have active sperm production (REP group), with elongated spermatids at spermiation (ES). a) Disorganisation of seminiferous epithelium in testes from a 5-year-old polar bear captured in March. Disorganisation score 3, more than 50% of the tubules were affected. Presence of giant cell (GiC). b) Normal testes from a 6-year-old polar bear captured in April. Stain haematoxylin-eosin (HE). Bar = 50 µm.

4.2.3 Semi-automatic morphometry of testes

Effect of prolonged pre-fixation time and freezing on morphometry (Paper I)

Tubular area was constant for up to 30 h of delayed fixation, but at 42 h the area decreased compared with the tissue fixed 6 h post mortem (p=0.048). Freezing the tissue decreased the tubular area compared with the tissue fixed immediately after euthanisation (0 h) (p=0.020) and the tissue fixed 6 h post mortem (p=0.006). Tubular diameter also decreased when the testicular tissue had been frozen prior to fixation, compared with that in the tissue fixed immediately post mortem (0 h) (p=0.020) and the tissue fixed 6 h post mortem (p=0.006). Tubular diameter decreased over time and showed a tendency to decrease (p=0.055) between the tissue fixed 6 h and 42 h post mortem. Epithelial height was affected by time and showed a significant increase in all groups (6, 18, 30 and 42 h, 6 h + frozen) compared with 0 h. To distinguish between the effects of time and freezing on epithelial height, a comparison of the 6 h and 6 h + frozen groups was performed, since the epithelial height increased significantly already after

6 h of delayed fixation. This comparison revealed significantly lower epithelial height in the 6 h + frozen group compared with the non-frozen 6 h group.

Relationship between bisphenol A and persistent organic pollutants and morphometry in testes (Papers III and IV)

There were no significant differences between BPA-treated rats and controls regarding morphometric measurements of epithelial height, area and diameter of seminiferous tubules. In polar bear testes, only area and diameter were measured due to the finding in Paper I that epithelial height was less reliable if the pre-fixation time was prolonged. No relationships between area or diameter of seminiferous tubules and any of the investigated POPs were found.

5 Discussion

This thesis investigated different aspects of histological evaluation of testes as a tool to measure the influence of environmental chemicals on male reproductive health. The effect on male reproduction in laboratory settings with exposure of rats to a single chemical was studied, reliable measurements and methods for mink to be used in field conditions were explored and real-life exposure and seasonality in polar bears were investigated. This discussion is divided in three sections. Section 5.1 discusses the methods used and the different factors influencing the investigated endpoints. Section 5.2 discusses the findings in relation to testicular effects of chemical exposure, while section 5.3 discusses differences between using laboratory and wild animals.

5.1 Methods to measure male reproductive health

5.1.1 Organ measurements

Organ measurements are commonly used endpoints in reproductive toxicology and acknowledged as sensitive endpoints that are easy to collect (Creasy, 2003; Morrissey *et al.*, 1988). In Paper I, it was demonstrated that testes weight is not affected by prolonged pre-fixation time, which makes this endpoint suitable to use under field conditions. However, testes weight decreased significantly after the samples had been frozen and thawed, which should thus be avoided. In the polar bear study (Paper IV), testes weight, length, height and width and baculum weight, length and density differed significantly between three groups of animals studied (REP, UND and DEG). This indicates the importance of considering reproductive group and/or capture month of seasonal breeders when investigating the effect of environmental exposure to chemicals. Seasonal differences have been reported previously in mink exposure data, again emphasising the importance of capture date (Persson *et al.*, 2013a; Persson *et*

al., 2013b). In midpubertal rats in Paper III, there were large differences between individuals even when they were euthanised at the same day in their development. Therefore, when investigating the effect of chemicals on midpubertal animals, organ weight is not a reliable method and it may be more accurate to use histology to describe testes development. This confirms the importance of knowledge about reproductive status when using organ weight to evaluate the impact of chemicals, in order to minimise false positive or false negative relationships.

5.1.2 Histopathology of testes

The endpoints used to describe morphological manifestations of testicular toxicity used in this thesis were missing germ cell layers or cell types like retained spermatids, multinucleate or apoptotic germ cells, and sloughing of spermatogenic cells into the lumen. Tubular dilatation/contraction was also studied. The tubular stages of the spermatogenic cycle were considered when identifying endpoints (Papers III and IV). All endpoints are described and recommended by the Society of Toxicologic Pathology (Lanning *et al.*, 2002). The recommended approaches for evaluation of testicular and epididymal toxicity are designed for laboratory animals in studies where the exposure is controlled (Lanning *et al.*, 2002). In the studies presented in this thesis, more factors influenced the results and must be considered.

