Selection in sperm and its consequences

Exploring haploid selection, ageing and epigenetic effects in sperm

COSIMA HERMANS NÉE HOTZY
Sexually reproducing eukaryotes are typically going through a biphasic life cycle with a diploid and a haploid phase. Unlike in plants where selection on haploid pollen genotypes is well established, the possibility of selection occurring in animal sperm is currently not known. One of the main reasons for this lack of knowledge is the general assumption that due to the shortness and the apparent absence of gene expression in haploid sperm, selection during that phase is unlikely to occur. The aim of this thesis was to fill this gap and address some of the main fundamental questions. I investigated the interaction between sperm phenotype and offspring phenotype with a focus on the trans-generational effects of (i) selection on the haploid sperm genotype, (ii) sperm ageing and (iii) sperm-mediated epigenetic effects. For one, we performed several experimental studies to investigate how selection on the sperm phenotype affects offspring performance in two externally fertilizing fishes, Atlantic salmon and zebrafish. We found that in Atlantic salmon, sperm of intermediate post-activation longevity sire offspring that hatch earlier. In zebrafish, longer living sperm sire more viable offspring with a higher fitness than their short-lived sibling sperm. We explored the mechanisms of these trans-generational effects and found that neither intrinsic post-ejaculation sperm ageing (Atlantic salmon and zebrafish) nor pre-ejaculation sperm ageing (zebrafish) affect offspring performance. However, we identified genetic differences between sperm pools that were obtained by selecting different phenotypes within ejaculates of zebrafish males. These results suggest a genetic basis for intra-ejaculate sperm phenotype variation and show that there is potential for haploid selection in sperm. In a separate experiment, we explored the role of sexual selection in shaping sperm-mediated epigenetic effects, and found that short-time changes in male-male competition affect offspring hatching time and survival. In conclusion, this thesis provides evidence that sperm phenotype affects offspring phenotype, and that sperm phenotype is affected by both epigenetic changes influenced by the male environment and differences in the haploid genome of sperm.

Keywords: sperm, evolution, selection, haploid selection, epigenetics, sperm-mediated effects, trans-generational effects, sperm ageing, sperm senescence

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ISSN 1651-6214
ISBN 978-91-554-9918-1
urn:nbn:se:uu:diva-320437 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-320437)
Für Claudia & Peter
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


* These authors contributed equally to the study.

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<td>deoxyribonucleic acid</td>
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1 Introduction

1.1 Biphasic life cycle
Sexual eukaryotic organisms are characterized by a biphasic life cycle that switches between a haploid and a diploid phase in each generation (Hofmeister, 1851; Strasburger, 1894). There are three types of biphasic life cycles that can be distinguished by the relative length of the two phases, and the intensity of growth and development during each phase. Animals and vascular plants have a diplontic life cycle with a dominant diploid phase where multicellular growth and somatic development occurs only during the diploid phase while the haploid phase is reduced to the gametic stage and only concerns the single-celled gametes (Mable and Otto, 1998). At the other end of the spectrum lies the haplontic life cycle found in green algae, brown algae, and yeast that is characterized by a very short diploid phase during the zygote stage and a dominant haploid phase (Mable and Otto, 1998). In the haploid-diploid life cycle of mosses, ferns, some fungi, red algae, most brown algae and many green algae, both phases experience extensive growth and development and produce distinct multicellular organisms (Richerd et al., 1993).

Due to the predominantly diploid life cycle of animals, most research has focused on the diploid organism. Most selection studies for example focus on the diploid stage or on the phenotypic variance in the diploid organism, while selection at the haploid stage has mostly been neglected (Joseph and Kirkpatrick, 2004). The primary reasons for this neglect are that the haploid phase of animals concerns the germ cells only during their short haploid phase, and that the haploid gametes are believed to be transcriptionally silent (Joseph and Kirkpatrick, 2004). Without haploid gene expression, phenotypic variation does not represent haploid genetic variation and so selection has no variation to act upon. Thus, many researchers believe that in animals, the opportunity for selection during the haploid phase is minimal. However, some evidence exists that this is not always the case and that selection at the gametic level may be important, as I will discuss in section 1.4. The relatively late discovery of spermatozoa and especially their function – as described in the next section – may also have contributed to the focus on the diploid organism in animals. During my PhD studies, I have instead focused on the haploid phase of the animal life cycle and selection that occurs during that stage.
1.2 History of sperm biology research

Although already the ancient Greeks realized that both males and females are involved in reproduction and that the semen (i.e. the ejaculate) of the male plays a role in that process, the mysteries of reproduction were far from answered and the existence of spermatozoa was not even known before 1677 (Birkhead et al., 2009; Cobb, 2012). The discovery of the spermatozoa by Antonie van Leeuwenhoek in 1677 was based on the fact that the microscopes he built were so superior to the other microscopes at that time that he could discover hundreds of new objects among which were the spermatozoa (Birkhead et al., 2009; Leeuwenhoek, 1678). He named them “animalculis” (Leeuwenhoek, 1678) and believed that they were the source of live (Leeuwenhoek, 1702). Many of his colleagues though did not believe that the animalculis were involved in reproduction. They argued that because spermatozoa were found in such vast amounts in the semen, they were more likely to be parasites, since they found it hard to believe that so many animalculis should be needed to fertilize only one or a few eggs (Cobb, 2006; Moore, 1986). The role of spermatozoa in fertilization was thus not understood at that time and it took many more decades until the diffuse picture of the function of sperm in reproduction became clearer.

The question of whether sperm were involved in fertilizing eggs was not resolved until 1824, when it became evident that the sperm and not the seminal fluid are required for fertilization (Prevost and Dumas, 1824; as cited in Moore, 1986; see also Wagner, 1837). Shortly after that, in 1842, rabbit sperm were observed inside eggs for the first time (Barry, 1843) and in 1847, three studies documented the interaction between sperm and ova during fertilization in the sea urchin (Derbès, 1847; Dufossé, 1847; von Baër, 1847; reviewed in Birkhead et al., 2009). But it took until the 1870s for Oscar Hertwig and Herman Fol to demonstrate that fertilization is based on the fusion of the nuclei from the sperm and the ovum (Fol, 1879; Hertwig, 1875).

Another essential discovery for our understanding of sperm biology and reproduction (and for our understanding of biology in general) was the discovery of deoxyribonucleic acid (DNA) by Friedrich Miescher in 1869 (Dahm, 2005, 2008; Miescher, 1871). He was the first to isolate DNA and called it “nuclein” because of its location in the cell nucleus (Miescher, 1871). Miescher investigated the existence of nuclein in different cell types and realized that sperm cells were especially useful for investigating nuclein because he could isolate purer and larger quantities of nuclein from them than from other cells (Dahm, 2005). He mostly studied nuclein in salmon sperm but also in the sperm of various other species (Miescher, 1874). Miescher was the first to propose that nuclein could be the substance responsible for the heritability of traits (Miescher, 1874). But at the same time he believed that other substances must be involved in heritability too, since he
could not imagine that a single substance could be diverse enough to carry the information needed for the diversity of traits found within and across species (Miescher, 1874). His publication in 1874 about nuclein in sperm of various vertebrates gained some attention, but neither Miescher nor the research community grasped the importance of his discoveries at the time (Dahm, 2005; Miescher, 1874). Even long after Miescher’s death, DNA received little attention and it was widely believed that proteins contained the information needed for the heritability of traits (Dahm, 2005).

