Di(2-ethylhexyl) Phthalate and Semen Quality in Boars

Effects of Pre-pubertal Oral Exposure on Sperm Production, Viability and Function Post-puberty

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To my family with love

"Great is the art of beginning, but greater is the art of ending."
Lazurus Long

Said about pigs…

“A cat will look down to a man. A dog will look up to a man. But a pig will look you straight in the eye and see his equal.”
Winston Churchill

“Never try to teach a pig to sing; it wastes your time and it annoys the pig.”
Robert A. Heinlein

“You should never try and teach a pig to read for two reasons. First, it's impossible; and secondly, it annoys the hell out of the pig!”
Will Rogers

“I learned long ago, never to wrestle with a pig, you get dirty; and besides, the pig likes it.”
G.B. Shaw

“I am very proud to be called a pig. It stands for pride, integrity and guts.”
Ronald Reagan

...and about science...

“The most exciting phrase to hear in science, the one that heralds new discoveries, is not Eureka! (I found it!), but rather, "hmm.... that's funny....”
Isaac Asimov

“Believe those who are seeking the truth; doubt those who find it.”
Andre Gide

Abstract

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Di(2-ethylhexyl) phthalate (DEHP), a plasticizer used in polyvinyl chloride (PVC) products, has been reported to have toxic effects on animal reproduction. However, these reports come from studies mostly using rodents as test species and using doses higher than the doses humans are, presumably, exposed to. In the present thesis work young boars were used as model animals to assess the effects of pre-pubertal DEHP exposure on the quality of fresh and cryopreserved spermatozoa post-puberty. Pairs (n=8) of cross-bred male boar siblings were used, with one brother per pair becoming, at random, the test animal while the other was the control. Test males were orally exposed to DEHP (300 mg/kg body weight (bw)) three times per week from 3 to 7 weeks of age, while controls were given placebo. Semen collections and analyses started when the boars were 6 months old and continued until they were 9 months old. Ejaculates collected between 8 and 9 months of age were also cryopreserved for analyses post-thaw. Ejaculates were evaluated for sperm numbers, motility, morphology and plasma membrane integrity (PMI). Post-thaw spermatozoa were assessed for sperm motility, PMI, the ability to capacitate *in vitro* when exposed to the effector bicarbonate, and to acrosome-react when exposed to calcium ionophore (Ca-ionophore), the ability of the nuclear deoxyribonucleic acid (DNA) to sustain denaturation *in vitro* using a sperm chromatin structure assay (SCSA), and the ability of post-thaw spermatozoa to *in vitro* penetrate homologous, *in vitro*-matured (IVM) oocytes. The spermiogram did not significantly differ between exposed and control boars, except for sperm morphology. Boars exposed to DEHP had fewer ($p<0.05$) spermatozoa with tailless, defective heads (at 7–8 months of age) and double-folded tails (at 6–7, 7–8 and 6–9 months) than did controls. Regarding post-thaw spermatozoa, there were no differences in PMI between groups, but the DEHP-exposed boars had significantly fewer linearly motile spermatozoa at 30 ($p<0.05$) and 120 ($p<0.001$) minutes post-thaw, depicting also a larger amplitude of lateral head displacement (LHD) 120 minutes post-thaw ($p<0.05$). Proportions of capacitated post-thaw spermatozoa were similar between groups (control: 3.7%; DEHP-exposed: 4.4%), and exposure to bicarbonate had similar effects on capacitation of stable spermatozoa (control: 24.0%; DEHP-exposed: 22.1%). Live post-thaw spermatozoa from either group were acrosome-reacted *in vitro* to similar rates after exposure to Ca-ionophore (control: 9.3%; DEHP-exposed: 8.9%). Chromatin structure stability was similar between groups, with low proportions of spermatozoa showing induced DNA-denaturation (DNA fragmentation index, DFI; control: 0.15; DEHP-exposed: 0.17). *In vitro* sperm penetration rate did not significantly differ between groups (control: 59%; DEHP-exposed: 50%), nor did the number of spermatozoa in the ooplasm (control: 1.7; DEHP-exposed: 1.5). In summary, the DEHP exposure in the present studies did not cause obvious adverse effects on sperm production or sperm quality in boars. Cryopreservation was only able to disclose minor post-thaw sperm kinematic deviations in DEHP-exposed boars. However, DEHP did not seem to affect the ability of spermatozoa to capacitate or acrosome-react or to damage the nuclear genome, nor did it seem to affect their *in vitro* fertilizing ability.

Key words: spermatozoa, di(2-ethylhexyl) phthalate (DEHP), sperm motility, computer-assisted sperm analysis (CASA), sperm morphology, plasma membrane integrity (PMI), freezing, capacitation, acrosome reaction (AR), sperm chromatin structure assay (SCSA), in vitro penetration assay, boar.

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Papers I–IV

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Spjuth, L., Ljungvall, K., Saravia, F., Lundeheim, N., Magnusson, U., Hultén, F. & Rodríguez-Martínez, H. 2006: Does exposure to di(2-ethylhexyl) phthalate in pre-pubertal boars affect semen quality post-puberty? *International Journal of Andrology* 29: 534–42.

II. Spjuth, L., Saravia, F., Johannisson, A., Lundeheim, N., & Rodríguez-Martínez, H. 2006: Effects of exposure of pre-pubertal boars to di(2-ethylhexyl) phthalate on their frozen-thawed sperm viability post-puberty. *Andrologia* 38:186–94.

III. Spjuth, L., Johannisson, A., Lundeheim, N., & Rodríguez-Martínez, H.: Early pre-pubertal exposure to low-dose oral di(2-ethylhexyl) phthalate does not affect sperm plasma membrane stability, acrosomal integrity or chromatin structure in the post-pubertal boar. (Submitted)

IV. Spjuth, L., Gil, M.A., Caballero, I., Cuello, C., Almiñana C., Martínez, E.A., Lundeheim, N., & Rodríguez-Martínez, H.: Pre-pubertal di(2-ethylhexyl) phthalate (DEHP) exposure of young boars did not affect sperm *in vitro* penetration capacity of homologous oocytes post-puberty. (Submitted)

Abbreviations

AI	artificial insemination
AO	acridine orange
AR	acrosome reaction
AR medium	AR-inducing medium
BSA	bovine serum albumin
BTS(+)®	Beltsville thawing solution
bw	body weight
C medium	capacitation-inducing medium
Ca-ionophore	calcium ionophore
CASA	computer-assisted sperm analysis
COC	cumulus-oocyte complex
COMP	cells outside the main population
DEHP	di(2-ethylhexyl) phthalate
DFI	DNA fragmentation index
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
EDTA	ethylenediamine tetra-acetic acid
FITC	fluorescein isothiocyanate
H33342	Hoechst 33342
HOST	hypo-osmotic swelling test
ICSI	intra-cytoplasmic sperm injection
IVF	*in vitro* fertilization
IVM	*in vitro*-matured
LHD	lateral head displacement
LSM	least-squares mean
M-540	Merocyanine 540
MEHP	mono(ethylhexyl) phthalate
n.s.	not significant
PI	propidium iodide
PMI	plasma membrane integrity
PNA	peanut agglutinin
PSA	*Pisum sativum* agglutinin
PVC	polyvinyl chloride
SCSA	sperm chromatin structure assay
SD/SE	standard deviation/standard error
sHOST	short hypo-osmotic swelling test
SM-CMA	Strömberg-Mika Cell Motion Analyser
ssDNA	single-stranded DNA
TDS	testicular dysgenesis syndrome
TNE	Tris-NaCl-EDTA
Tris	Tris (hydroxymethyl) aminomethane
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight linear velocity

Introduction

For the past 50–60 years there has been an intense debate about whether human male fertility, in terms of semen quality and particularly of sperm counts, has declined over time. The matter has been explored in many studies all over the world (USA: Paulsen, Berman & Wang, 1996; Fisch *et al.*, 1996; Swan, Elkin & Fenster, 1997; 2000; Belgium: Comhaire *et al.*, 1995; Van Waeleghem *et al.*, 1996; Spain: Andolz, Bielsa & Vila, 1999; Scotland: Irvine *et al.*, 1996; Japan: Itoh *et al.*, 2001), but the results are still inconsistent. While some researchers have found a significant decrease in different semen parameters, including sperm concentration and motility (Auger *et al.*, 1995; Irvine *et al.*, 1996), others have not seen this deterioration (Fisch *et al.*, 1996; Paulsen, Berman & Wang, 1996; Vierula *et al.*, 1996). Geographical differences between countries (Jørgensen *et al.*, 2001) and between regions within a country (Fisch *et al.*, 1996) seem to exist, and this could be due to different exposure to environmental chemicals or due to differences in lifestyle (Jørgensen *et al.*, 2001). Possible causes for the potential decline in human sperm quality and, ultimately, in male fertility have been discussed (Harrison, Holmes & Humfrey, 1997; Giwercman & Bonde, 1998). Lifestyle factors, such as alcohol consumption, smoking and a trend towards a more sedentary lifestyle, as well as elevated exposure to environmental chemicals have been proposed as perhaps the most likely causes. Genetic causes have also been mentioned, but are unlikely since the changes have happened relatively fast (for a review, see Sharpe, 2000; Fisher, 2004).