Effect of prolonged pre-fixation time, freezing and poor fixation

Paper I identified endpoints in testes histopathology that could be affected by chemicals and by prolonged pre-fixation time and freezing, and should therefore be evaluated with extra caution in experiments when these problems have occurred. One example is sloughing of cytoplasm and germ cells into the seminiferous tubular lumen, which was seen in mink after only 6 h of storage between euthanisation and fixation. However, Bryant and Boekelheide (2007) observed no sloughing of the apical seminiferous tubular epithelium over up to 48 h in a study examining time-dependent post mortem histopathology in rat testes. Any affected cell in the testicular tissue can be sloughed into the lumen, regardless of mechanism of toxicity (Creasy, 1997). It may also be an effect of Sertoli cell dysfunction (Vidal & Whitney, 2014). Checking for exfoliation of germ cells into the lumen of seminiferous tubules in routine testicular histopathology is recommended (Lanning *et al.*, 2002). Several studies report germ cells sloughing into the tubular lumen as a sign of toxic damage after exposure to toxicants such as cadmium, nickel and di (n-butyl) phthalate (Toman *et al.*, 2012; Alam *et al.*, 2010; de Souza Predes *et al.*, 2010). In the polar bear

study (Paper IV), 78% of the polar bears with active spermatogenesis had exfoliated germ cells in the lumen of seminiferous tubules. Rat and mink testis tissues appeared to react differently as regards sloughing of germ cells into the lumen when storage time prior to fixation was prolonged, and it is not known how polar bear testis responds. It is therefore not possible to conclude whether the reason for the large number of polar bears with germ cells in the lumen is impaired reproduction or poor fixation of samples post mortem.

The selection of polar bear testes of sufficient quality for histological evaluation (Paper IV) was based on knowledge gained during the evaluation of mink in Paper I about the effect on cells of prolonged pre-fixation time (increasing rearrangement of chromatin in the germ cells) and freezing (pyknotic cells with condensed chromatin).

Young animals

In Paper III, rats were evaluated at PND 35, in midpuberty, after exposure to BPA *in utero* and during lactation, and no link to BPA treatment was found. Both testis morphology and weight showed considerable variation between rats. This is consistent with previous descriptions of the first wave of spermatogenesis (Creasy, 2003). The findings also reflect the very rapid increase in testicular weight in rats between PND 18 and 90 (Picut *et al.*, 2015; Sharpe *et al.*, 1999). It was decided in Paper III to assess the degree of first-wave spermiogenesis by evaluating round and elongated spermatids using Gata-4 immunoreactivity as a marker for acrosome development. A similar approach has been used previously, where seminiferous tubules containing spermatids with acrosomal vesicles and acrosomes were counted (Brouard *et al.*, 2016). It is interesting to assess the effect of BPA on pubertal development, but evaluation of testis toxicity in midpuberty must be done with great care. Immature testes are characterised by tubules containing almost no germ cells and pubertal testes contain large numbers of exfoliated degenerated cells, resulting in a generalised appearance of disorganisation within the seminiferous tubules, which complicates examination for toxic damage (Creasy, 2003; Lanning *et al.*, 2002). There is no simple way of assessing puberty, but to evaluate how far testis maturation has progressed in rats at a certain day, the method used in this thesis may be a more useful way to study pubertal development in detail than just assessing the day of preputial separation.

In samples from polar bears, the presence of giant or mononucleated inflammatory cells in testes histopathology from animals with undeveloped testes was evaluated. This was a heterogenic group, with animals of various ages, and the technique developed in rats could therefore not be used. Reliable and validated endpoints to evaluate testicular toxicity in young and developing

animals are, to our knowledge, hard to find in the literature. In the polar bears studied, it was found that higher oxychlordane concentrations were significantly correlated with lower testes and baculum measurements in sexually mature animals. The concentrations of chlordane in polar bears were highest in animals with undeveloped testes. However, toxic effects on male reproduction in young animals may have been overlooked due to the lack of suitable and reliable endpoints.