This changed in 1944 when Avery and his colleagues proofed that the DNA is the source of heritability and carries the genetic information (Avery et al., 1944). Due to this finding, spermatozoa were then understood as DNA transmitters who deliver the second half of the genetic information to the zygote and this is – no doubt – one of their most important functions in fertilization. But the research community reduced the function of sperm to this role (Marshall, 2015; Miller et al., 2005). This means that until the early 1990s, sperm were believed to deliver nothing else (at least nothing of importance) to the eggs but paternal DNA.

In the early 1990s, it became clear that sperm actually have functions beyond the transmission of DNA. It was shown that sperm transfer ribonucleic acid (RNA) (Pessot et al., 1989), proteins (Wilson et al., 2006), epigenetic marks (Puri et al., 2010) and their centrioles (Sathananthan et al., 1996; Simerly et al., 1995) to the zygote and that those agents affect fertilization (Jodar et al., 2015), offspring development (Liu et al., 2012; Sone et al., 2005) and offspring phenotype (Chen et al., 2016b; Gapp et al., 2014). Over time our picture of the contributions of sperm to fertilization and the zygote has become clearer and more detailed – but still many pieces of the puzzle are missing and we are far from fully understanding the processes. For instance – and this is just one of the many questions that are left – it is still unclear what role the phenotype of individual sperm plays. Does sperm phenotype reflect sperm genotype and does it affect the diploid offspring phenotype? To what extent does the haploid set of genes of the sperm affect sperm phenotype? This leads me to the topics I was studying during my PhD studies, during which I have investigated whether and how sperm phenotypes shape offspring phenotypes and how artificial selection of sperm affects offspring fitness. In particular, I was interested in three processes that are related to these trans-generational effects of sperm phenotype: (i) the role of haploid selection in sperm, (ii) the role of sperm-mediated epigenetic effects, and (iii) the effects of sperm ageing on offspring fitness.

### 1.3 Selection in sperm

Gametes are under strong selection, which manifests itself particularly in the unique phenotypic diversity found in sperm (Snook, 2005). In fact, sperm
are one of the most diverse cell types in the animal kingdom, which is astonishing given the fact that this cell type has essentially the same function across all animals (Afzelius, 1975; Baccetti and Afzelius, 1976; Birkhead et al., 2009; Jamieson, 1987). Sperm vary greatly in their size (e.g. flagellum length, mid-piece length, head size etc.), their shape (e.g. head shape, mid-piece shape etc.), their motility (ranging from very motile to immotile), their survival time after ejaculation (ranging from a few seconds to decades) and essentially in every possible morphological trait across species (Afzelius, 1975; Baccetti and Afzelius, 1976; Birkhead et al., 2009; Jamieson, 1987). Nematode sperm for example are amoeboid and have no tail (Ward and Carrel, 1979), while each sperm of the Australian termite has about 100 separate tails (Baccetti and Dallai, 1978)! The two externally fertilizing fish I used during my PhD studies, the Atlantic salmon (papers I, II) and the zebrafish (papers III, IV), on the other hand, are very similar to the sperm stereotype that everyone is familiar with and have a classic morphology with a round head, a small midpiece adjacent to the head and a flagellum. Most interestingly, the diversity of sperm phenotypes does not end at the species level. Sperm vary also greatly within species, both across males (Álvarez et al., 2003; Bauer and Breed, 2006; Birkhead et al., 2005; Chawanji et al., 2006; Morrow and Gage, 2001; Oppliger et al., 1998; Simmons et al., 1999; Ward, 1998; Ward and Hauschteck-Jugen, 1993) and even within males (Álvarez et al., 2003; Chawanji et al., 2006; Fitzpatrick et al., 2010; Söderquist et al., 1996; Swallow and Wilkinson, 2002). Moreover, sperm of the same male do not only differ across ejaculates (Cornwallis and Birkhead, 2007; Söderquist et al., 1996), but also within a single ejaculate (Chawanji et al., 2006; Fitzpatrick et al., 2010; Holt and Look, 2004; Immler et al., 2008; Swallow and Wilkinson, 2002). My PhD research focused on both intra-ejaculate variation of sperm (papers I, II, III) and sperm variation across males of the same species (paper IV).

Why are sperm so diverse? The proximate sources of sperm variation are genetic differences, epigenetic differences and sloppy spermatogenesis. Variation in sperm phenotype is thus based on differences of the diploid genomes across individuals, on differential gene expression across and within males, and on production errors during spermatogenesis. The haploid genome of the sperm might also contribute to sperm variation within ejaculates but the relative importance of haploid gene expression is still under debate. There is some evidence for post-meiotic gene expression (Barreau et al., 2008; Erickson, 1990; Schultz et al., 2003; Vibranovski et al., 2010) and haploid selection (Hiraizumi and Nakazima, 1967; Joseph and Kirkpatrick, 2004; Lyon, 1984; Silver, 1993) in the sperm of mice and D. melanogaster, but to date only few studies have investigated haploid selection in sperm and most of them have focused on segregation distorters (Hiraizumi and Nakazima, 1967; Lyon, 1984; Lyttle, 1991; Silver, 1993). In contrast, the importance of diploid gene expression and the paternal genome for sperm
phenotype variation is well established. Production errors during spermatogenesis that are caused by sloppy spermatogenesis may also contribute to the variation among sperm (Cohen, 1969, 1973; Holt and Look, 2004). However, lax spermatogenesis may only account for random sperm variation within the same male. Furthermore, the variation it causes is not heritable and thus not subject to selection. This contrasts with the sperm variation that is based on genetic and epigenetic variation, which is inherited and thus may affect offspring fitness and the variation of sperm found in the subsequent generations.

While genetics, epigenetics and production errors during spermatogenesis explain sperm variation at the proximate level, they cannot explain the ultimate causes for sperm diversity. The ultimate causes for sperm cell variation are post-copulatory sexual selection, and natural selection. I briefly discuss these selection pressures in the following paragraphs.

**Post-copulatory sexual selection**
The main force driving the remarkable diversification in sperm phenotypes is thought to be post-copulatory sexual selection (Sivinski, 1980; Snook, 2005; Werner and Simmons, 2008), which comes in two forms, sperm competition (Parker, 1970, 1982) and cryptic female choice (Eberhard, 1996).

Sperm competition takes place when sperm of several males directly compete for the fertilization of the ova of one female (Parker, 1970, 1982). Sperm competition occurs in most sexually reproducing species since polyandry is extremely widespread (Birkhead and Möller, 1998; Pizzari and Wedell, 2013; Taylor et al., 2014). Thus, in most species, a male that produces sperm of a superior phenotype, which out-competes the sperm of its rivals, will have a greater fitness and its alleles will increase in frequency until another male produces sperm of an even more superior phenotype. This race between sperm leads to the rapid evolution that is typical for sperm and ejaculate traits (Birkhead et al., 2009; Eady, 2001; Manier et al., 2013; Sivinski, 1980; Swanson and Vacquier, 1998; Vacquier et al., 1997; Wyckoff et al., 2000). While there is a large body of research on sperm competition between males (e.g. Birkhead et al., 1999; Byrne et al., 2003; Chapman et al., 2000; Eady, 1994; Gage, 1991; Gage et al., 2004; Hotzy and Arnvqvist, 2009; Manier et al., 2010; Price et al., 1999; Radwan and Witaliński, 1991; Scaggiante et al., 1999), little is known about competition between sperm cells within a single ejaculate from one male. Such sib-sperm competition (or intra-male sperm competition) is not expected, as sperm phenotype is assumed to be under diploid control by the male and the sperm phenotype of an individual sperm within an ejaculate therefore should not reflect its haploid genetic/epigenetic content (Eddy, 2002; Higgginson and Pitnick, 2011; Immler, 2008). However, theoretical work by Haig and Bergstrom (1995) predicts that genetic or epigenetic differences among sperm of the same ejaculate can lead to sperm competition between the different sperm pheno-
types and result in differences in offspring fitness. During my PhD studies, I tested whether there is opportunity for sib-sperm competition by selecting for sperm phenotypes within ejaculates and measuring the effects of artificial intra-ejaculate selection on offspring fitness.