Most studies in humans have been done on retrospective data, making it hard to avoid bias, caused for example by the use of different methodologies in different studies or changes in methods used over time (Fisher, 2004). Controlled longitudinal studies on farm animals are, unfortunately, few despite our ability to use available databases on artificial insemination (AI) sires. Using this possibility, a retrospective study evaluating farm animal data collected between 1932 and 1995 is the most complete thus far. The author concluded that no obvious decrease in sperm counts could be seen (Setchell, 1997), thus opening the question about the degree of exposure to potential environmental chemicals, and their effects, in animals other than laboratory animals and humans.

Some chemicals have the potential to act as endocrine disruptors, meaning they alter hormone action within the body (for a review, see Sharpe & Irvine, 2004). These endocrine disruptors can affect the reproductive health of both genders, and depending on how they act they can exert different effects on females and males (Brevini *et al.*, 2005). So far, most studies on endocrine disruptors have been done on wild animals and laboratory rodents, and little is known about the effects on species such as farm animals. Farm animals can ingest endocrine disruptors through food, water and soil, but it is still unclear whether the levels are high enough to cause the same effects as seen in experimental studies on rodents, or whether the effects are the same in all species. It could well be that while exposure to one endocrine disruptor is not high enough to cause any effects, exposure to a

mixture of chemicals could lead to adverse effects on areas of the body such as the reproductive system (Rhind, 2005).

One of the chemicals that has been found to act as an endocrine disruptor, in laboratory rodents for example, is di(2-ethylhexyl) phthalate (DEHP), a plastic softener used in polyvinyl chloride (PVC) products such as packaging material (*e.g.* plastic bags), indoor construction material and medical devices (*e.g.* blood bags). This phthalate is not chemically bound to the PVC polymer (Latini, 2000; Fromme *et al.*, 2002; Latini, De Felice & Verrotti, 2004), but can leach out from the PVC products into the environment, leading to human, farm animal and wildlife exposure through air (Oie, Hersoug & Madsen, 1997; for a review, see Wensing, Uhde & Salthammer, 2004; Bornehag *et al.*, 2005), food and drinking fluids (Tsumura *et al.*, 2001a; 2001b; Biscardi *et al.*, 2003; Yano *et al.*, 2005) or water (Fromme *et al.*, 2002; Casajuana & Lacorte, 2003). Some groups of people are at risk of higher exposure to DEHP than the general population, for example patients undergoing intensive medical treatments who are exposed to DEHP because of the migration of the chemical from the walls of soft PVC bags into the contents, including blood, glucose or saline solutions (Nassberger, Arbin & Ostelius, 1987; Smistad, Waaler & Roksvaag, 1989; Faouzi *et al*., 1999; Loff *et al*., 2000; Kambia *et al.*, 2001; review by Tickner *et al.*, 2001; Inoue *et al*., 2005; Weuve *et al.*, 2006) as well as to air inhaled through plastic respiratory tubes (Hill, Shaw & Wu, 2003). Also, plastic industry workers are a high-risk group through their occupational exposure (Dirven *et al.*, 1993; Oie, Hersoug & Madsen, 1997).

The degree of exposure for domestic animals is, considering these routes, presumably lower, although it has not been investigated in detail. The multifunctional DEHP has been thoroughly investigated regarding its effects on laboratory rodents and has been shown to cause adverse effects on male reproduction, including semen quality. For instance, in a study performed by Agarwal *et al.* (1986) on adult male rats, dietary DEHP exposure (0–20,000 ppm) led amongst other effects to a dose-dependent reduction in testis and epididymal weight, and a decreased epididymal sperm density and motility. Adverse effects of DEHP exposure in pre-pubertal laboratory animals have been found in several studies (Gray *et al*., 2000; Moore *et al.*, 2001). For example, reduced testis weight relative to body weight (bw) was seen after *in utero* or lactational exposure of DEHP in rats (Arcadi *et al.*, 1998). Li, Jester & Orth (1998) assessed the effects of mono(ethylhexyl) phthalate (MEHP), the primary metabolite of DEHP, and found that it caused adverse effects *in vitro* on cultured Sertoli cells from neonatal rats. Acute effects on Sertoli cells have also been seen after a single, relatively low dose of MEHP in pre-pubertal rats (Dalgaard *et al.*, 2001).

The effects found after phthalate exposure in rodents resemble those seen in testicular dysgenesis syndrome (TDS) in men (Fisher, 2004; for a review, see Skakkebaek, Rajpert-De Meyts & Main, 2001). However, as already mentioned, most studies on the effects of DEHP have so far been carried out in laboratory rodents, leaving it uncertain whether the effects are the same in other species, including humans and domestic animals. It is known that humans are exposed to DEHP in everyday life, but it is not known whether the exposure levels are high

enough to cause the same effects as seen in laboratory rodents, or whether humans are even affected in a similar way. Possible similarities of exposure have not been addressed in farm animals. Species differences in kinetics, for example, could potentially lead to differences in effects as well. Pigs seem to absorb less DEHP after oral exposure compared with rodents (Ljungvall *et al.*, 2004), and the same has been shown for non-human primates, in which experimental exposure to DEHP has not yet been found to have any detrimental effects on male reproduction (Rhodes *et al.*, 1986; Pugh *et al.*, 2000). In comparative studies on rats and marmosets using the same dosage of DEHP/kg bw, inter-species differences in effects were found, with DEHP causing testicular atrophy in rodents, but not in primates. Levels of DEHP metabolites also differed between rats and marmosets 24 hours after exposure in that particular study, with marmosets having lower levels compared with rats, a possible cause for the difference in effects (Rhodes *et al.*, 1986). Similar differences in metabolism have been seen in other studies as well (Kessler *et al*., 2004). In a study by Pugh *et al.* (2000) on young adult cynomolgus monkeys, no effects could be seen on behaviour, organ weights, reproductive hormones or histopathology findings.

Investigations have been done on urine metabolites of DEHP in humans, and metabolites were found in all participating individuals tested (Brock *et al*., 2002; Koch, Drexler & Angerer, 2003; Koch *et al.*, 2003). Moreover, DEHP metabolite levels were higher in children than in adults (Koch, Drexler & Angerer, 2004). Studies investigating levels of phthalate metabolites in relation to semen quality in humans have found correlations for different sperm parameters, including sperm concentration and motility (Duty *et al.*, 2003), but have, to the best of my knowledge, not been able to confirm that DEHP has major adverse effects (Jönsson *et al.*, 2005; Hauser *et al*., 2006; Zhang *et al*., 2006). However, there is a lack of controlled studies in other species with a presumably low exposure risk, such as domestic animals, evaluating in particular the effects of DEHP on semen quality, for instance sperm production, viability and function.

Disruption of spermatogenesis in the post-pubertal male can originate from disturbances in the development of the reproductive system during late embryonic, foetal and/or neonatal life (Norgil Damgaard *et al*., 2002). There are differences in susceptibility to disruptor exposure (such as DEHP) by age, with younger animals apparently being more sensitive than older ones. For example, administration of DEHP to 4-week-old, but not 15-week-old, rats produced effects such as reduction in testis, seminal vesicles and prostate weight (Gray & Gangolli, 1986). Sex hormones are crucial for the normal development of the reproductive organs, and DEHP exposure during foetal and early neonatal life has been shown to disrupt sexual differentiation in male rats by causing a reduction in testosterone levels. This is probably related to the decrease in testicular weight observed in DEHP-exposed animals compared with controls at different stages during exposure (Parks *et al.*, 2000). This DEHP effect on testis weight has also been reported after *in utero* and lactational exposure (Moore *et al*., 2001). Since such effects persist into adult life, this suggests long-term effects of DEHP, which could be monitored by assessment of hormone production or sperm output and quality, and which could, ultimately, lead to a decrease in fertility. However, such effects of DEHP

on fertility have not been recorded when mating adult male rats that had been neonatally exposed to DEHP, with untreated females (Dostal *et al*., 1988). True long-term studies on the effects of different chemicals in rodents are, however, difficult to conduct considering their short life span and, hence, their short pre-pubertal period.

Using animals other than rodents may help in assessing the diversity of effects or sensitivity between species. Farm animals, such as pigs, are already being used for comparative studies in human medicine. Pigs are often used in experimental surgery because their gastro-intestinal anatomy and physiology, for instance, resemble those of humans (Swindle, Smith & Hepburn, 1988; Smith & Swindle, 2006). They also have the advantage of having a longer and more well-defined pre-pubertal period than rodents, making them suitable for studies on long-term effects, for example to investigate the effect of DEHP on testicular development and function and, in particular, its effects on potential target cells such as the Sertoli cells.

Sertoli cells are crucial for spermatogenesis, giving both physical and metabolic support to the germ cells (for a review, see Sharpe *et al*., 2003). There is evidence suggesting that the final number of Sertoli cells present and active during adulthood is determined during the foetal and peri-natal period (Orth, 1982). Since each Sertoli cell only can hold a certain number of germ cells, the number of Sertoli cells is correlated to the number of spermatozoa that an adult is able to produce (Orth, Gunsalus & Lamperti, 1988). In boars, Sertoli cell proliferation has been reported to take place in two distinct phases, the first being from birth until about one month of age, and the second between about 3 and 4 months of age, *i.e.* just before onset of puberty (Franca *et al*., 2000). These authors also found that testicular weight increased in a similar pattern, but with the second phase being registered between 4 and 5 months of age instead. Germ cell numbers increased the most between 4 and 5 months of age, and tended to stabilize when the boars were 7 months old.