Short- or long-term exposure

In Paper III, rats were exposed *in utero* and during lactation to BPA and euthanised at 12-months, resulting in a long recovery time. Depending on which cell the toxin targets, recovery time can vary (Meistrich, 1986). In the polar bear study (Paper IV) it was not possible to control the timing and duration of the exposure. It could be a mix of acute and chronic exposure over years, probably with varying levels of exposure. There are few reliable endpoints in chronic exposure, especially in non-conventional animal species. Vidal and Whitney (2014) describe maturation depletion, when successive populations of post-spermatogonial developing germ cells are eliminated, after prolonged toxic compound exposure. Chronic exposure of Sertoli cells to toxicant can result in disorganisation of the seminiferous epithelium (Vidal & Whitney, 2014), as frequently observed in the polar bear testes examined in this thesis.

Some of the endpoints described may be less relevant to measure in wild animals or after chronic exposure. In this thesis, a search was made for missing pachytene spermatocytes or round spermatids in stage VII, which is described as a response to decreased intratesticular testosterone levels (Vidal & Whitney, 2014; Lanning *et al.*, 2002; Creasy, 1997). Ramaswamy and Weinbauer (2014) observed a morphological testicular response after exposure to a GnRH antagonist, resulting in lower testosterone levels, at different time points after exposure. In their study, missing cell types in stage VII were seen after 14 to 28 days in rats, non-human primates and dogs. In all these species, a complete lack of spermatogenesis will occur if the decreased testosterone levels persist for over four weeks. In wild animals, chronic exposure might be more likely and the expected morphology at low testosterone levels would then be no spermatogenesis, rather than the lack of a certain cell type in stage IV. If wild animals suffer from temporarily low testosterone levels, recovery to a normal testis may be seen (Ramaswamy & Weinbauer, 2014). This could also be the case in the rat, where the recovery time after the end of exposure was more than 11 months. There is a risk that changes induced due to chronic exposure are different than the endpoints evaluated, which could result in toxic damage being overlooked.

5.1.3 Staging

The number of stages differs between species. In mink, 12 stages are defined by Pelletier (1986). This description is based on changes in the development of the acrosome in the spermatid, in accordance with the method proposed by Leblond and Clermont (1952), who defined 14 stages in rat. The validity of the manual staging method in mink (Paper II) defining the 12 stages had 70.5% inter-observer agreement with a kappa value of 0.67, which is considered substantial, and an intra-observer agreement of 83.6% with a kappa value of 0.81, which is considered almost perfect (Watson & Petrie, 2010; Viera & Garrett, 2005). Manual staging using 12 stages in mink can therefore be considered reproducible. The question is whether 12 stages are needed? The finer the division into stages, the more likely it is that a tubule will be close to the boundary between stages, and may be confused with neighbouring stages. In Paper II, pooling of the 12 stages in mink into five different groups (A to E) was tested. It was found that this pooling raised the intra- and inter-observer agreement. Pooling of stages is not new. In rat, McClusky *et al.* (2007) pooled the 14 stages defined by Leblond and Clermont (1952) into seven different groups, while Hess *et al.* (1990) pooled these 14 stages into four groups. If the stages were pooled into even fewer groups, the kappa value would probably be higher, but this would also lead to a marked reduction in information transmitted (Cross, 1998). In polar bear, four stages are described in this thesis. To my knowledge, this is the first time that details of the cycle of seminiferous epithelium in polar bears have been described. Pooling of the stages in polar bear was similar to the pooling proposed in mink, but stages A and B in mink were further pooled into stage AB in polar bears. The inter- and intra-observer agreement in polar bear analyses has not yet been tested. In Paper I, it was found that the acrosome remained marked with Gata-4 even if the pre-fixation time was prolonged up to 42 hours and even after freezing in mink. There was a change in distinctiveness of the acrosome stain over time, with increased background stain and difficulty in distinguishing which stained acrosome belonged to which nucleus. The robustness of Gata-4 as an acrosome marker justifies the use of this stain in wild animals, where non-optimal fixation may be more of a problem. In this thesis, Gata-4 was used on polar bear samples to identify stages in the cycle of the seminiferous epithelium, though it was found that long-term storage of tissues in fixative resulted in difficulties with sections falling from glass slides. The computerised staging system for mink tissues developed in Paper II was designed to advance and objectify manual staging, as discussed below.