In many species, sperm do not only directly compete against the sperm of other males but also have to be chosen by the female to win the fertilization race (Birkhead et al., 1993; Córdoba-Aguilar, 2006; Eberhard, 1996; Hellriegel and Bernasconi, 2000; Higginson et al., 2012; Rosengrave et al., 2008; Welke and Schneider, 2009). Females may increase their fitness by choosing sperm of superior males for fertilization (Eberhard, 1996) or by resisting sperm with certain phenotypes (Arnqvist and Rowe, 2005). Female preference for long sperm for instance, has led to extraordinarily long sperm in some Drosophila species (Lüpold et al., 2016; Miller and Pitnick, 2002a). This process is called cryptic female choice because it occurs hidden within the female after copulation, in contrast to female mate choice that occurs before copulation (Eberhard, 1996). Females have evolved various mechanisms to choose among the sperm of different males (Carré and Sardet, 1984; Córdoba-Aguilar, 2006; Edvardsson and Göran, 2000; Hellriegel and Bernasconi, 2000) and this in turn shapes sperm evolution (Higginson et al., 2012; Lüpold et al., 2016; Miller and Pitnick, 2002b; Presgraves et al., 1999; Rosengrave et al., 2008).

Natural selection
Recent research on selection in sperm focuses on sexual selection, while natural selection is mainly mentioned parenthetically (Byrne et al., 2003; Pitnick et al., 1999) and receives very little attention (Fitzpatrick et al., 2012; Reinhardt et al., 2015). Nevertheless, natural selection is an important selection pressure acting on sperm that shapes sperm phenotype by selecting for primary sperm function and sperm survival via the environmental conditions sperm experience during their “lifetime” (Fitzpatrick et al., 2012; Franzén, 1956, 1977a, 1977b). The fertilization environments and thus the corresponding selection pressures acting on sperm differ greatly across species and between internal and external fertilizers in particular. The sperm of external fertilizers – such as many fish and marine invertebrates – have to adapt to the fresh or salt water conditions they encounter after their release from the male (Alavi and Cosson, 2005, 2006; Albright and Mason, 2013; Billard, 1986; Cosson et al., 2010; Morisawa et al., 1983; Purchase et al., 2010; Purchase and Moreau, 2012; Wang et al., 2011). Likewise, the sperm of internal fertilizers, such as birds, insects and mammals, have to adapt to the environment they encounter inside the female reproductive tract and female sperm storage organs (Birkhead et al., 1993; Reinhardt et al., 2015; Werner and Simmons, 2008), which can for instance involve facing obstacles such as female immune responses (Austin, 1957; Bedford, 1965; Parish et al., 1967; Witkin and David, 1988). Sperm must also adapt to the often
highly specific processes involved during fertilization. In some broadcasting externally fertilizing species for instance, sperm have to travel long distances to find an egg and fertilization can take place hours or even days after sperm release (Kupriyanova, 2006; Powell et al., 2001; Williams and Bentley, 2002), thus prolonged sperm longevity becomes a crucial fitness factor. In other species – such as the Atlantic salmon – rapid sperm velocity is more important as fertilization takes place within seconds after sperm release (Cosson et al., 2008; Yeates, 2005).

In the following three sections I provide the background for the three core themes that I investigated during my PhD studies: the role of haploid selection in sperm, sperm-mediated epigenetic effects and the consequences of sperm ageing on offspring phenotype.

1.4 Haploid selection in sperm

Haploid selection is defined as selection that acts on a single set of genes/chromosomes (Joseph and Kirkpatrick, 2004). This contrasts with diploid selection, which acts on the two copies of a diploid set of genes/chromosomes. Haploid selection occurs for instance in the sex chromosomes of the heterogametic sex where only one copy of each sex chromosome is available for selection (Joseph and Kirkpatrick, 2004). Another example of haploid selection in animals is genomic imprinting, where only the paternal or the maternal allele is expressed and hence under selection (Reik and Walter, 2001). Selection on a haploid genome is much more direct as recessive alleles cannot hide, and hence any allele expressed in a haploid stage will be under selection. Such a difference in response to selection may have substantial consequences for major evolutionary processes such as the rate of adaptation (Orr and Otto, 1994), mutation load (Charlesworth and Charlesworth, 1992) and inbreeding depression (Charlesworth and Charlesworth, 1987). For example, Orr and Otto (1994) showed in a theoretical study that at low recombination rates, selection during the haploid phase of the life cycle can speed up the adaptation rate of recessive or partially dominant mutations because under these circumstances even brief haploid selection is more efficient in removing deleterious alleles and preserving beneficial alleles than diploid selection.

Haploid selection in sperm refers to selection that acts on the haploid genome of sperm during the haploid phase of the life cycle. To date, haploid selection in sperm is a controversial topic that has received relatively little attention (Joseph and Kirkpatrick, 2004). A common objection is that haploid selection in sperm is improbable because it leads to a conflict between a male and its sperm due to their diverging evolutionary interests (Haig and Bergstrom, 1995; Immler, 2008; Parker and Begon, 1993; Reiss, 1987). While a male normally shares exactly 50% of its genome with all of its gam-
etes, the relatedness among its sperm varies hugely due to meiosis. Thus, for the male it does in most cases not matter which of its sperm fertilizes an egg, while this is different for the sperm. Each individual sperm benefits most evolutionarily by being the one who fertilizes the egg – although sperm may also gain indirect fitness benefits if fertilization is achieved by a sperm with whom they share many alleles (Hamilton, 1964). This conflict may lead to male suppression of haploid gene expression (Hosken and Hodgson, 2014; Immler, 2008).

The magnitude of this conflict is thought to depend on the intensity of sperm competition among males (Parker and Begon, 1993). Under monogamy, the extent of such a male-sperm conflict over haploid selection depends on the outcome of the competition among sibling sperm. A male can gain from haploid selection if this leads to increased fertilization by sperm that generate offspring with higher fitness. But if sperm producing offspring with lower fitness outcompete the sperm that would produce offspring of higher fitness, it should be in the interest of the male to suppress haploid selection. Under intense inter-male sperm competition, the main goal for both the males and their sperm becomes to outcompete the sperm of the other males and thus competition among sibling sperm – and thereby also haploid selection – will become less important (Parker and Begon, 1993). Therefore, there should be less potential for haploid selection under intense inter-male sperm competition.

In line with the assumption that sperm phenotype is controlled by the diploid male, post-meiotic sperm are assumed to be transcriptionally silent due to their highly condensed DNA (Steger, 1999). However, research in mice and fruit flies has shown that some genes are actually expressed in post-meiotic sperm, despite their densely packed DNA (Barreau et al., 2008; Schultz et al., 2003; Vibranovski et al., 2010). Yet, sperm have also been shown to share gene products over cytoplasmic bridges (Braun et al., 1989; Caldwell and Handel, 1991), and thus can become phenotypically diploid even if haploid gene expression takes place. A more recent study, however, suggests that haploid selection might be possible nevertheless by demonstrating in mice that not all post-meiotic gene products are shared between the spermatids (Zheng et al., 2001). Such evidence suggests that haploid selection is theoretically possible, but we have currently no understanding of the importance of haploid selection in animals.