Intramuscular, low-dose DEHP exposure in pre-pubertal boars in one study caused elevation of plasma testosterone levels with an increased Leydig cell surface area post-puberty (*i.e.* at 7.5 months of age), suggesting delayed effects of DEHP on the testes of pigs (Ljungvall *et al.*, 2005). However, these effects could not be found when measuring testosterone levels after oral exposure, either immediately after exposure or at 9 months of age, *i.e.* post-puberty. The mating behaviour and the morphology of reproductive organs post-puberty were also evaluated, but no differences could be seen between DEHP-exposed and control boars (Ljungvall *et al.*, 2006). However, even small effects on Sertoli cells, not necessarily affecting their numbers, may have affected their ability to sustain spermatogenesis, leading to less visible long-term effects on sperm numbers, sperm viability and – ultimately – fertility. Spermatozoa may also be affected at a more functional level, and it is possible that DEHP affects the fertility of a male in such a way that he is still able to reproduce, but that the offspring, or its development, is somehow affected (for a review, see Wyrobek, 1993). Therefore, the possible effects of pre-pubertal DEHP exposure on sperm production and

fertility when boars have reached puberty are of high interest. Semen collected from the same boars as in the thesis work by Ljungvall (2006) was the tissue included in the present thesis.

Male genital function can be evaluated either using chemical (*e.g.* seminal plasma compounds) or endocrine (*e.g.* testosterone production by the testis) markers, or based on the ability of the organs to produce large numbers of morphologically and functionally normal spermatozoa, which maintain fertilizing ability even following handling *in vitro* (Rodríguez-Martínez & Larsson, 1998). Evaluation of semen, which is considered an essential part of any andrological screening, has helped researchers and clinicians identify clear-cut cases of infertility, or even of potential sub-fertility in farm animals. Evaluation of a semen sample usually includes recordings of volume, sperm concentration and motility, and, less often, sperm morphology and screening for presence of foreign cells, as well as measurement of general characteristics of the spermatozoa (*e.g.* motility patterns or organelle integrity). All these factors and attributes are essential to fertility if maintained until the spermatozoa are confronted with the oocyte (for a review, see Silva & Gadella 2006; Rodríguez-Martínez, 2007). Morphological deviations are grouped by origin in order to determine underlying testicular or epididymal pathology, or are classified as artefacts caused by mishandling the semen. To the best of my knowledge, the ejaculates of boars exposed to DEHP have not been previously evaluated in terms of either sperm counts or sperm quality, variables essential in determining whether DEHP affects spermatogenesis.

However, there are other, more complicated spermatological methods that are more important when spermatozoa have diminished quality, but do not have clear-cut deficiencies that can be visualized by a simple spermiogram. Among these methods are those which attempt to mimic the interactions between the spermatozoon and the female genital tract *in vitro* and the oocyte vestments and the process of fertilization *in vivo* (Comhaire, 1993; review by Rodríguez-Martínez, 2003; Aitken, 2006; Rodríguez-Martínez & Barth, 2006).

Spermatozoa are labile cells with a terminal specialization, and undergo destabilizing changes during handling. Extension of the sperm suspension, even under controlled forms and in suitable extenders, and cooling or freezing-thawing are processes known to be stressful to spermatozoa and, in the worst case, compromise cell survival and/or function (Watson, 1990). If DEHP only subtly affects boar spermatozoa, it seems logical to stress these cells by controlled handling and evaluate their intactness and readiness to fertilize thereafter. A good alternative is to examine spermatozoa following cryopreservation, *i.e.* post-thaw.

What sperm attributes need to be examined? Motility patterns are relevant for sperm interaction with the female genital tract and the oocyte vestments. Owing to the complexity of sperm motion, computer-assisted sperm analysis (CASA) instruments have been devised and are now widely used. These instruments digitize microscope images of sperm trajectories, providing information on proportions of motile spermatozoa, motility patterns and other kinematic variables. A functionally intact plasma membrane is a prerequisite for sperm life and

function. The plasma membrane maintains a chemical gradient of ions and solutes by its semi-permeable features. It also contains specific structural proteins that act as transporters for water, energy source substrates and signalling receptors – all relevant for sperm metabolism and the sperm's ability to interact with its surroundings. Loss of this functional integrity threatens sperm function and life to various degrees, from decreased fertilizing capacity to cell death. Plasma membrane integrity (PMI) is usually assessed with membrane-impermeable dyes, using the rationale that spermatozoa that can exclude these dyes are alive. Examples of impermeable dyes include eosin and several deoxyribonucleic acid (DNA)-binding fluorescent probes (Rodríguez-Martínez *et al.*, 1997). A similar rationale lies behind the hypo-osmotic swelling test (HOST) (Jeyendran *et al.*, 1984), in which spermatozoa with membranes able to react to a hypo-osmotic environment have a functional membrane. If the plasmalemma is intact, but functionally unstable, the spermatozoon is unable to interact with its environment, and unable to fertilize. Cooling, freezing and re-warming also cause changes in the stability of the plasma membrane of boar spermatozoa (Maxwell & Johnson, 1997) and sperm membrane structure and function can be affected without the membrane being eroded. Subtle changes in the lipid bi-layer destabilize the plasma membrane and compromise its function without immediately causing cell death – however, jeopardizing the fertilizing ability of the spermatozoon (Harrison, 1996). Lipid scrambling, an increased disorder in the lipid bi-layer of the plasma membrane, relates to the earliest stages of sperm capacitation (Harrison & Gadella, 2005) and can be detected using specific markers and flow cytometry (Silva & Gadella, 2006). The acrosome needs to be intact for sperm penetration of the zona pellucida during fertilization. Acrosome integrity can be examined by microscopy or flow cytometry after using fluorescent-conjugated lectins that bind to specific carbohydrate moieties of acrosomal glycoproteins (Gillan, Evans & Maxwell, 2005). The most commonly used lectins are derived from peanuts (*Arachis hypogaea*; peanut agglutinin, PNA), for assessment of the outer acrosomal membrane, or from green peas (*Pisum sativum* agglutinin, PSA), for labelling of acrosomal matrix glycoproteins. Acrosome integrity can also be challenged by exposure to calcium ionophores (Ca-ionophores), to assess how reactive the potentially fertile spermatozoa are (Januskauskas *et al.*, 2000).

Boar spermatozoa have a highly condensed chromatin containing protamines that tightly pack and protect the haploid DNA (Rodríguez-Martínez *et al.*, 1990). Optimal sperm DNA packing appears to be essential for full expression of male fertility potential (Spano *et al.*, 2000), and spermatozoa resulting from defective spermatogenesis usually have damaged nuclear chromatin, in the form of single-stranded DNA (ssDNA) (for a review, see Evenson & Wixon, 2006). The sperm chromatin structure assay (SCSA) has been designed to determine DNA damage (for reviews, see Fraser, 2004; and Evenson & Wixon, 2006) and characterizes sperm nuclear chromatin stability based on the increased susceptibility to *in situ* denaturation of altered DNA when exposed to very low pH. The degree of denaturation within each sperm nucleus is quantified by flow cytometry (Evenson, Darzynkiewicz & Melamed, 1980). Obviously, it is of utmost interest to assess spermatozoa collected from boars exposed to DEHP using the above mentioned

techniques. The effects can be reinforced by stressing the spermatozoa by handling and preservation.

The ultimate test of a spermatozoon, and of how it is affected by DEHP exposure, is to assess its fertilizing capacity. However, despite the availability of AI as a suitable and proven alternative to natural mating, the measurement of fertility using *in vivo* methods requires large numbers of confirmed pregnancies or, even better, offspring, before attempting to establish relationships with fertility (Amann, 2005). Such *in vivo* strategies are constrained by the costs and the time needed to accurately measure fertility through the AI of large numbers (often hundreds) of females. Fertility can also be evaluated *in vitro* using the so-called "oocyte penetration test" (Martínez *et al.*, 1993), in which the presence of spermatozoa or male pronuclei in the ooplasm of homologous oocytes determines the success of the test. Though not as accurate as fertilization *in vivo*, this method has several advantages. The use of offal ovaries from which oocytes can be collected diminishes female fertility variation, making it possible also to repeat the examination of semen samples from the same boar at a low cost. It appears, therefore, to be a suitable alternative for testing spermatozoa from boars exposed to DEHP.

Aims

The general aim of this thesis was to investigate, using the pig as an animal model, the potential effects of oral exposure to a relatively low dose of DEHP in pre-pubertal boars on the quality of their semen post-puberty. Freshly ejaculated and cryopreserved spermatozoa were studied to increase the chances of detecting effects of the phthalate on sperm structure and function.

More specifically, the aims were to determine –

- the quality of the ejaculates of DEHP-exposed and control siblings, in terms of ejaculate volume, sperm counts, sperm motility, sperm morphology and plasma membrane integrity;

- the plasma membrane integrity and sperm kinematics of corresponding spermatozoa post-thaw in relation to DEHP exposure;

- the ability of these post-thaw spermatozoa to undergo capacitation and acrosome reaction (AR) *in vitro*, or show a higher degree of chromatin instability following acid denaturation in relation to DEHP exposure; and

- the potential effects of DEHP on the ability of frozen-thawed spermatozoa to penetrate *in vitro*-matured (IVM) homologous oocytes *in vitro*.