5.1.4 Image analysis

Morphometry using image analysis

The first step in image analysis of testis sections was to segment seminiferous tubules. It proved difficult to develop a fully automated method. Even if it is very clear to the human eye which cells belong to the tubular structure, it was a challenging task for the computer to correctly identify these cells. Two automated segmentation methods were tried. One of these involved using PAS-stained tubules in rooster testis, where first the border of the lumen was delineated, followed by segmentation of the border of tubules. This method does not rely on identifying individual cells forming the tubules. It uses the colour of the stain to detect as much interstitial tissue as possible and thereafter find boundaries for individual tubules using lumen and interstitium as hints (Fakhrzadeh *et al.*, 2012). In the other automated segmentation approach tested, attempts were made to find a segmentation method that could be used even if the pre-fixation time was prolonged up to 42 hours in mink testicular tissue, to make it robust for field studies. Gata-4 stain was used to mark the cell nuclei, a graph was constructed on top of the cells and finally a graph-cut optimisation was used to cut the link between cells of different tubules (Fakhrzadeh *et al.*, 2013). This segmentation method showed 85% correctly segmented cells in the mink testes with optimal fixation, but neither of the fully automated methods could be used and a semi-automatic method was therefore developed.

The semi-automatic, livewire segmentation method described in Paper I was used in all four papers in this thesis. In this method, the user adds a seed point on the boundary of a tubule and an algorithm calculates the cost of the optimal boundary between this seed point and all other pixels in the image. The semi-automatic method is less sensitive to stain. In Paper I, a variant of PAS stain was used. Regular PAS and HE was used in Paper III and IV, respectively, while in Paper II Gata-4 stain counterstained with haematoxylin was used. It would be preferable to achieve fully automatic segmentation of testicular tubules.

Computerised automatic staging

In this step, it was first necessary to calculate relevant features for all tubules and then use a proper classifier to classify them. A large number of algorithms were tried, and a combination of Local Ternary Pattern (for feature calculation) and Support Vector Machines (for the classifier) gave the best results (Fakhrzadeh, 2015). The Local Ternary Pattern method looks at the epithelium in cross-sections as a textured pattern, not relying on correct identification of nuclei or acrosome. The Support Vector Machine classifier was used to learn

which textures correspond to each stage. Different techniques have been used to facilitate staging by marking acrosome development. PAS is probably the most commonly used, but the low colour contrast of the stain makes it very challenging for the computer program to reliably differentiate the colours. Higher contrast and more distinct colour differences were needed to simplify image analysis and the computer program. The key to the computerised staging was the distinctive dark brown Gata-4 stain of acrosome development. McClusky *et al.* (2009) proposed immunolocalisation of Gata-4 to facilitate staging in rat testis, and it was also used to stain the acrosome in mink and polar bear in this thesis.

The 12 manually identified stages were not equally distributed, for example, 18% of all tubules were in stage VI, whereas other stages were scarce, *e.g.* only 2% of all tubules were in stage X (Paper II). This, in combination with very similar morphology in neighbouring stages, was problematic when training the classifier. The classifier does not take this imbalance into account and is likely to be biased towards the more populous stages (Fakhrzadeh, 2015). The pooled five-group classification proposed for mink (Paper II) was easier for the computer program because the grouping of tubules resulted in more examples of tubules for each category. This led to better generalization of these examples by the classifier. The more examples of a stage, the better the program was at identifying that stage. Pooling of stages raised the agreement between the consensus staging and the computer to a substantial value of 76.7% with a weighted kappa value of 0.77.

5.1.5 Use of staging in evaluation of toxic damage

The Society of Toxicologic Pathology recommends evaluation of cell associations, defining the stages, since these can be disrupted if animals are exposed to chemicals affecting reproduction (Lanning *et al.*, 2002). Knowledge of staging is required to identify these cell-specific and stage-specific effects (Creasy, 1997) and it is time-consuming to manually find sufficient tubules in certain stages to evaluate. The computerised staging does not need to be 100% accurate to be useful. For example, an important endpoint that requires staging to be detected is sperm retention, whereby spermatids do not leave into the tubular lumen as is normal during stage VII in mink. To detect sperm retention, it is recommended to observe tubules in stages VIII-IX in mink, to ensure that they only contain one population of elongated spermatids. This would correspond to group D in the pooled staging system, which is found correctly by the computerised staging tool in 67% of cases.

The recommendations do not include collecting statistics on the distribution of tubules across stages (Lanning *et al.*, 2002). However, stage frequency can be altered by endocrine disrupting chemicals, which increases the importance of this endpoint (McClusky *et al.*, 2007; Aravindakshan *et al.*, 2004). The reason for this not being recommended might be the large number of tubules and animals that need to be examined to obtain a sufficiently precise estimate of the stage distribution for comparative statistics (Hess, 1990). Here, computerised staging may facilitate handling of large amounts of data. The computerised staging approach has the potential to modernise the tedious staging process required in toxicological evaluation of testicular tissue.