In papers I, and III, we investigated the potential for haploid selection in sperm of Atlantic salmon and zebrafish. We performed intra-ejaculate sperm selection experiments to test whether and how intra-ejaculate sperm variation affects offspring fitness and explored the mechanisms of these trans-generational effects.
1.5 Sperm-mediated epigenetic effects

Besides their genome, sperm transmit epigenetic information from the father to the offspring by passing on RNAs (Kawano et al., 2012; Sone et al., 2005) and the structure modifications of their chromatin (Hammoud et al., 2009; van der Heijden et al., 2006, 2008). The definition of epigenetics is highly debated (Bird, 2007; Haig, 2004; Ptashne, 2007). Throughout this thesis, I refer to epigenetics as the mechanisms that lead to mitotically and/or meiotically heritable phenotypic variation due to differential gene regulation instead of genomic differences (Russo et al., 1996). Epigenetic mechanisms play an important role in development (Li et al., 1992; Okano et al., 1999; Reinhart et al., 2000) and cell differentiation (Conaco et al., 2006; Lagos-Quintana et al., 2002; Lee et al., 2004) and they are crucial for the incorporation of environmental signals (Breton et al., 2009; Gage, 1991; Kucharski et al., 2008). The epigenetic states of an individual depend thus partly on its environment and can lead to environment-specific inheritance (Chen et al., 2016a; Gapp et al., 2014; Heijmans et al., 2008; Wei et al., 2014).

In paper IV, we studied such sperm-mediated environment-specific inheritance in zebrafish. We investigated how short-term manipulation of sperm competition intensity (as part of the social environment) affects the sperm and the offspring of the males. Both sperm chromatin modifications and/or sperm RNA content may lead to environment-specific paternal inheritance and I briefly introduce them below.

Chromatin modifications

DNA and histone modifications play an important role in gene regulation by changing the accessibility of DNA regions to transcription (Rothbart and Strahl, 2014; Vaissière et al., 2008). Some chromatin modifications are maintained in the germline and are transmitted to the offspring, which leads to inheritance of those modifications and their regulatory attributes (Jiang et al., 2013; Potok et al., 2013; Szfy, 2015). Among all DNA modifications, DNA methylation has been studied the most. DNA methylation leads to transcriptional repression, both directly by blocking the binding of transcription factors to promoters that are methylated (Bell and Felsenfeld, 2000; Holmgren et al., 2001; Szabó et al., 2000) and indirectly by recruiting proteins that suppress transcription (Hendrich and Bird, 1998; Nan et al., 1993, 1997; Prokhortchouk et al., 2001). The knowledge about the inheritance of DNA methylation is limited. In mammals, it has been shown that the DNA is almost entirely demethylated after fertilization and only few modifications are preserved in the offspring (Blewitt et al., 2006; Daxinger and Whitelaw, 2012; DeChiara et al., 1991; Monk et al., 1987). In zebrafish on the other hand, the DNA methylome is largely paternally inherited (Jiang et al., 2013; Potok et al., 2013). At the DNA packaging level, post-translational histone modifications regulate gene expression by generating less or more tightly
packaged DNA regions (Rothbart and Strahl, 2014). During sperm development of many species, these histones are replaced to varying degrees by protamines that allow very tight DNA packaging (Calvin, 1976; Carrell, 2012; Lewis et al., 2003). However, some histones are retained and those may play an important role in sperm-mediated epigenetic inheritance and development (Brykczyńska et al., 2010a; Daxinger and Whitelaw, 2012; Hammoud et al., 2009). Moreover, post-translational modifications of protamines have been demonstrated and these could function in gene regulation and epigenetic inheritance in sperm (Brunner et al., 2014). In zebrafish, histones are not replaced by protamines during sperm development (Wu et al., 2011), which could indicate that sperm chromatin structure modifications might play a particularly important role in paternal epigenetic inheritance in this species.

Regulatory RNAs
Sperm RNA was first discovered in the late 1980s (Pessot et al., 1989) and we now know that sperm contain and pass along a substantial variety of RNAs from the father to the zygote. Some sperm RNAs have been shown to affect fertilization (Bourc’his and Voinnet, 2010; Jodar et al., 2015) and embryo development (Kawano et al., 2012; Liu et al., 2012; Sone et al., 2005). Moreover, sperm RNAs play an important role in environment-specific inheritance or in the transmission of paternally acquired traits to the offspring (Brennecke et al., 2005; Chen et al., 2016a, 2016b; Gapp et al., 2014; Villota-Salazar et al., 2016). A substantial amount of micro RNAs (miRNAs) are found in sperm and there is evidence that they are involved in embryo development and environment-specific inheritance (Chen et al., 2016b; Gapp et al., 2014; Liu et al., 2012). In mice, one specific sperm-born miRNA (microRNA-34c) is necessary for the first cell division of the zygote (Liu et al., 2012). tRNA fragments (tsRNAs), another type of regulatory RNA, are also very abundant in sperm and have been shown to transmit paternally acquired traits to the offspring (Chen et al., 2016b, 2016a; Sharma et al., 2016). For example, zygotic injection of sperm tsRNA from male mice fed with a high-fat diet triggered metabolic disorders in the offspring (Chen et al., 2016a).

1.6 Sperm ageing
A process that may affect the genome and epigenome of sperm by introducing new mutations and altering epigenetic profiles is sperm ageing. Sperm ageing is the deterioration of sperm cells or their precursors over time. Fertilization by aged sperm has been shown to negatively affect offspring performance and survival (Han, 2014; Reinhardt et al., 2005; Rochebrochard and Thonneau, 2002; Wagner et al., 2004; White et al., 2008). Ageing pro-
cesses that affect sperm are found both pre-meiosis and post-meiosis (as reviewed by Pizzari et al., 2008). Pre-meiotic sperm ageing is related to the age of males. It is caused by the accumulation of mutations in the germline and thereby increases the probability of older males to sire offspring that carry genetic disorders (Lewis and Aitken, 2005; Rochebrochard and Thonneau, 2002; Velando et al., 2011; Zhu et al., 2005). Older males often produce sperm of lower quality than younger males (Ford et al., 2000; Kidd et al., 2001; Kühnert and Nieschlag, 2004; Lewis and Aitken, 2005). In contrast to pre-meiotic sperm ageing, post-meiotic sperm ageing occurs during the “lifetime” of individual sperm and affects both young and old males, although it can impact the sperm of older males more strongly (Zubkova and Robaire, 2006). Post-meiotic sperm ageing takes place both during storage of the sperm in the male before the sperm are released and after ejaculation – either within the female in internal fertilizers or in the environment in external fertilizers. Like pre-meiotic sperm ageing, post-meiotic sperm ageing has been shown to negatively affect the fertilization potential of the male and the viability and fitness of the resulting offspring (Wagner et al., 2004; White et al., 2008).

Sperm ageing is caused by extrinsic and intrinsic factors. Extrinsic sperm ageing is caused by environmental factors like temperature stress or osmotic stress (Reinhardt, 2007). Intrinsic factors are related to the metabolism of the cell. Two examples of such intrinsic factors are oxidative stress and ATP depletion (Aitken and Baker, 2004; de Lamirande and Gagnon, 1992; MacLeod, 1943). Oxidative stress due to accumulation of reactive oxygen species (ROS) is thought to be one of the main factors generating deterioration of sperm quality (Aitken et al., 2012; Reinhardt, 2007). ROS accumulate in the cell as a byproduct of the aerobic metabolism and small amounts of ROS are not dangerous to sperm cells (de Lamirande and Gagnon, 1995a; Finkel, 1998). In fact, in mammalian sperm cells a certain amount of ROS is required to trigger capacitation (de Lamirande and Gagnon, 1995b; O’Flaherty et al., 2006, 1999). But high ROS concentrations within sperm cells have been shown to damage to the plasma membrane (Alvarez et al., 1987; Twigg et al., 1998), to cause DNA fragmentation (Twigg et al., 1998), and to alter the DNA methylation profile (Tunc and Tremellen, 2009). Thus, oxidative stress can not only affect the performance or fertilization potential of sperm but also the resulting offspring. Furthermore, sperm are thought to be particularly sensitive to oxidative stress because of their high metabolic activity, large amount of polyunsaturated fatty acids, and small cytosol (Baker and Aitken, 2004; Reinhardt, 2007).