Materials and Methods

Animals

Twenty male piglets (Swedish Yorkshire x Swedish Landrace) from ten different litters were used, a pair of siblings from each litter. The piglets were weaned at 3 weeks post-partum and were, from then until 5 months of age, housed in two communal indoor boxes, one for each group (DEHP-exposed or control) at the Lövsta research station of the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. The boxes had a heating area with straw bedding in one corner. During the first 2 weeks after weaning the pigs were fed a milk substitute (Piggolact; Granngården, Sweden) and thereafter they were fed a commercial pig feed, according to Swedish standards (Simonsson, 1994), and provided with water *ad libitum*. One month before semen collection started, *i.e.* at 5 months of age, the animals were moved to individual indoor pens with straw bedding at the Division of Comparative Reproduction, Obstetrics and Udder Health, SLU, Uppsala, Sweden. All young boars were housed in one stall, without female pigs in the vicinity. The experimental protocol had previously been reviewed and approved by the Ethics Committee for Experimentation with Animals, Uppsala, Sweden.

Experimental design

A split-litter design, with two boar piglets from each of the ten litters, was used in the study. One sibling from each pair at random became the test animal (*i.e.* became exposed to DEHP) while the other acted as control. From 3 weeks of age, *i.e.* at the time of weaning, until 7 weeks of age the animals in the DEHP group were orally exposed, using a blunt syringe, to 300 mg/kg (*i.e.* 0.3 mL/kg) bw of 99.5% pure DEHP (Sigma; Sigma-Aldrich Chemicals, Stockholm, Sweden) three times a week. The control group received the same amount of placebo (water; 0.3 mL/kg bw) and underwent the same handling routines during the same time period as the test group. Two animals were lost between 7 weeks and 6 months of age, one control pig to endocarditis and one DEHP-exposed pig to acute myositis. The corresponding sibling in the other treatment group was therefore excluded from the experiment and the remaining number of animals in the experiment was 16 (eight in each group).

Collection of semen started when the boars were 6 months old and continued until 9 months of age (when they were euthanized). The boars were exposed to a dummy sow in a neighbouring pen and each boar was allowed 5 minutes for mounting attempts on each occasion. If mounting did not occur, the boar was returned to his pen. The ejaculate was collected, using the gloved-hand method, in a plastic bag inside an insulated, pre-heated (37°C) thermos flask covered with gauze to separate particles of the gel fraction. Ejaculates were collected twice weekly during the collection period.

Semen evaluation (paper I)

Semen samples were evaluated for volume, sperm concentration, total sperm count, sperm motility, morphology and PMI. The volume of the ejaculate was measured in a pre-warmed gradient vial. Sperm concentration was determined by counting cells in two separate counting chambers of a Bürker haemocytometer (as described by Bane, 1952) and total sperm count was calculated by multiplying sperm concentration/mL by ejaculate volume. The observer was blinded to all semen evaluations performed in this study.

Sperm motility assessment

Sperm motility was assessed both subjectively (within one hour of collection) using a light microscope equipped with phase contrast optics, and objectively (between 8.5 and 9 months of age) using a CASA instrument, the Strömberg-Mika Cell Motion Analyser (SM-CMA; MTM Medical Technologies, Montreaux, Switzerland). The CASA assessment was done using a microscope (Optiphot-2; Nikon, Japan) equipped with a thermal plate (38°C) and phase contrast optics. For each sample, a minimum of 200 spermatozoa were counted. Total percentage of motile spermatozoa and percentages of linearly motile, locally motile, circularly motile and non-linearly motile spermatozoa were recorded, as were the sperm velocity variables straight linear velocity (VSL; µm/s), average path velocity (VAP; µm/s) and curvilinear velocity (VCL; µm/s); and the amplitude of lateral sperm head displacement (LHD; µm).

Morphological evaluation

Sperm morphology was evaluated once weekly. The frequencies of abnormal acrosomes and mid-pieces, as well as frequencies of coiled tails and proximal and distal cytoplasmic droplets were recorded in wet preparations of semen fixed in buffered formalin (Bane, 1961). Head abnormalities were monitored in air-dried smears stained with carbol-fuchsin (Williams, 1920), and heads that were pear-shaped, narrow at the base, abnormal in contour, tailless, tailless and defective, undeveloped, narrow or variable in size were registered. The number of spermatozoa showing each class of abnormality was expressed as a percentage of the number of cells evaluated. Presence of cells other than spermatozoa was assessed in step-wise thick smears stained with Papanicolau and the results scored along a gradient ranging from 0 (no cells present) to +++ (considerable presence).

Sperm membrane integrity

Between 8 and 9 months of age, PMI of the spermatozoa in the collected, Beltsville thawing solution (BTS(+)®; IMV, L'Aigle, France)-extended semen samples was evaluated using a short HOST (sHOST) as described by Perez-Llano *et al.* (2001). This method measures the ability of an intact plasma membrane to react to extra-cellular media with an osmolality lower than that of spermatozoa. Semen was added to a hypo-osmotic solution consisting of BTS(+)® and distilled water, mixed to obtain an osmolality of 75 mOsm/kg. The samples were incubated at 38ºC for 5 minutes and fixed in 1,000 µL of hypo-osmotic BTS(+)® solution with 5% added formaldehyde. Spermatozoa were counted in a phase contrast

microscope (Laborlux 12; Leitz, Jena, Germany) using the guidelines given by Jeyendran *et al.* (1984). Two to three operators counted each sample and the intra-sample variation was not allowed to be >10%. An average of the counts for each sample was used for statistics.

Handling of semen, including freezing and thawing (papers II–IV)

At between 8 and 9 months of age, ejaculates were frozen in order to be stored until further analysis. The complete ejaculate, except for the gel fraction, was always collected and the semen was extended in pre-heated (38°C) BTS(+)® before cooling, centrifugation and further extension to a final concentration of 2 x 10^9 spz/mL. Spermatozoa were thereafter packaged in 0.5 mL plastic medium straws (Minitüb, Tiefenbach, Germany) and transferred to a chamber of a programmable freezer (Mini Digitcool 1400; IMV, L'Aigle, France). After cooling/freezing, the samples were plunged into liquid nitrogen (LN_2; -196°C) for storage, where they were kept until analysed. For analyses post-thaw, straws were thawed in circulating water at 50°C for 12 seconds.

Assessment of sperm motility and viability (paper II)

Sperm plasma membrane integrity, assessed using the short hypo-osmotic swelling test and flow cytometry after loading spermatozoa with SYBR-14/propidium iodide

The above described sHOST was used to assess PMI in samples prepared at 0, 30 and 120 minutes post-thaw. An average of the counts for each sample was later used for the statistical analyses. Within 30 minutes post-thaw, PMI was also assessed by flow cytometry using the LIVE/DEAD® Sperm Viability Kit L-7011 (Molecular Probes Inc., Eugene, OR, USA) consisting of a combination of the DNA-binding fluorophores SYBR-14 and propidium iodide (PI). After thawing, spermatozoa were re-extended in pre-warmed (38ºC) BTS(+)®. Five µL of SYBR-14 (which is able to penetrate an intact plasmalemma and bind to the DNA) were added to the spermatozoa and samples were incubated at 37°C for 10 minutes. Thereafter, 5 µL of PI (which penetrates eroded plasma membranes) were added and samples were incubated for another 10 minutes (37°C). After incubation, spermatozoa were examined using a laser flow cytometer (Becton Dickinson, San José, CA, USA). Fluorescent data from 100,000 gated events per sample were collected in list mode.

Sperm motility assessment

Computer-assisted sperm analysis as described above was used to assess sperm motility variables. Measurements were done at three time points after thawing, at 0, 30 and 120 minutes. Each sample was analysed three times at each time point and a mean value was later used for statistical calculations.

Assessment of sperm plasma membrane stability, acrosomal status, ability to undergo acrosome reaction by exposure to Ca-ionophore, and chromatin integrity by flow cytometry (paper III)

One straw/collection occasion and boar was thawed and used for each of the flow cytometric analyses. Flow cytometry of post-thaw spermatozoa loaded with the Merocyanine 540 (M-540) stain was used to assess the stability of the sperm plasma membrane and its ability to destabilize under capacitation conditions, as described by Januskauskas *et al.* (2005). After thawing and re-extension in pre-heated (38°C) BTS(+)®, each sample was split in two. One of the splits was immediately re-suspended in BTS(+)® supplemented with Yo-PRO-1 (Y 3603; Molecular Probes Inc., Eugene, OR, USA), M-540 (M 24571; Molecular Probes), and Hoechst 33342 (H33342; Molecular Probes) working solutions, and then incubated at 38°C for 10 minutes prior to analysis with a flow cytometer to examine the degree of lipid membrane stability. The other split sample was re-suspended in a capacitation-inducing medium (C medium) consisting of BTS(+)®, calcium chloride (2382; Merck; Merck, Darmstadt, Germany), sodium bicarbonate ($NaHCO_3$; S-6014; Sigma), caffeine (C-0775; Sigma) and bovine serum albumin (BSA; A4378; Sigma), and incubated for 30 minutes in a humidified incubator at 38°C and 5% CO_2 with air, before staining as above. Measurements were carried out on a laser flow cytometer (Becton Dickinson, San José, CA, USA). Non-sperm events were gated out based on H33342 fluorescence (DNA content). Three sperm populations were differentiated: viable with low M-540 fluorescence; viable with high M-540 fluorescence; and dead cells (stained with Yo-PRO-1).