5.2 Relationships between chemicals and male reproduction in rat and polar bear

This thesis presents results from two studies evaluating the relationship between chemicals and impaired male reproduction. One was a laboratory study investigating the effect of BPA on male reproduction in rats under very controlled settings. The other was based on analysis of polar bear tissues collected during 16 years from East Greenland, data on persistent organic pollutants in adipose tissue and possible correlations to male reproductive organs.

In the BPA exposure study, the only affected endpoint was a significantly higher score of mild inflammatory cell infiltrate in cauda epididymis in 12-month-old Fischer 344 rats exposed to 50 µg BPA/kg bw/day *in utero* and during lactation. However, the inflammatory cell infiltrate observed was mild and only present in 12-month-old rats and not in younger rats (PND 35), which would have strengthened the validity of this result. The mild inflammatory cell infiltrate observed could be a chance finding that is age-related and not related to exposure, but it still does not explain why the control and the exposed group differed. The effect of BPA on male reproduction has been debated and the large number of studies addressing the issue report both negative and positive findings. A recent study, conducted as part of the Consortium Linking Academia and Regulatory Insights on BPA Toxicity (CLARITY-BPA) tried to shed further light on the male reproductive toxicity of BPA (Dere *et al.*, 2018). Effects of BPA on all male reproductive endpoints studied were only found at the highest investigated dose, 25,000 µg BPA/kg bw/day (Dere *et al.*, 2018).

In captured wild polar bears, there was a significant negative relationship between oxychlordane concentrations in adipose tissue and testes and baculum measurements, indicating a potential impact on male reproduction. The negative association between the concentration of oxychlordane and testis and baculum

measurements is in accordance with findings in several other studies on various POP effects on male reproduction in polar bears, as summarised in reviews by Sonne (2010) and Letcher *et al.* (2010). Half the polar bears with active sperm production that were examined in this thesis showed disorganisation of testes. This might be a sign of disrupted reproduction. However, no correlations to investigated chemicals were detected. Investigation of relationships between chemicals and male reproduction in wild animals involves a variety of confounders, *e.g.* poor handling of the tissues, possible diseases, genetics and differences in nutritional status, that have the potential to interact with the results. These confounders are difficult to control, which adds a certain uncertainty when interpreting the results.

5.3 Laboratory animals versus wild animals

In Paper III, direct effects of BPA on male reproduction were investigated. To investigate the effect of a single chemical on male reproduction, laboratory settings using rats would likely be the primary choice. However, the main interest is often to search for chemicals affecting reproduction in humans and/or wildlife, in order to protect both groups. Environmental scientists are interested in identifying potentially dangerous chemicals so that they can be banned, but the interactions and synergistic effects of chemical mixtures are also of great concern. Laboratory animals are used widely in all kinds of reproductive studies. Wildt *et al.* (2010) reviewed reproduction-oriented articles over 11 years and found that 44% involved studies on rats, mice and cows. Those authors point out the benefits of using other, non-conventional species for advanced reproductive science (Wildt *et al.*, 2010). Rats are often used for practical reasons, as they are small, cheap, have a short reproductive cycle, are easy to keep and give high comparability between studies. However, it is not always easy to transfer results from rats to humans. Perel *et al.* (2007) reviewed the agreement between animal and clinical studies showing evidence of treatment effects in clinical trials and found that 50% of the interventions had similar outcomes in the clinical trial and the animal studies. They concluded that some of the disagreement between studies may be due to the failure of the animal studies to mimic the complexity of real-life disease. It has also been shown that morphological response in testicular tissue can differ between species after the same exposure (Meistrich, 1986). According to Abbott (2005), many laboratory animal tests overestimate or underestimate human toxicity. Carcinogenic substances are an example of false-positive animal testing (Gold *et al.*, 2005). Wild animals provide a great opportunity to study real-life exposure and how the complex interactions between chemicals affect male reproductive health. On the other hand, studying

wild animals adds great complexity, since reproductive season, diseases, genetics and practical difficulties in tissue collection can affect the results. The issues raised in the rational for this thesis, whether histological examination of testes using commonly described endpoints is applicable in studies on wild animals and laboratory animals in different set-ups, and whether chemically induced changes can be revealed by such examinations are touched upon in this thesis, but many questions remain unanswered. Both laboratory and wild animal studies must thus be considered important to gain accurate knowledge of chemical effects on male reproductive health. However, no reliable answers will be found using unreliable methods.

6 Concluding remarks

Overall, this thesis demonstrates that handling of tissues prior to fixation, reproductive season and status are crucial factors to consider when evaluating testis histology and organ weight, in order to prevent the results being misinterpreted and incorrect conclusions drawn. It also demonstrates that computerised image analysis is a promising objective tool when evaluating testis histology in both wild and laboratory animals.