In papers II and III, we performed assays to assess the effects of post-meiotic sperm ageing on offspring performance. In paper III, we investigated the effects of pre- and post-ejaculation sperm ageing on offspring survival (i.e. before and after sperm release from the male) in zebrafish. In paper II, we focused on intrinsic post-ejaculation sperm ageing and investigated
whether the short active life span of salmon sperm is long enough for ROS damage to occur.
2 Main methods

2.1 Artificial selection

In papers I and III, we used artificial selection to study heritability of sperm traits, gametic selection and the evolution of sperm. In artificial selection experiments, selection for certain phenotypes or trait values is applied directly by the experimenter. This means the experimenter selects a subset of individuals from a lab population that express a specific phenotype or trait value to sire the offspring of the next generation and continues to do so for several generations (Hartl and Clark, 2007). Several selection methods can be distinguished (Fuller et al., 2005). In truncation selection experiments for instance, only the individuals with trait values greater/smaller than a set threshold (e.g. only individuals longer than 4.6 cm) are used to sire the next generation (Fuller et al., 2005; Hartl and Clark, 2007). In relative selection experiments, a certain percentage of the population that expresses the highest or lowest trait values (e.g. the 25% of individuals with the highest weight) are selected (Fuller et al., 2005). Independent of the specific selection method, artificial selection shifts the population mean of the selected trait in the direction of selection and towards the mean of the parents that are selected as long as the trait is heritable (i.e. that it is based on genetics or epigenetics and there is variation for the trait within the selection line/population; Hartl and Clark, 2007). Artificial selection is a powerful tool for understanding the heritability of traits, for measuring how evolvable and adaptive traits are and to understand evolutionary processes (Hartl and Clark, 2007; W G Hill and Caballero, 1992). In papers I and III, we applied a variant of artificial selection, in which we selected at the gametic level instead at the individual level. We selected for sperm phenotypes within ejaculates. Thus, one could say our populations were ejaculates and our individuals were spermatozoa. We then measured how selection at the gametic level affected the offspring.

2.2 In vitro fertilization

In papers I, II, III, and IV, we performed in vitro fertilization (IVF) experiments combined with selection of different sperm phenotypes or male treatments. IVF is the process of artificially combining sperm and ova for fertilization. IVF is a useful experimental tool because it allows the control of the
exact timing of fertilization, the control of the precise number of eggs and sperm used for the IVF, and the elimination of pre-mating and mating effects during fertilization. In externally fertilizing fish, such as the Atlantic salmon used in papers I and II, and the zebrafish used in papers III and IV, it is moreover straightforward to perform IVFs. One just has to collect the gametes and combine them in the right medium (in our case fresh water of the right temperature). IVF experiments furthermore allow the application of split-clutch experiments (see below).

Split-clutch design IVF experiments
Split-clutch design IVFs refer to a breeding design where the gametes of at least one parent are split into several parts and used for IVF with gametes of either different individuals or differently treated gametes of one single individual (Barber and Arnott, 2000). This allows accounting for maternal, paternal or parental effects depending on whether the egg clutch, the ejaculate or both are split. Such a split design ensures that the observed effects are actual treatment effects rather than parental artifacts. By combining sperm and eggs of different parental pairs, one can moreover account for effects caused by different degrees of genetic compatibility of parental pairs. In papers I, II, III, and IV, we split both the egg clutches of the females and the ejaculates of the males into several parts before we conducted IVFs with sperm that were exposed to different treatments. In papers I, II, and III, these sperm treatments were directly applied to the sperm before the IVFs. Moreover, we applied all sperm treatments to the sperm of each male and thus were able to replicate each treatment within each parental pair. In paper IV, we exposed different males to different sperm competition treatments and fertilized egg sub-clutches of several females with the sperm of the same male to be able to account for maternal and paternal effects.

2.3 Molecular work
In paper III we performed genomic work and sequenced zebrafish sperm genomes to study selection in sperm.

Genomics is the study of complete genomes or the complete set of genetic information of an organism or cell (Lander and Weinberg, 2000). Genomics is often used to study genetic differences across individuals, populations or species. In paper III, we were interested in potential genetic differences of haploid sperm phenotypes found within single ejaculates. We sequenced and analyzed the genomes of two sperm cohorts that were artificially selected from single zebrafish ejaculates. The sperm were artificially selected by the distance they swam during their active life phase. We then sequenced the whole genomes of the sperm in the resulting sperm pools and investigated
whether the two sperm pools differed at the genomic level. The aim of this investigation was to test whether the phenotypic intra-ejaculate variation across the two sperm cohorts was based on genetic differences.

To sequence our samples we used Illumina sequencing (Illumina HiSeq 2500 system). Illumina sequencing is one of several next generation sequencing technologies. Next generation sequencing technologies have changed the fields of genomics and transcriptomics tremendously because they can produce vast volumes of data at a reasonable prize and have led to an enormous expansion of our knowledge about genes and their expression (Mardis, 2008; Metzker, 2010; Ozsolak and Milos, 2011).

The main steps of Illumina sequencing are: library preparation, cluster generation, and sequencing. During library preparation, the DNA samples are randomly fragmented and adapters are ligated to the fragments. In the next step, the cluster generation, the library is loaded into a flow cell. The fragments are captured by surface bound oligonucleotides complementary to the adapters of the library and the fragments are amplified. During sequencing, fluorescently labelled nucleotides are incorporated into the DNA templates in cycles of DNA synthesis. The nucleotides are identified during each cycle by their fluorophore excitation. The use of fluorescently labelled nucleotides for sequencing is not new, but in next generation sequencing millions of fragments are analyzed simultaneously (whereas with older sequencing techniques only a single DNA fragment was processed at a time), which accelerates the sequencing process.
3 Study systems

We used two different model species for the studies included in this thesis, depending on the specifics of the study question, the experimental design and the methods we wanted to apply. Now, I introduce each model species and motivate why we chose them for the respective experiments.