Acrosomal intactness was assessed using flow cytometry and the acrosome-specific fluorochrome fluorescein isothiocyanate-labelled PNA (PNA-FITC) (Sigma Chemical Co., St. Louis, MO, USA). After re-extension in pre-heated (38°C) BTS(+)® each sample was split in two. One of the splits was further extended in BTS(+)® and then immediately stained with PI, PNA-FITC and H33342 and incubated for 10 minutes in the dark at 38°C before it was analysed by flow cytometry as below. The other split sample was re-suspended in AR-inducing medium (AR medium) consisting of BTS(+)®, calcium chloride and Ca-ionophore A23187 (C7522; Sigma). Thereafter it was stained, incubated and analysed using a FACStar^{PLUS} flow cytometer (Becton Dickinson Immunocytometry Systems, San José, CA, USA) equipped with standard optical equipment. Viable (PI-negative) spermatozoa with an intact outer acrosome membrane were PNA-FITC-positive, while PNA-FITC-negative spermatozoa were considered to be acrosome-reacted. The spontaneous reaction rates (control base values), the induced reaction rates (Ca-ionophore-challenged) and the difference between the two, *i.e.* the proportion of spermatozoa in the population capable of acrosome-reacting in the presence of Ca-ionophore (AR index), were evaluated.

Chromatin integrity was assessed using the SCSA, and abnormal chromatin structure was defined as the increased susceptibility of some sperm nuclear DNA

to undergo acid-induced denaturation *in situ*. The degree of chromatin stability following exposure of the prepared DNA to acridine orange (AO) was quantified by flow cytometric measurement. The metachromatic shift from green (stable, double-stranded DNA (dsDNA)) to red (denatured, ssDNA) AO fluorescence was evaluated and expressed as the function alpha t (α_t), which is the ratio of red to the total (*i.e.* red and green) fluorescence intensity, thus representing the amount of denatured ssDNA relative to the total cellular DNA. Alpha t was calculated for each spermatozoon in a sample and the results were expressed as the mean (x α_t, recently renamed "*x*-DNA fragmentation index" (*x*-DFI)), the standard deviation (SD) of the α_t distribution (SD α_t; now called "SD-DFI") and the percentage of cells with high α_t values (*i.e.* "% cells outside the main population" (% COMP α_t), now termed "DFI"), representing the cells with an excess of ssDNA. In the following we use the new nomenclature. The procedure used was originally developed by Evenson, Darzynkiewicz & Melamed (1980), and further described by Evenson & Jost (2000) and Januskauskas, Johannisson & Rodríguez-Martínez (2001). The thawed spermatozoa were step-wise re-extended with temperated TNE buffer (consisting of NaCl (S-5886; Sigma), Tris (hydroxymethyl) aminomethane (Tris)-HCl (15566-100; Merck) and ethylenediamine tetra-acetic acid (EDTA) (E-6758; Sigma), pH 7.4, 38°C) to a final sperm concentration of approximately 2 x 10^6 spz/mL, and immediately frozen in LN_2 vapour before transfer to a -70°C freezer for storage until flow cytometry analysis.

Samples were thawed on crushed ice and a 0.2 mL aliquot was subjected to partial DNA denaturation *in situ* by mixing with 0.4 mL of a low-pH detergent solution (0.17% Triton X-100 (X-100; Sigma), 0.15 M NaCl and 0.08 N HCl (30317-25; Kebo; Kebo, Stockholm, Sweden)), pH 1.4, followed 30 seconds later by staining with AO (1333; Merck). The samples were analysed by flow cytometry within 3–5 minutes of staining, using a FACStar^{PLUS} flow cytometer (Becton Dickinson Immunochemistry Systems, San José, CA, USA) equipped with standard optics. Acridine orange intercalated to dsDNA fluoresces green (530 ± 30 nm), while AO associated with ssDNA fluoresces red (~630 nm). A total of 10,000 events were measured for each sample and the results for each of the spermatozoa measured in the sample were analysed using FCS Express Ver 2 (De Novo Software, Thornhill, Ontario, Canada) to provide values of DFI, *x*-DFI and SD-DFI.

Assessment of the ability of spermatozoa to penetrate *in vitro*-matured homologous oocytes (paper IV)

The ability of spermatozoa to penetrate, *in vitro*, homologous IVM-oocytes was assessed, as described by Gil *et al.* (2005). Cumulus-oocyte complexes (COCs) were matured *in vitro* and prepared according to the protocol before semen was added. Semen straws were thawed, washed and re-suspended in *in vitro* fertilization (IVF) medium before being added to the medium that contained the oocytes. Each oocyte was exposed to 4,000 spermatozoa and after 6 hours of co-incubation with the spermatozoa, oocytes were transferred and cultured at 39ºC in 5% CO_2 in air for 10–12 hours. The oocytes were mounted on slides, fixed and then stained and examined under a phase contrast microscope. The fertilization

variables evaluated were sperm penetration rates (% of the number of oocytes penetrated/total number inseminated) and the number of spermatozoa/oocytes (mean number of spermatozoa in penetrated oocytes) per frozen-thawed sperm sample. At least 50 oocytes per sample were evaluated. Semen from an extra boar routinely used for IVF in the laboratory was included in each IVF session as a positive control.

Statistical analyses

The Statistical Analysis System (SAS) program software version 8 (SAS Institute, Cary, NC, USA) was used to handle and analyse data (**papers I-IV**). Traits were analysed by analysis of variance (ANOVA) using the PROC MIXED procedure. Results are presented as least-squares means (LSMs) and standard deviations (SDs, by treatment group; **papers I-III**) or standard errors (SEs; AR analyses, **paper III**, and IVF analyses, **paper IV**), or as medians and quartiles (q1 and q3) for motility variables assessed by CASA (**papers I-II**). In **paper I**, the statistical model for analysing sperm output, subjectively assessed motility and morphology included the fixed effects of treatment group (two groups), age and the interaction between group and age. In **paper I** regarding CASA-assessed motility and PMI (using the sHOST), and **papers II-IV**, the statistical model included only the fixed effect of treatment group. In all statistical models, also the random effect of boar nested within group was included.

In **paper I**, the two groups (control vs. DEHP) were compared both per month (6–7, 7–8 and 8–9 months) and for the entire collection period (6–9 months), except with regard to sperm PMI (sHOST) and motility (CASA), for which the analyses were done only for recordings made between 8 and 9 (sHOST) and 8.5 and 9 months (CASA). In **papers II-IV**, evaluations were done only between 8 and 9 months. In **paper IV**, the potential effect of "laboratory" where IVF analyses were done was evaluated, but, since the results did not differ between them, all data were analysed together.

For all studies, pair-wise differences between LSMs were considered statistically significant when $p<0.05$.

Results

Semen from 14 of the 16 boars was successfully collected over the entire collection period. All eight boars in the DEHP-exposed group and six boars out of eight in the control group could be routinely collected (not significant (n.s.)). Age at first successful collection of the entire ejaculate ranged from 6 to 9 months in the DEHP group and from 6 to 8.5 months in the control group (mean 6.5 months in both groups; n.s.). Average numbers of successful collections/boar during the collection period were 6.5 and 7.5 for the DEHP-exposed and control groups, respectively (n.s.).

Spermiogram of collected ejaculates (paper I)

There were no significant differences between the groups (*i.e.* control v. DEHP-exposed) regarding semen volume (6–7 months: 107 mL v. 145 mL; 7–8 months: 132 mL v. 139 mL; 8–9 months: 123 mL v. 136 mL), sperm concentration (6–7 months: 312 v. 265; 7–8 months: 368 v. 387; 8–9 months: 460 v. 430 x 10^6 spz/mL), total sperm count per ejaculate (6–7 months: 32 v. 40; 7–8 months: 45 v. 49; 8–9 months: 53 v. 53 x 10^9 spermatozoa) or subjectively assessed motility (6–7 months: 80% v. 70%; 7–8 months: 80% v. 80%; 8–9 months: 90% v. 90%). The large individual variation between boars for these analysed variables may have prevented statistical differences between groups. No differences in sperm motility between the semen from either group were detected using CASA.

When sperm morphology and presence of foreign cells were evaluated in the collected ejaculates, the mean proportions of total abnormalities were low for both control and DEHP-exposed animals (6–7 months: 21% v. 23%; 7–8 months: 17% v. 12%; 8–9 months: 13% v. 9%), values which were expected for boars of these ages. There were only minor, albeit significant, differences between groups in the percentage of tailless, defective sperm heads (at 7–8 months of age; $p<0.05$) and double-folded tails (at 6–7, 7–8 and 6–9 months of age; $p<0.05$). Interestingly, it was the DEHP-exposed group that had the lowest percentage of spermatozoa with the above morphological abnormalities. There were no obvious differences between groups with regard to presence of cells other than spermatozoa in the ejaculates.

The mean (range within brackets) percentages of freshly ejaculated spermatozoa responsive to the sHOST, *i.e.* with an intact plasmalemma, ranged widely in both groups, averaging 46.7% (range 22.5–58.5%) in the control group and 48.1% (range 13.5–84%) in the DEHP-exposed group (n.s.).

Viability of frozen-thawed spermatozoa (paper II)

Freezing and thawing clearly diminished the total proportion of spermatozoa depicting motility, which gave a median of 28% in controls and 31% in DEHP-exposed boars directly after thawing, 28% in controls and 33% in the DEHP group 30 minutes post-thaw and 22% in controls and 22% in the DEHP group 120 minutes post-thaw. Most kinematic variables recorded were similar (n.s.) between

control and DEHP-exposed boars, exceptions being a significantly lower percentage of linearly motile spermatozoa in the DEHP group compared with controls at 30 (control group: 19.3%; DEHP group: 11.3%; $p<0.05$) and 120 minutes (control group: 23.1%; DEHP group: 10.0%; $p<0.001$) post-thaw, and a larger amplitude of LHD in the DEHP group compared with the control group at 120 minutes after thawing (control group: 4.1 μm; DEHP group: 5.0 μm ; $p<0.05$).