Specific conclusions are that:

- When investigating mink, area and diameter of seminiferous tubules, length and weight of testis and acrosome marked with Gata-4 are particularly useful endpoints, as they proved to be robust up to 30 h prolonged pre-fixation time. However, freezing of testicular tissues prior to fixation should be avoided, since most endpoints evaluated were severely affected by this.

- Gata-4 as an acrosome marker can be used to evaluate stages in mink and polar bear seminiferous tubules and assess the degree of first-wave spermatogenesis in midpubertal rats. Pooling of the 12 stages in mink into five groups made the staging system more reliable and in wild polar bears four staging groups were described.

- Computerised image analysis can be a useful tool to segment seminiferous tubules in order to measure area and diameter in both laboratory and wild animals. The proposed computerised method in mink with stages pooled into five groups reached substantial agreement and is an objective method to assess testicular tissue. The method has potential to modernise the tedious staging process required in toxicological evaluation of testicular tissue

- No long- nor short-term effects on reproductive health were detected when rats were exposed to low doses of BPA *in utero* and during lactation, apart from higher prevalence of mild inflammatory cell infiltrate in cauda epididymis in adult rats. Due to large morphological variation during puberty, care should be taken when evaluating histopathology at this age and reliable assessment methods must be used.

- It is possible to identify polar bear reproductive status even if data on capture date or age are lacking. Furthermore, if reproductive organ measurements are to be used to investigate the effect of POPs on male reproduction, reproductive status must be considered.

- Male reproduction in East Greenland polar bears may be impaired, since disorganised testes were detected in half of the bears tested. However, no correlations to investigated POPs were detected. Oxychlordane may have a potential impact on reproduction in polar bears, as increasing concentrations of this chemical were associated with decreasing baculum and testes measurements.

7 Perspectives and future research

This thesis investigated different aspects of histological evaluation of testes, as a tool to measure the effect of environmental chemicals on male reproductive health, in laboratory and wild animals. However, several questions remain to be answered in future studies.

The semi-automated computerised staging method developed in Paper II needs some improvements to be useful on a larger scale. To fully automate the computerised image analysis segmentation method, an immuno- mediated delineation of the basement membrane by laminin should be employed as the next step (Abd-Elmaksoud, 2009). In combination with Gata-4, performed as a double stain, it would then be possible to achieve a fully automated staging method. Furthermore, a whole image scan would facilitate interaction between the computerised program and pathologist observations, in order to identify important stages for histopathological endpoints. In future studies, the method need to be tested in rats, mice and possibly other species, including wild animals, to prove its usefulness.

Validated and reliable methods to detect toxic damage in testis from animals of different ages are needed. Researchers have already used various methods to assess effects on rat testis during pubertal development (Brouard *et al.*, 2016; Atanassova *et al.*, 2000). However, the large variation between animals, probably due to the rapid growth during this period (Picut *et al.*, 2015; Sharpe *et al.*, 1999), complicates the evaluation. In this thesis, a method to evaluate testis maturation was developed. This method needs to be evaluated further with intra- and inter-observer agreement and tested on other animal species. This would also be useful when evaluating testis from non-sexually mature polar bears and other wild species.

Robust endpoints to evaluate testicular histology were identified in mink. Future projects collecting samples from wild animals should make great efforts to place tissues for histological purposes in fixative as soon as possible, to minimise difficulties and uncertainties in the evaluation. One difficulty when

capturing small wild animals, *e.g*. mink, is that they are often captured in traps that are checked only on a daily basis, for practical reasons (Persson *et al.*, 2013a; Persson *et al.*, 2013b). This obviously delays the pre-fixation time. New technology whereby a message is sent to the trapper's phone when an animal is caught has the potential to shorten this time. Traps capturing animals live, in combination with a phone alert system, have been tested (Ziegler *et al.*, 2018). However, this may cause stress for the trapped animal, which in turn may affect the results. It is obvious that great care should be taken when designing experiments that involve the capture of wild animals, to identify the most optimal conditions.

The final question is what is required to assure a chemical as sperm-safe. Testing a new chemical can require up to 12,000 laboratory animals (Abbott, 2005). Laboratory studies may not give the correct answer, considering the chemical cocktail and species differences, which are just two factors known to influence the results. There are no easy answers to the question, but both laboratory and wild animals are likely needed and reliable methods must be used when evaluating both.