3.1 Atlantic salmon

The Atlantic salmon *Salmo salar* is a teleost fish that belongs to the Salmonidae. The Atlantic salmon is found in the northern Atlantic Ocean and in rivers that flow into the northern Atlantic Ocean (Klemetsen et al., 2003). Most populations are anadromous and migrate between fresh water and salt water, but there are also salmon populations that are non-anadromous and never leave their fresh water habitats (Power, 1958). Atlantic salmon that follow an anadromous migration pattern have their feeding and growth peak in the ocean but migrate to their native rivers for spawning (Fleming, 1996; Klemetsen et al., 2003; Power, 1958). Atlantic salmon are external fertilizers and release their gametes into the water for fertilization. Males may adopt two distinct mating strategies to increase their fitness: large anadromous males fight against each other for access to females, whereas small mature parr males sneak into spawnings of anadromous males to achieve fertilizations (Fleming, 1996; Gage et al., 1995). Consequently, anadromous males invest in traits that help them to monopolize females, such as big body sizes, red body coloration, and hook-like lower chaws during the breeding season for fighting other males (Fleming, 1996). Small mature parr males on the other hand, invest more into traits that are important in sperm competition, such as bigger relative testes size, denser ejaculates, and more motile and longer living sperm (Gage et al., 1995). Spawning usually takes place in autumn and typically sperm competition is intense with several males – one or several large anadromous males and one or several mature parr males – fertilizing the eggs of one female (Fleming, 1996). The female chooses the nesting site (or redd) and may excavate several gravel nests in her redd by using her caudal fin as a paddle (Fleming, 1996). At spawning the female, the anadromous males and the potential mature parr males release their gametes simultaneously for fertilization. Fertilization happens within a few seconds after gamete release and the female uses her caudal fin to cover the
eggs with gravel immediately after fertilization (Fleming, 1996; Yeates, 2005). After the eggs have been covered, the female will move to the next nest accompanied by the males and release another egg bout (Fleming, 1996). This process is repeated until the female has laid all her eggs. Which anadromous males and mature parr males will fertilize the eggs of each individual nest is determined by their relative vicinity to the current nest, the relative dominance of the males and of course their sperm competition ability (Fleming, 1996). The resulting embryos develop in the redd during the winter. The alevins (newly hatched juveniles with unabsorbed yolk sacs) hatch in early spring, absorb their yolk sac and then – as so called fry – leave the gravel to establish themselves on the feeding grounds of the river (Dill, 1977). As the fry grow they develop into parr and when they are big enough they leave the rivers and migrate to the sea (Klemetsen et al., 2003; Power, 1958). At which age they migrate varies within and across populations (Klemetsen et al., 2003). During their one to four year long stay in the sea they experience a rapid growth phase and grow into adults (Myers, 1986; Power, 1958). As mentioned before, some parr males already become sexually mature before migrating and reproduce as sneaker males until they migrate eventually (Myers, 1986). Atlantic salmon may return to the sea several times, though most of them only migrate once or twice (Klemetsen et al., 2003).

The Atlantic salmon emerged as a very suitable model system for some of the experiments of my PhD studies because its external fertilization allows that IVFs can be performed easily. Furthermore, the vast amount of male and female gametes they produce allowed us to not only use a split-clutch design where we could replicate our treatments within each parental pair, but also to conduct many different treatments within this split-clutch design. However, Atlantic salmon is not very suitable to be kept in the lab for a whole generation (due to its size, its generation time, its life stages, and its migration pattern), and we therefore chose another study species for the experiments that ran over several generations.

### 3.2 Zebrafish

The zebrafish, *Danio rerio*, is also a teleost fish and belongs to the Cyprinidae. It is a small, beige-black-striped, fresh water fish with a maximal length of about 4 cm. Zebrafish are native to the Indian subcontinent where they mostly occur in shallow, slow-flowing water ponds or ditches that are created by flooding of floodplains (Engeszer et al., 2007b; Spence et al., 2008). They are sometimes also found in slow flowing breaches of streams (Engeszer et al., 2007b; McClure et al., 2006). Zebrafish are omnivorous but feed primarily on small insects (McClure et al., 2006; Spence et al., 2008), and are a shoaling species where fish live in social groups (Engeszer et al.,
Zebralsh are external fertilizers, just as the Atlantic salmon, and spawning takes place during a short period at dawn and is induced by the light (Rowena Spence et al., 2007). During spawning, sperm and eggs are released simultaneously and the eggs are directly deposited into the bare substrate (Spence et al., 2008). The spawning is partitioned into several bouts and the female deposits up to 20 eggs per bout (Spence et al., 2008). Zebralsh are group spawners, i.e. couples usually do not spawn alone but in groups of three to seven fish (Spence et al., 2008). Zebralsh provide no parental care, which is thought to be a possible reason for females to be choosy about oviposition sites. Females prefer gravel over silt, as gravel is better aerated but still provides protection against predation (Rowena Spence et al., 2007). Males adopt two distinct mating tactics, territorial defense or active pursuit of females (Spence and Smith, 2005). Territorial males defend territories surrounding good oviposition sites against other males. Non-territorial males focus on courting females. In nature, zebras are an annually breeding species, with the reproductive period starting just before the monsoon time (R. Spence et al., 2007). This makes sense as zebras have their highest growth rate during the first three months of their life and food availability is highest during the monsoon months when it rains a lot and the temperature is high (Spence et al., 2008). But the breeding season appears to be more related to food availability than to the actual season, since zebras breed all year around in the lab. In the lab, mating intervals range from one to six days (Spence and Smith, 2006). Females can lay up to several hundred eggs per spawning. Zebras are relatively large and transparent, which allows for easy observation of embryo development under a dissecting microscope (Kimmel et al., 1995). Development is furthermore very quick: all major organs develop within 36 h and the larvae hatch about two to three days post fertilization (dpf) and start feeding at five dpf (Kimmel et al., 1995).

Like the Atlantic salmon, the zebrafish is a suitable model system for sperm selection experiments because its external fertilization allows for straightforward IVF experiments and the application of split-clutch designs. Additionally, zebras are easy to keep in the laboratory and have a relatively short generation time of three to four months. They are therefore well suited for selection experiments that run for several generations and for studying trans-generational effects. Furthermore, they are a model organism for developmental biology, genetics, biomedicine and neurophysiology and thus much is already known about many aspects of their biology (e.g. their genome, function of their genes, their development, how they best are kept in the lab etc.).
4 Research aims

The overall aim of my PhD studies was to investigate selection in sperm and the trans-generational effects of sperm phenotype; whether and how sperm phenotype affects offspring phenotype, and especially which role haploid selection plays in this context. More specifically the research aims of the respective papers were:

I Investigating whether and how different sperm longevity cohorts found within single ejaculates affect offspring performance in the Atlantic salmon *Salmo salar*.

II Investigating the effects of intrinsic post-ejaculation sperm ageing on offspring fitness in the Atlantic salmon *Salmo salar*.

III Studying the (long-term) effects of intra-ejaculate sperm selection on offspring fitness in the zebrafish *Danio rerio*, and to explore the mechanism of these trans-generational effects.

IV Investigating the effects of short-term variation in sperm competition on both ejaculate traits and offspring performance in the zebrafish *Danio rerio*. 
5 Summaries of the papers

Paper I

Sperm variation within a single ejaculate affects offspring development in Atlantic salmon

It is generally believed that a sperm’s phenotype does not reflect its haploid genotype and that the variation of sperm phenotypes within a single ejaculate should have no effect on offspring performance (Eddy, 2002; Higginson and Pitnick, 2011; Immler, 2008). Although there is recent evidence that selection on the phenotype of individual sperm does affect offspring performance (Crean et al., 2012), sperm are still considered to be products of the diploid genome of the male (Hecht, 1998).