The PMI was clearly lower in frozen-thawed spermatozoa than in freshly ejaculated spermatozoa. The mean proportion of viable spermatozoa, *i.e.* with an intact sperm plasma membrane as determined by the sHOST, directly after thawing (*i.e.* at 0 minutes) was 23.6% for the control group and 21.1% for the DEHP group. After 30 minutes of incubation the control and DEHP group had 25.2% and 22.4% viable spermatozoa, respectively, and after 120 minutes the percentage of viable spermatozoa was 25.0% in controls v. 24.9% in the DEHP group. The PMI assessed by SYBR-14/PI and flow cytometry approximately 30 minutes post-thaw yielded means of 53.1% of spermatozoa from the control group while 52.6% of the DEHP group had intact plasma membranes. Although there were clear differences between the two methods used (sHOST and SYBR-14/PI), the second being more discriminative, there were no significant differences between the groups in the proportions of spermatozoa with an intact plasma membrane assessed by either method at any of the measuring times.

Capacitation status and ability of frozen-thawed spermatozoa to undergo capacitation after *in vitro* exposure to bicarbonate (paper III)

The proportions of spermatozoa with a viable and stable plasma membrane (*i.e.* non-capacitated spermatozoa with low M-540 fluorescence) was 28% in controls and 25% in the DEHP group (LSMs) (n.s.), while the number of capacitated-like spermatozoa (*i.e.* viable spermatozoa with high M-540 fluorescence) was very low, 3.7% in the control group and 4.4% in the DEHP group (LSMs), without significant differences between groups. Nor did the groups differ significantly with regard to the proportions of dead spermatozoa. When spermatozoa were challenged by bicarbonate, the proportion of uncapacitated spermatozoa decreased by 24.2% (LSMs) in controls and by 22.7% in the DEHP group. Regarding capacitated-like spermatozoa, corresponding figures were +20.3% among controls and +17.7% in the DEHP group. No significant difference between groups could be found for any of these variables.

Ability of frozen-thawed spermatozoa to undergo acrosome reaction after *in vitro* exposure to the calcium ionophore A23187 (paper III)

Frozen-thawed spermatozoa were evaluated for presence of AR before and after exposure to Ca-ionophore using PNA-FITC, PI, and H33342 with flow cytometry. Most live spermatozoa (48% in the control group and 41% in the DEHP group) were acrosome-intact, but exposure to Ca-ionophore *in vitro* induced a more than 20-fold increase in AR in both groups. Live post-thaw spermatozoa from either

group were acrosome-reacted *in vitro* to similar rates after exposure to Ca-ionophore (control: 9.3%; DEHP-exposed: 8.9%). There were, however, no significant differences between groups in the proportions of live, non-acrosome-reacted and acrosome-reacted spermatozoa, or of dead, either non-acrosome-reacted or acrosome-reacted spermatozoa when tested prior to (0 sample) or after exposure to Ca-ionophore (AR sample).

Chromatin structure in frozen-thawed spermatozoa (paper III)

The DFI values were low (0.15 in controls and 0.17 in the DEHP-exposed group) and there were no significant differences for DFI, *x*-DFI or SD-DFI between the control and treatment groups, indicating that most boar spermatozoa had a normal chromatin structure and sustained the acid denaturation applied *in situ* well.

Sperm ability to *in vitro* penetrate *in vitro*-matured homologous oocytes (paper IV)

Both the rate of sperm penetration and the number of spermatozoa per oocyte were considered to be within acceptable limits and within the expected ranges for frozen-thawed boar semen of good quality. The penetration rate was at or above 50% (control: 59%; DEHP: 50%) and did not significantly differ between the groups, possibly owing to a large variation between boars and replicates. The number of spermatozoa in the ooplasm was low and similar (n.s.) between groups (control: 1.7; DEHP-exposed: 1.5; $p>0.05$). There were no significant differences in the results obtained when the penetration assay was performed in the participating IVF laboratories.

General discussion

This thesis aimed to evaluate the potential effects of early, pre-pubertal oral exposure to DEHP in boars on their semen quality later in life, post-puberty. The study was motivated by the lack of controlled studies on species with a presumably low exposure risk. One such species is the boar, a domestic animal whose anatomical and physiological features are convenient for comparative studies, particularly longitudinal comparative studies, for instance when evaluating long-term effects of DEHP exposure on semen quality, including sperm production, viability and function.

Di(2-ethylhexyl) phthalate is frequently used as a plastic softener in PVC plastic products and can easily leach out into the environment (Latini, 2000; Fromme *et al.*, 2002; Latini, De Felice & Verrotti, 2004), leading to potential exposure of wildlife, domestic animals and humans (Brock *et al.*, 2002; Koch, Drexler & Angerer, 2003; 2004; Koch *et al.*, 2003). Studies performed mainly on laboratory rodents have shown that DEHP can adversely affect reproduction, including semen quality (Gray *et al.*, 2000; Moore *et al*., 2001). Pre-pubertal exposure to DEHP has been shown to cause effects such as reduced testis weight (Arcadi *et al.*, 1998), and MEHP, the primary metabolite of DEHP, has also been assessed and shown to damage *in vitro* cultured Sertoli cells from neonatal rats (Li, Jester & Orth, 1998). Since rodents are physiologically different from humans, extrapolations may be unreliable and it is still uncertain whether DEHP affects non-rodent animals, including humans, in the same way.

The pig is in many respects similar to humans, particularly regarding anatomy and physiology, and was therefore chosen as animal model for the present study. Moreover, pigs have the advantage of having a longer and easy distinguishable pre-pubertal period compared with animals with a shorter lifespan, which makes them well suited for long-term studies evaluating chronic effects of a compound. Also, they give rise to large litters, making it possible to minimize genetic variation. Their anatomy and physiology are well known and they also have similarities with humans in terms of adult organ size, blood volume, an omnivorous diet *etc*. Although pigs have been used as models in other areas, for example, surgical experiments (Swindle, Smith & Hepburn, 1988; Smith & Swindle, 2006), their use in toxicological studies is still limited. Pigs (Ljungvall *et al.*, 2004) and non-human primates do not seem to absorb as much DEHP after oral exposure as rodents do, and studies on non-human primates (Rhodes *et al.*, 1986; Pugh *et al.*, 2000; Kessler *et al.*, 2004) do not report the effects seen in rodents after DEHP exposure.

To ensure that there was a clear difference in exposure between test and control animals a dose of 300 mg/kg bw of DEHP was chosen. Exposure was intended to begin before spermatogenesis started in order to establish whether this chemical would affect Sertoli cells, gonocytes or Leydig cells to such an extent that this would be later reflected in disturbances in spermatogenesis, epididymal function or accessory sex glands. As such, this would also be reflected in the ejaculate,

either as effects on semen volume, or in the sperm counts, sperm morphology or sperm viability (as sperm motility or PMI) (**paper I**). The experimental rationale was based on previous results of an accompanying thesis within the same research project (Ljungvall, 2006), where low-dose DEHP exposure in pre-pubertal boars resulted in affected Leydig cells and increased plasma testosterone levels post-puberty (at 7.5 months of age) (Ljungvall *et al.*, 2005).

Moreover, spermatozoa were subjected to stress by handling, cooling and freezing-thawing and were studied for other variables post-thaw, such as motility patterns, plasma membrane and acrosome integrity, and stability of the lipid bi-layer (capacitation status) and of the chromatin (DNA). Some of these variables were further challenged *in vitro*, for example by exposure to capacitation effectors or Ca-ionophore to test whether the cells could undergo capacitation or AR, respectively, both prerequisites for fertilization. Finally, spermatozoa from both groups (DEHP-exposed and control) were tested for their ability to penetrate IVM homologous oocytes, and confirm their capacity to fertilize *in vitro* (**papers II–IV**).

The exposure dose used in this work was set at a relatively realistic level compared with what some groups of people, who are at higher risk of exposure than the general population, are exposed to. For example, people undergoing intensive medical treatments are exposed to DEHP through the chemical's migration into blood, glucose and saline solutions from soft PVC storage bags (Smistad, Waaler & Roksvaag, 1989; Loff *et al*., 2000; review by Tickner *et al.*, 2001; Inoue *et al.*, 2005; Weuve *et al.*, 2006). Exposure levels in these persons can be considerably higher than in the general population (review by Tickner *et al.*, 2001), and while exposure levels of DEHP in the general population have been estimated to be up to 0.27 mg/day in the United States, neonates undergoing intensive medical care, such as exchange blood transfusions, have been found to be exposed to up to 22.6 mg/kg bw per day (review by Tickner *et al.*, 2001; FDA report, 2001). However, it is not clear whether it is the exposure dose or the moment when this exposure appears that may cause deleterious effects. Most likely, there is a need for a concerted action of these variables to cause damage.