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Popular science summary

Poor sperm quality, increased frequency of testicular cancer and increased prevalence of malformations in newborn boys are among the reproductive disturbances that have increased in some countries in recent decades. Various reproductive disorders have also been observed in wild animals, including fish, birds and mammals. During the same period, the production of new chemicals has increased dramatically and many of these may end up in the environment. Some chemicals have hormone-disrupting properties, leading to concerns that chemicals are responsible for the increase in male reproductive disorders.

To investigate how a potentially hormone-disrupting chemical, bisphenol A, affects male reproduction, rats were exposed to this chemical through their drinking water during pregnancy and lactation. Mild inflammation in the epididymis in the adult offspring was the only effect observed. Reproduction in rats during puberty was also studied, but reliable methods for measurements during puberty are lacking. Therefore a new method was developed to describe how testicles develop during this period. Puberty is problematic to evaluate, as it is a period of rapid development, which made it difficult to detect whether bisphenol A affected the cells in testicles or not. In laboratory experiments, everything can be controlled, from light, temperature, material and food to exposure of the chemical. However, the real world is different, involving exposure to a mixture of many chemicals through water, food and air. In addition, several hormone-disrupting chemicals have been shown to produce greater combined effects than the effect from each chemical alone at the same concentration.

One way to investigate how a chemical mixture affects male reproduction is to use wild animals, either as a model for humans or to detect how the animal itself is affected by the chemicals. Mink and polar bear were studied in this thesis. Both species are at the top of the food chain, where chemicals accumulate. Material from polar bears was collected during legal hunting in Greenland,

where chemicals are transported through the air from industrialised countries and accumulate in the bears in high concentrations.

When using wildlife, there is often a long transport phase between the place where the animals were killed and the laboratory where the testicle cells are investigated. Before testicular tissue can be examined under a microscope, it must be fixed in a solution that maintains structures and cells in as close to live status as possible. It is important that testicular tissue samples are placed in fixative as quickly as possible, as otherwise the cells will be damaged. Healthy mink from a fur farm were used to investigate the effect of prolonging the time between euthanisation and fixation on cells in the testicles. Testicular length and weight and area and diameter of the tubes where the spermatozoa are produced were among the parameters that proved to be robust, and can be used even if the testicle has been stored before fixation. However, it was concluded that samples should not be frozen before they are sent to the laboratory, as almost all measured parameters were adversely affected by freezing, especially at microscopic level.

In order to measure damage to the testicle, it is important to know what normal testicular cells look like. Therefore, a method was developed for assessing developmental stages of the testicles, from stem cell to mature spermatozoa, in healthy mink. In order to improve the method, a computer-assisted image analysis method was designed and found to facilitate and streamline the work of assessing testicular cells.

The reproduction of polar bears and the seasonal variation that occurs in polar bear testicles are described in this thesis. This seasonal variation proved to be important when assessing whether chemicals affect reproduction, because the weight of the reproductive organs differed depending on the season in which the polar bears were caught. The cells in the testicles of more than half the polar bears studied were disorganised in a way that suggests that sperm production may be affected. However, this damage was not correlated with any of the chemicals detected in fat from polar bears. Bears with a higher concentration of oxychlordane, a chemical used to control pests, had smaller reproductive organs, indicating that oxychlordane may have a negative impact on male reproduction. In this thesis, a number of important factors that affect the measured parameters used to assess male reproduction were identified. When examining how the mixture of chemicals in the environment affects male reproduction in wildlife, consideration should be given to the reproductive capture season, the transport time of the organs and whether the tissue has been frozen before fixation. Otherwise, the results may be misinterpreted and incorrect conclusions drawn. The method for assessing sperm development in mink testicles presented in the thesis can be useful in future work. Moreover, the computerised image analysis

method developed can objectify and eventually streamline the time-consuming manual work of assessing damage to testicular tissue. In future work, new methods should be developed to assess testicular damage in animals during puberty. The method presented in this thesis for describing testicle development in pubertal rats can be a starting point for this work.

Populärvetenskaplig sammanfattning

Sämre spermiekvalité, ökad frekvens av testikelcancer och fler missbildningar hos nyfödda pojkar är några av de reproduktionsproblem som ökat i vissa länder. Det har även observerats en rad reproduktionsstörningar hos vilda djur, hos såväl fiskar som fåglar och däggdjur. Samtidigt har produktionen av nya kemikalier ökat kraftigt och många av dessa hamnar i miljön. En del kemikalier har hormonstörande egenskaper vilket lett till en stark oro för att det är kemikalier som ligger till grund för den ökade ohälsan bland män och handjur.