In paper I, we tested whether within-ejaculate variation in sperm phenotype affects offspring performance in the Atlantic salmon, *Salmo salar*. We performed two split-clutch design IVF experiments in which we replicated our sperm treatments within each parental pair. In the first experiment, we selected for different sperm longevity cohorts (i.e. sperm with different active lifespans) within single ejaculates by varying the time between activation of the sperm and fertilization, with waiting times of 0 s, 20 s and 40 s. We then used the resulting sperm longevity cohorts from each male to fertilize three sub-clutches of one female each. The sperm of the 0 s treatment consisted of all types of sperm phenotypes and any motile sperm had a chance to fertilize an egg. In the 20 s treatment, only sperm that survived for more than 20 seconds post activation were able to fertilize the eggs (ca. 50% of the sperm were still active) and in the 40 s treatment, only sperm that survived for longer than 40 seconds were able to fertilize the eggs (only very few sperm are still active at this time point). Since this led to different concentrations of active sperm across our treatments, we performed a sperm dilution experiment to test for the effects of sperm density and the potentially resulting effects of differential egg competition in our treatments. In this second experiment, we manipulated the sperm concentration by diluting 1000 µl, 200 µl, 50 µl or 5 µl sperm of the same ejaculate in 100 ml water and then used these different sperm concentrations for fertilization. We then measured hatching time, standard length at hatching, and growth rate of the larvae after hatching for the offspring of both experiments.
We found that within-ejaculate variation in sperm longevity affected the hatching time and that sperm of the intermediate longevity cohort (20 s treatment) sired offspring that developed faster and hatched significantly earlier than the offspring of the other two longevity cohorts (0 s and 40 s treatment). While the dilution effect was statistically significant the actual effect was weaker and opposite to the effect observed in our first experiment. We thus conclude that the effect of sperm longevity on offspring developmental time is not confounded by egg competition effects or sperm dilution effects. Furthermore, we found that within-ejaculate variation in sperm longevity affected the standard length of the larvae. The larvae of the 40 s treatment were significantly larger than those of the other treatments. But this result could at least partly be explained by egg competition effects, since the larvae in the 5 µl treatment were also significantly larger than those in the 1000 µl treatment.

There are at least four non-mutually exclusive mechanisms that could explain our results. First, there is some evidence for haploid gene expression in sperm (Vibranovski et al., 2010; Zheng et al., 2001), and thus haploid selection in the sperm could have caused our result. Second, pre- and/or post-meiotic sperm ageing (Pizzari et al., 2008; Reinhardt, 2007) could partially explain our results if older sperm behave differently (e.g. have a different sperm phenotype, different motile lifespan) and also carry more mutations than younger sperm. One interesting aspect of our results is that the relationship between offspring development and sperm longevity cohort was non-linear. Thus, while within-ejaculate selection for longer lived sperm of better quality (Birkhead et al., 1993; Keller and Reeve, 1995) could be responsible for the shortened developmental time of the offspring of the 20 s treatment, sperm ageing effects could explain the increased developmental time of the offspring of the 40 s treatment (Pizzari et al., 2008; Reinhardt, 2007; White et al., 2008). Third, epigenetic effects that alter sperm phenotype could be transferred to the zygote and then alter offspring performance (Dadoune, 2009; Jenkins and Carrell, 2012). Fourth, ‘sloppy’ spermatogenesis could have an impact on offspring performance if abnormal sperm that are still able to fertilize eggs were more likely to carry DNA defects or to transfer abnormal epigenetic information to the zygote (Holt and Look, 2004).

At this point we cannot say which of these mechanisms is most likely to explain our results. Further investigations are necessary to ascertain that. However, our results show that the variation in sperm phenotype within a single ejaculate affects offspring performance in the Atlantic salmon and provide evidence for trans-generational effects of sperm phenotypes on offspring performance.
Intrinsic post-ejaculation sperm ageing does not affect offspring fitness in Atlantic salmon

Sperm ageing at any stage of sperm development is thought to impair offspring fitness (Pizzari et al., 2008; Reinhardt, 2007). It has been shown that fertilization by aged sperm lowers the rate of embryonic development, reduces embryonic viability and decreases offspring performance (Wagner et al., 2004; White et al., 2008). In paper II, we focused on the effects of post-ejaculation sperm ageing, which is caused both by intrinsic factors (including oxidative stress and ATP depletion), and extrinsic factors (such as temperature stress and osmotic stress). We were interested in the effects of intrinsic sperm ageing (Aitken et al., 2012; Cabrita et al., 2014; Reinhardt, 2007) where oxidative stress – caused by the accumulation of reactive oxygen species (ROS) within the cell – is considered to be the major factor leading to sperm ageing (Cabrita et al., 2014; Reinhardt, 2007). High ROS concentrations lower sperm motility, induce breaks in the cell membrane, decrease the fertilization capacity of sperm, and disrupt the genome integrity and RNA stability of the sperm (Cabrita et al., 2014). Thus, oxidative stress affects both the fertilization potential of sperm and the viability and fitness of the resulting offspring. To date most post-ejaculation sperm ageing research has focused on internal fertilizers (den Boer et al., 2009; Firman et al., 2015; Han, 2014; Lewis and Aitken, 2005; Reinhardt et al., 2005; Wagner et al., 2004; White et al., 2008; Zubkova and Robaire, 2006), and little is known about post-ejaculation sperm ageing in external fertilizers.

In paper II, we investigated post-ejaculation sperm ageing in an externally fertilizing fish, the Atlantic salmon. We performed in vitro fertilizations with four different post-ejaculation sperm ageing treatments and tested how these affected offspring performance. We used a split-clutch design for the IVFs by splitting all egg clutches and ejaculates into four parts and thus replicated all four sperm ageing treatments within each parental pair. After sperm activation, we waited for a treatment-specific amount of time (0 s, 20 s, 40 s, 60 s) – while minimizing extrinsic sperm ageing in our treatments – and then used the sperm for the IVFs. We chose this time interval because it reflects the natural active lifespan of Atlantic salmon sperm, which is about one minute post activation (Yeates, 2005). To track offspring fitness, we measured embryo survival, hatching time, standard length at hatching, and growth rate after hatching to see whether our sperm ageing treatments affected offspring performance.

However, we found no effect of intrinsic post-ejaculation sperm ageing on offspring fitness. Within the natural sperm activity window of the Atlantic salmon, none of the offspring traits we had measured were affected by our sperm ageing treatments. The most probable explanation for the absence
of any effect is that the negative effects of ROS accumulation take longer than the time available in Atlantic salmon under natural conditions. According to the literature, salmon sperm are expected to both contain high ROS concentrations during their active lifespan (between ejaculation and fertilization) and to be very sensitive to ROS induced damage (Vladić and Petersson, 2016). Nevertheless, we did not find an effect of post-ejaculation sperm ageing on offspring fitness, which indicates that the brief fertilization window may be too short for intrinsic sperm ageing effects to take place. It would be interesting to test whether intrinsic post-ejaculation sperm ageing affects offspring fitness in external fertilizers with extended fertilization windows.

Paper III

**Haploid selection within a single ejaculate increases offspring fitness**

Selection during the haploid phase can have important consequences for the evolutionary dynamics (Charlesworth and Charlesworth, 1987, 1992; Orr and Otto, 1994; Otto et al., 2015). While haploid selection is well studied in plants (Hormaza and Herrero, 1992), little is known about haploid selection in animal gametes. The current dogma is that sperm performance is under diploid control (Eddy, 2002; Higginson and Pitnick, 2011; Immler, 2008) and that offspring performance is not affected by sperm phenotype or sperm genotype. Yet, sperm of the same ejaculate are astonishingly variable (Holt and Look, 2004), and we know very little about the consequences of this variability and how it affects both competition among the sperm of the same ejaculate, and offspring performance of the resulting offspring (Haig and Bergstrom, 1995; Parker and Begon, 1993). Theoretical work by Haig and Bergstrom (1995) predicts that genetic/epigenetic variation among sperm of the same ejaculate can both lead to competition between the sperm and affect offspring fitness. Furthermore, there is some evidence for haploid gene expression and hence the potential for haploid selection in sperm (Joseph and Kirkpatrick, 2004; Martin-DeLeon et al., 2005; Schultz et al., 2003; Vibranovski et al., 2010; Zheng et al., 2001). Moreover, two recent empirical studies demonstrated that sperm phenotype affects offspring phenotype (Crean et al., 2012; Immler et al., 2014). But it has not been shown yet whether the link between sperm phenotype and offspring phenotype is genetic, epigenetic or related to sperm ageing. In paper III, we performed a series of experiments and show that different phenotypes of sperm within a single ejaculate that differ in their longevity also differentially affect offspring fitness, and that phenotypic differences in sperm are linked to variation in genotypes of the sperm.