The question of whether male reproductive abnormalities and infertility problems may have their origin in foetal life has been raised during the past years (Skakkebaek, Rajpert-De Meyts & Main, 2001; Skakkebaek, 2002; 2003; Sharpe & Skakkebaek, 2003). It is possible that a chemical can give rise to adverse effects without giving any clinical symptoms in childhood, but leading to impaired fertility in adulthood (Norgil Damgaard *et al.*, 2002). Studies have shown, for instance, that the reproductive system is extra sensitive to disturbances during its development and that a disruption in the proliferation of Sertoli cells during this period can cause disturbances in spermatogenesis later in life (Li, Jester & Orth, 1998; Li *et al*., 2000). Exposure to DEHP causes more damage in younger individuals than in older ones (for a study on rodents, see Gray & Gangolli, 1986), including reduction in testis weight, disruption of sexual differentiation, and hormonal imbalance (Parks *et al*., 2000; Moore *et al*., 2001). Most of these disruptions persist into adult life, with low sperm output, presence of sperm

aberrations, hormonal imbalance that could cause malfunction of accessory sex glands or behavioural problems, and – above all – diminished sperm fertilizing ability. Obviously, investigations of DEHP effects require long-term studies of the type devised in the present thesis.

As mentioned above, in a previous study intramuscular low-dose DEHP administration in pre-pubertal boars increased the surface area of Leydig cells and elevated plasma testosterone levels, suggesting delayed effects of DEHP on the reproductive system (Ljungvall *et al*., 2005). However, this could not be found in a follow-up study in the orally exposed boars used in this thesis when measuring testosterone levels either immediately after exposure or at 9 months of age, *i.e.* post-puberty. Neither could any effects be found on mating behaviour post-puberty or on testes weight or number of Sertoli cells at 9 months. The weight of the bulbo-urethral glands at 9 months of age was, however, significantly higher in the DEHP group compared with controls (Ljungvall, 2006).

In this thesis, semen variables in boars that had been pre-pubertally exposed to DEHP were analysed using a wide range of methods, from relatively rough and subjective methods to methods that are mainly objective and able to reveal more subtle effects on spermatozoa. Sperm production, motility and viability were analysed in fresh semen. Hardly any semen variable deviated from what would be considered normal in a young boar and, interestingly, DEHP did not seem to adversely affect the spermiogram of the boars. The increased bulbo-urethral gland weight found in the DEHP-exposed boars (Ljungvall, 2006) would, in theory, have led to an increased semen volume in the treatment group, but no such effect was seen in these boars (**paper I**). There were some minor differences in the proportions of spermatozoa with abnormal morphology, in which the DEHP-exposed animals were actually superior. However, values for both groups were within normal limits and the differences disappeared by the time the boars were >8 months old. It was hypothesized that DEHP exposure could have damaged either the gonocytes or the Sertoli cells of the developing testes during pre-puberty. If this had been confirmed, sperm output would have been lower than normal and there would have been a higher proportion of morphologically abnormal spermatozoa. As indicated by the results obtained, the hypothesis proved to be false, since no significant deviations from control values were found.

Cooling and freezing-thawing are stressful to spermatozoa and may, in the worst case, cause irreversible damage to their plasma membrane, leading to cell death or functional defects in a large number of spermatozoa (Holt, 2000). Freezing and thawing of spermatozoa leads to an influx of ions (especially calcium), protein aggregations and a disorder in the lipid components of the plasma membrane, causing effects resembling those seen during sperm capacitation (Watson, 2000). Cooling also leads to fusion events between the plasma membrane and the underlying acrosomal membrane, which resemble the AR (Ortman & Rodríguez-Martínez, 1994). One of the hypotheses tested in the present thesis was that DEHP exposure may cause changes in spermatogenesis without leading to cell death or other major abnormalities, *i.e.* causing changes that are not detectable by conventional sperm analyses routinely used to assess

boar semen (as subjective motility assessment). These changes may be of a more subtle nature and cause effects that do not lead to cell death, but impair functionality of the spermatozoa. Specific motility patterns or the stability of the sperm plasma membrane or even nuclear DNA integrity may be modified in spermatozoa produced by boars exposed to DEHP. However, such changes may not be detectable unless spermatozoa are further challenged by external stimuli, either when stress is imposed on them (through processes such as cryopreservation) or when the cells are exposed to specific effectors (bicarbonate, Ca-ionophores, an acidic environment, *etc.*). Such exposures have to be clearly controlled, especially considering the effects of cryopreservation, since different individuals usually present variations in their capacity to sustain freezability of their semen. The use of siblings in this study would have minimized potential individual differences. Moreover, the semen from both groups of sibling boars (DEHP-exposed and control) was handled in the same way using the same freezing and thawing protocols, and therefore differences solely due to freezability should have been minimal. Consequently, any detected differences between groups should primarily have been caused by exposure to DEHP.

In frozen-thawed spermatozoa significant differences ($p<0.05$) in motility patterns were seen in DEHP-exposed animals compared with controls, with a lower percentage of linear sperm motility in the DEHP group, a difference that increased with time post-thaw (**paper II**). Linear motility has been found to be correlated with fertility in several species (Zhang *et al.*, 1998; 1999; Al-Qarawi *et al.*, 2002), which is logical since only progressively motile spermatozoa can migrate through the cervical mucus and penetrate the oocytes. Lateral head displacement correlates with fertility in species such as humans (Hirano *et al.*, 2001) and the dromedary (Al-Qarawi *et al.*, 2002). In the present studies, a difference between the groups was seen in LHD at 120 minutes after thawing ($p<0.05$), with higher values in the DEHP group (**paper II**). However, whether these differences would have any impact on the sperm fertilizing ability *in vitro* or *in vivo* is yet to be established.

More functional aspects of the spermatozoa, such as the ability to undergo capacitation and AR as well as the chromatin structure of the spermatozoa, all required for a spermatozoon to be able to successfully fertilize an oocyte, were also assessed after freezing-thawing, after loading spermatozoa with specific probes, using flow cytometry (**paper III**). The capacitation process starts when the spermatozoa are separated from the seminal plasma and continue on their way through the female reproductive tract. This process leads to changes in the sperm plasma membrane and to a new motility pattern, hyperactivation, which is thought to facilitate the sperm's way to the oocyte (for a review, see Aitken, 2006). Before the spermatozoon is able to penetrate the oocyte, the AR, in which acrosomal enzymes are released, has to take place. The spermatozoon binds to the zona pellucida and the release of acrosomal enzymes enables it to penetrate the oocyte, and the male-derived nuclear DNA is incorporated into the oocyte (for a review, see Ohl & Menge, 1996; Silva & Gadella, 2006). Today, more and more couples use IVF techniques to achieve fertility, with an increasing use of intra-cytoplasmic sperm injection (ICSI), where a spermatozoon is chosen based mostly on its

morphology and motility, and in some cases not even these attributes. More functional aspects are usually not considered, which means that if the spermatozoon has damaged DNA, for example, it may still be used, and if the damage is not large enough to cause infertility it may still be able to fertilize the oocyte, but may give rise to developmental effects on the foetus and may cause negative effects on the offspring (Evenson & Jost, 2000).

Even small effects on Sertoli cells, not necessarily affecting their numbers, may have affected the ability to sustain spermatogenesis, leading to less visible, long-term effects on sperm viability and function and, ultimately, fertility. Such assumption was part of the hypothesis tested in the present thesis. However, pre-pubertal exposure to DEHP did not adversely affect the semen quality of post-pubertal boars, in terms of sperm motility in fresh ejaculated semen (**paper I**), PMI and stability (**papers I-III**), acrosome integrity or DNA structure (**paper III**). Even following a stressful procedure such as cryopreservation, no adverse effects were found, with the exception of minor post-thaw sperm kinematic deviations in DEHP-exposed boars (**paper II**). Moreover, DEHP did not seem to affect the sperm ability to capacitate or acrosome-react *in vitro* (**paper III**).

The overall results of the present thesis thus show that the boars from both groups were normal in the sense that they had a normal semen picture and acceptable sperm production, and that their spermatozoa were able to undergo capacitation and AR when exposed to stimuli, and had very low levels of abnormal DNA structure (**papers I–III**). The final test for the spermatozoa of either control or DEHP-exposed boars was the *in vitro* penetration assay, in which the ability of sperm to penetrate IVM homologous oocytes was assessed. The use of IVF to assess fertility has the advantage over *in vivo* methods, *i.e.* natural mating or AI, in that the confounding "female factor" is greatly diminished. Also, IVF is easier and faster to execute and makes it possible, furthermore, to assess a larger number of samples per boar within a shorter time interval. The results of IVF analyses have been found to significantly correlate with *in vivo* fertility (Gadea, Matás & Lucas, 1998), and studies have also shown that boars that are good freezers, *i.e.* that have good motility and sperm viability post-thaw, among other variables, also have good IVF results, and vice versa (Gil *et al.*, 2005). Since no adverse effects of DEHP exposure were found when analysing sperm production, motility, viability or function, apart from the few changes that could be seen in the motility pattern after freezing-thawing, this indicates that the spermatozoa should be able to successfully fertilize oocytes (**papers I–III**). This was also shown in **paper IV** where no significant differences in either sperm *in vitro* penetration rate or number of sperm per oocyte could be found. Evidently, although the kinematic changes registered by CASA could potentially have been due to the DEHP exposure, the lack of effects in all remaining semen attributes evaluated, including *in vitro* oocyte penetration, raises doubts regarding the influence they would have on the *in vivo* fertility of these boars, if tested.

There may be several explanations for the lack of adverse effects of pre-pubertal DEHP exposure in the semen of boars, when evaluated post-puberty, seen in this thesis work. Firstly, it may be that the DEHP dose used was not effective in

inducing changes in the testes and/or the endocrine system controlling them, or in the accessory sex glands. Hence, no effects on sperm quality would be seen. However, the dose used is considered to be relatively realistic when comparing it with levels that children undergoing medical treatments are exposed to, for example (for a review, see Tickner *et al.*, 2001). Alternatively, pigs may be more resistant to DEHP compared with other species, or to absorb less DEHP after oral exposure compared with rodents, for instance (Ljungvall *et al.*, 2004). Such possibilities need further comparative kinetic studies in order to be proven.