För att undersöka hur en potentiellt hormonstörande kemikalie, Bisphenol A, påverkar hankönsreproduktionen exponerades råttor genom sitt dricksvatten under dräktighet och digivning. Den enda påvisade effekten var mild inflammation i bitestikeln hos råttornas vuxna ungar. Vi undersökte även råttorna under puberteten. Då det saknas bra mätparametrar under puberteten utarbetades en ny metod för att beskriva hur testiklarna utvecklas under denna period. Puberteten är en svår period att utvärdera då det är en fas med snabb utveckling och det är svårt att uttala sig om Bisphenol A påverkar testikelcellerna.

I laboratorieförsök kan man kontrollera allt från ljus, temperatur, bomaterial, mat och hur mycket av kemikalien som råttan exponeras för. Men i verkligheten blir vi utsatta för en blandning av kemikalier via vatten, mat och luft. Dessutom har flera hormonstörande kemikalier visat sig ge större sammanlagda effekter än effekten de ger var för sig i samma koncentration. Ett sätt att undersöka hur denna kemikaliemix påverkar reproduktionen är att använda vilda djur, dels som modell för människor, men också för att se hur djuret själv påverkas. Både mink och isbjörn har används i denna avhandling. Båda arterna befinner sig högt upp i näringskedjan där kemikalierna ackumuleras. Material från isbjörn är insamlat under laglig jakt på Grönland där kemikalier som transporterats via luften från industrialiserade länder ansamlas i björnarna i höga nivåer.

När man använder vilda djur är det ofta lång transporttid mellan platsen där djuren avlivats och laboratoriet där testikelcellerna ska analyseras. Innan man

gör vävnadssnitt av testikeln för analys i mikroskop fixeras den i en lösning som bevarar celler så lika den levande vävnaden som möjligt. Det är viktigt att vävnaden placeras i lösningen så fort som möjligt då cellerna annars påverkas. Friska minkar från en uppfödare användes för att undersöka hur den förlängda tiden mellan avlivning och fixeringen påverkade cellerna i testikeln. Testikellängd och vikt samt area och diameter på rören där spermierna bildas är de mätparametrar som visade sig mest robusta och kan användas även om testikeln legat en tid innan fixering. Vi kom även fram till att djuren inte får frysas innan de skickas till laboratoriet då nästan alla mätparametrar påverkades negativt av frysning, särskilt om man vill titta på celler i mikroskop.

För att kunna mäta skada på testikeln är det viktigt att veta hur normala celler ser ut. På friska minkar utarbetades därför en metod som bedömer utvecklingsstadierna i testikeln från stamcell till färdig spermie. För att förbättra metoden ytterligare designades en bildanalysmetod som med datorns hjälp kan underlätta och effektivisera arbetet med att bedöma testikelcellerna. Även isbjörnarnas reproduktion och de säsongsvariationer man ser i deras testiklar presenteras. Säsongsvariationen visade sig viktig när man ska bedöma om kemikalier påverkar reproduktionen eftersom vikterna på reproduktionsorganen ändrades med säsong. Cellerna i testiklarna på mer än hälften av isbjörnarna var oorganiserade på ett sätt som antyder att spermieproduktionen kan vara påverkad. Vi kunde dock inte koppla denna skada till någon av de kemikalier vi mätte i isbjörnarna. Däremot fann vi att björnar med en högre koncentration av oxyklordan, en kemikalie mot skadedjur, hade mindre reproduktionsorgan. Detta kan tyda på att oxyklordan påverkar reproduktionen negativt.

I denna avhandling har vi kommit fram till en rad viktiga faktorer som påverkar de mätparametrar som används för att bedöma hankönsreproduktion. När man undersöker hur blandningen av kemikalier i miljön påverkar vilda djur bör man ta hänsyn till vilken reproduktiv säsong djuren fångats i, transporttiden av organen och om de varit frysta innan fixering, annars kan resultaten feltolkas och felaktiga slutsatser dras. Den metod för att bedöma spermieutvecklingen i testikeln hos mink som presenterades visade sig vara användbar och med hjälp av datoriserad bildanalys kan metoden bli mer objektiv och på sikt effektivisera det tidskrävande manuella arbetet med att bedöma skada på testikelvävnad. Vidare bör man utarbeta nya metoder för att undersöka testikelskada under puberteten. Ett steg på vägen kan vara den metod vi presenterar i avhandlingen som beskriver hur långt råttan nått i testikelutvecklingen.

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