Secondly, it is possible that the exposure period was not right to induce any detrimental effects on the developing reproductive organs. The administration of DEHP took place between 3 and 7 weeks of age and it is possible that this exposure period was too late to affect the first Sertoli cell proliferation (first month post-partum), and too early to affect the second (3–4 months post-partum) (Franca *et al*., 2000). However, an earlier exposure was not performed in the present thesis since it would have clashed with the weaning of the piglets. Weaning had to take place before exposure began in order to apply the split-litter design and diminish individual variation. A later exposure was not chosen because the period between exposure and semen sampling would have been too short to evaluate long-term effects of DEHP exposure.

Thirdly, it is possible that the number of animals used was not large enough to reveal any significant differences between groups. The cost for animals and management is higher when working with domestic animals and makes it impossible to use as many animals as when performing laboratory rodent studies. However, the present studies used a split-litter design to minimize genetic variation and consequently allowed for a smaller number of experimental animals.

Fourthly, it may also be that the diagnostic methods used were not able to reveal potential adverse effects. The methods applied ranged from relatively rough and subjective (conventional spermiogram) to more objective techniques, of the kind that enable disclosure of subtle, often functional defects in spermatozoa. Sperm motility was assessed in both fresh and frozen-thawed spermatozoa using both subjective, ocular screening and the more objective CASA instrumentation. Similarly, sperm PMI, an indicator of sperm viability, was analysed both by sHOST, and by use of specific fluorophores (SYBR-14/PI) and flow cytometry analysis. In this manner, detection of subtle membrane damage could be compared between methods. Use of the fluorophores SYBR-14/PI was evidently superior to the sHOST in detecting spermatozoa with an intact plasma membrane. Flow cytometry, which allows analysis of a large number of cells in a short time and detection of changes in fluorescence intensity undetectable by microscopy (Gillan, Evans & Maxwell, 2005), was therefore used to screen substantial numbers of spermatozoa for sperm capacitation, acrosome intactness and chromatin structure. These variables are all prerequisites for fertilization and embryo development, and analysis of relevant numbers of cells was therefore essential. Moreover, the ability of intact, alive spermatozoa to undergo capacitation or AR was tested using specific effectors, in order to confirm that these cells were equipped with these essential attributes.

Knowing the variation present among IVF laboratories, the sperm penetration test in the present thesis was repeated at two different laboratories. Since the results did not vary between them it was assumed that the methodology used was repeatable and the results were considered reliable enough to indicate that the DEHP exposure pre-puberty, as carried out in the present thesis work, did not affect the *in vitro* fertilizing capacity of the spermatozoa produced by these boars later in life, not even following cryopreservation. However, *in vitro* methodology can always be biased by laboratory practices, results of sperm evaluation can be blurred by the heterogeneity of the sperm suspension under scrutiny, and even fertility results can be masked by insemination using excessive sperm numbers (Rodríguez-Martínez, 2003). Therefore, further studies would have been desirable to test the *in vivo* fertility of these boars, using either natural mating or AI with fresh semen.

Conclusions

- Spermiograms (including ejaculate volume, sperm counts, sperm motility, and sperm morphology) or PMI did not significantly differ between DEHP-exposed and control boars, except for some minor differences in sperm morphology. Boars exposed to DEHP had fewer ($p<0.05$) spermatozoa with tailless, defective heads at 7–8 months of age and double-folded tails at 6–7, 7–8 and 6–9 months than did controls.

- In post-thaw spermatozoa, there were no differences in PMI between groups, but the DEHP-exposed boars had significantly fewer linearly motile spermatozoa at 30 and 120 minutes, and a larger amplitude of LHD at 120 minutes, post-thaw compared with their control siblings.

- Proportions of non-capacitated and capacitated spermatozoa post-thaw were similar between groups. Capacitation could be *in vitro*-induced by bicarbonate, but at similar rates in both groups. There were no differences in proportions of live, acrosome-reacted spermatozoa, and live spermatozoa from both groups could be induced to acrosome-react *in vitro* to similar rates by exposure to Ca-ionophore. The chromatin structure stability was similar between groups, with low proportions of spermatozoa showing DNA-induced denaturation.

- The *in vitro* sperm penetration rate did not differ significantly between the groups, nor did the number of spermatozoa in the ooplasm.

In summary, under the conditions of the present experimental design, pre-pubertal exposure to relatively low and realistic levels of DEHP *per os* did not seem to adversely affect the semen quality of post-pubertal boars. Adding a stressful procedure such as cryopreservation prior to analysis of sperm attributes essential for fertilization only disclosed minor post-thaw sperm kinematic deviations in DEHP-exposed boars. However, DEHP did not seem to damage the sperm nuclear genome or to affect the ability of the spermatozoa to capacitate, acrosome-react, or fertilize *in vitro*.

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Populärvetenskaplig sammanfattning

Under de senaste 50-60 åren har det pågått en debatt om huruvida manlig fertilitet, med betoning på spermiekvalitet, har försämrats över tiden. Flera forskargrupper världen över har undersökt detta genom att analysera data från olika databaser exempelvis från fertilitetskliniker och spermabanker. Det är dock svårt att jämföra äldre data med nyare, p.g.a. att det har skett ändringar i analysmetoder *etc*. Vissa studier har kunnat visa att det har skett en försämring av spermiekvaliteten, medan andra inte har sett denna förändring. Man har också sett att det finns skillnader i spermiekvalitet mellan olika länder, samt mellan olika områden inom samma land. Detta kan bero på t.ex. skillnader i livsstil samt olika stor exponering för miljökemikalier i olika delar av världen. Om det är så att det har skett en försämring av spermiekvaliteten skulle detta t.ex. kunna vara orsakat av att vi har gått till en mer stillasittande livsstil, ökad alkoholkonsumtion, högre andel rökare samt att det finns en större mängd kemikalier i miljön.

Vissa kemikalier kan verka hormonstörande och på så sätt påverka kroppen, t.ex. reproduktionssystemet. En av dessa kemikalier är di(2-ethylhexyl) phthalate (DEHP), som används bl.a. som plastmjukgörare i olika PVC-plastprodukter. Man kan hitta DEHP i plastpåsar och andra mjuka plastförpackningar, medicinsk utrustning som blodpåsar samt i en del byggmaterial. Kemikalien är inte kemiskt bunden till plasten utan kan läcka ut i omgivande miljö, vilket leder till att människor utsätts för den genom mat, dryck, luft och vatten samt genom viss medicinsk behandling. Studier har visat att i princip alla människor utsätts för DEHP i varierande grad och att vissa grupper av människor exponeras för större doser än andra, t.ex. genom intensiv medicinsk behandling som blodtransfusioner. Även plastindustriarbetare är en högriskgrupp.

I studier gjorda på framför allt råttor och möss har man kunnat påvisa negativa effekter av DEHP på reproduktionssystemet hos hanar. Eftersom det finns stora artskillnader mellan råttor och människor är det svårt att säga om effekterna är de samma hos människor. I den här studien använde vi oss av grisen som modelldjur då den har flera fördelar jämfört med råttor, bl.a. genom att den liknar oss människor rent anatomiskt och fysiologiskt, samt att den har en längre livslängd vilket gör att man kan använda den för att studera långtidseffekter av olika ämnen.

I denna studie användes 8 brödrapar galtar. Den ena brodern i varje par exponerades för DEHP i en relativt låg och realistisk dos vid upprepade tillfällen (3 ggr/vecka) mellan 3 och 7 veckors ålder, medan den andra fick placebo (vatten). Spermiekvaliteten undersöktes sedan mellan 6 och 9 månaders ålder. Detta upplägg gjordes för att undersöka om exponering för DEHP tidigt i livet, före puberteten, hade någon påverkan senare i livet, när djuren hade uppnått puberteten. Olika spermieparametrar undersöktes, bl.a. spermieproduktionen (t.ex. spermavolym och antal spermier/mL sperma), motiliteten (hur stor andel spermier som rör sig och om rörelsemönstret är normalt), morfologin (hur spermierna ser ut och om deras utseende avviker från det normala), viabiliteten (hur stor andel av spermierna som lever och har ett funktionellt spermiemembran) samt flera

funktionella parametrar som är nödvändiga för att spermien ska kunna befrukta ägget. Även spermiernas förmåga att befrukta ägget *in vitro* undersöktes.

Resultaten visade att DEHP inte har någon större påverkan på spermiekvaliteten hos galtar som exponerats på detta sätt. Små skillnader i rörelsemönster, som i teorin skulle kunna påverka spermiernas befruktningsförmåga, kunde ses och då var det de DEHP-exponerade grisarna som hade sämst resultat. Även små skillnader i morfologin kunde ses, men här var det istället kontrollgruppen som hade sämst resultat. Eftersom inga skillnader kunde ses i de andra, mer funktionella testerna, inklusive spermiernas förmåga att befrukta ett ägg *in vitro*, så är det inte troligt att dessa små förändringar skulle ha någon påverkan om man skulle använda dessa galtars sperma för att t.ex. inseminera suggor. Detta skulle vara mycket intressant att undersöka, men skulle kräva inseminering av ett stort antal suggor och vara mycket kostsamt och tidskrävande.