We used the zebrafish as study organism in paper III. To test the effect of different sperm phenotypes on offspring performance, we performed IVF
experiments where we selected sperm based on their longevity by manipulating the time between sperm activation and fertilization. More specifically, we applied a split-clutch design dividing both eggs and sperm into two parts and conducted IVFs with sperm that were exposed to two treatments. In our “short activation time” (SAT) treatment, fertilization took place immediately after sperm activation. In our “long activation time” (LAT) treatment, we waited until about half of the sperm had become immotile before performing the IVFs. Thus, we directly selected against short-lived sperm in our LAT treatment. We measured several fitness traits in the resulting offspring and tested whether the traits were affected by our sperm selection treatments. We measured embryo survival, the number of apoptotic cells in the embryo eight hours post fertilization (hpf) (which is an indicator for embryonic fitness), sperm swimming velocity in the F1 sons, and the reproductive performance of the F1 offspring when mated to non-experimental fish. Furthermore, we performed an experiment to test whether pre- and post-ejaculation sperm ageing could have caused the effects we found in the first assays by performing very similar IVF experiments where we used sperm of different ages. In a last experiment, we examined whether sperm phenotype variation is linked to genomic variation in the sperm. We separated two different sperm pool types within ejaculates based on their ability to cover a certain distance during their motile phase and then tested for allele frequency differences between the two separated sperm pool types.

We found that the offspring of longer lived sperm (LAT sperm) were more likely to survive and had a reduced number of apoptotic cells eight hpf than offspring of shorter lived sperm (SAT sperm). Sons sired by LAT sperm exhibited furthermore a higher fitness than their SAT brothers: they produced denser ejaculates and faster swimming sperm, and had an increased fertilization success. Females mated to males sired by LAT sperm produced more eggs than when they mated to males sired by SAT sperm. The offspring of the daughters sired by LAT sperm (F2 generation) were in addition more viable than the offspring of their sisters that were sired by SAT sperm. Offspring of both sexes that were sired by LAT sperm produced a higher percentage of normal embryos than the ones sired by SAT sperm. Neither pre- nor post-ejaculation sperm ageing had any consequences on offspring viability and sperm ageing can therefore not explain the effects on offspring survival we observed in the first experiments. We checked also whether fertilization rate could explain the differential embryo survival, but it had no impact on our results. When comparing the genomes of the two selected sperm pool types, we found allele frequency differences between the selected pools.

Our results show that phenotypic variation among intact fertile sperm within an ejaculate affects offspring fitness in the zebrafish, and that these effects persist even in the F2 generation without further selection. The results further indicate that the phenotypic variance of our selected sperm pool
types is based on genetic variation. Overall, we demonstrate that there is a link between sperm phenotype variation within ejaculates and both genotypic variation of the sperm and offspring fitness. Our findings suggest that there is opportunity for haploid selection in sperm, which has crucial implications for our thinking about the evolutionary processes that are important both at and after fertilization.

Paper IV

Short-term variation in sperm competition causes sperm-mediated epigenetic effects on early offspring performance in the zebrafish

Epigenetic effects have been shown to play an important role in evolutionary processes and to be responsible for non-genetic inheritance (Bonduriansky, 2012; Bonduriansky and Day, 2009; Danchin et al., 2011). While non-genetic maternal effects on offspring have been extensively studied (Groothuis and Schwabl, 2008; Howell et al., 2001; Kappeler and Meaney, 2010; Mousseau and Fox, 1998), relatively little is known about non-genetic paternal effects on offspring fitness. But there is growing evidence for sperm-mediated epigenetic effects, and that they are important in early embryo development (Arico et al., 2011; Brykczyńska et al., 2010b; Hackett and Surani, 2013; Hammoud et al., 2009). However, the mechanisms causing variation in epigenetic effects and how fitness of the offspring is affected by this variation are not fully understood. One potential mechanism that could affect the variation in epigenetic effects in the offspring is sperm competition. Males alter their ejaculate depending on the sperm competition risk they encounter: the higher the sperm competition risk, the more competitive ejaculates and sperm they produce (Birkhead et al., 1999; Cornwallis and Birkhead, 2007; Gage, 1991; Pilastro et al., 2002). These ejaculate trait changes have been shown to happen over very brief time periods (Cornwallis and Birkhead, 2007; Crean and Marshall, 2008; Immler et al., 2010). However, still relatively little is known about how these changes affect offspring performance (but see Adler and Bonduriansky, 2013; Crean et al., 2013). The aim of paper IV was to test whether short term changes in ejaculate traits caused by varying levels of sperm competition affect sperm/ejaculate traits and offspring performance in the zebrafish.

We chose the zebrafish as study system, because this allowed us to perform IVF experiments with a split-clutch design, and to thereby account for parental effects and the compatibility between parental pairs. Prior to the IVFs, we manipulated the sperm competition intensity experienced by males over a relatively short time period of two weeks. The males were randomly allocated to two treatments: a high sperm competition treatment (two males were kept with one female, only one male was used for IVF) and a low
sperm competition treatment (one male was kept with two females). We then performed IVFs using the males from our treatments and stock females that were kept in a 1:1 sex ratio and tested whether our treatments affected ejaculate traits and early offspring performance. The ejaculate traits and sperm traits that we measured were sperm motility and number/proportion of motile sperm, velocity, sperm density, sperm longevity, ejaculate volume. For the IVFs, we either used whole egg clutches (if ≤ 20 eggs) of the female for one IVF, or split the egg clutches into two to six equal parts depending on the egg clutch size. The ejaculate of each male was split into two parts and used for fertilization of eggs of two different females. After fertilization, we measured the following traits of offspring performance: the proportion of hatched larvae within each sub-clutch over the whole hatching period, and offspring survival until one week post fertilization.

We found that males of the high sperm competition treatment produced faster swimming sperm, a higher proportion of motile sperm, and sired offspring that hatched over a narrower time period than the males exposed to the low sperm competition treatment. But offspring of the high sperm competition males were less likely to survive the first week after fertilization than the ones of the low sperm competition males. Sperm density, ejaculate volume, and sperm longevity did not differ between our treatments.

Our results provide evidence that epigenetic adjustments to encountered sperm competition intensities not only affect ejaculate traits of males, but also the performance of their offspring, and that these paternal effects manifest themselves within a rather short time (in our case only two weeks or two spermatogenic cycles). This suggests that short-term changes of the social environment of males may create epigenetic changes that are inherited by the offspring and that affect both offspring fitness and survival and in turn also female fitness (since female fitness decreases with increasing mortality of the offspring).

Currently, we cannot disentangle which sperm-mediated inheritance pathways have led to our results. There are two main epigenetic pathways that could alter both ejaculate traits and offspring performance. First, differentially expressed RNAs that are transferred from the sperm into the zygote could explain the observed effects (Dadoune, 2009). There is evidence that variation in the sperm RNA profiles of humans is linked to variation in sperm motility (Chen et al., 2012; Lambard et al., 2004) and in mice, sperm RNA is needed to trigger embryo development (Sone et al., 2005). Second, the variation in sperm competition in our treatments could have led to differential histone pre-marking or altered DNA methylation patterns that could explain the differences in the ejaculate traits and offspring performance we observed (Lindeman et al., 2011; Wu et al., 2011). In zebrafish, methylation patterns are largely paternally inherited (Jiang et al., 2013; Potok et al., 2013) and thus gene expression in the offspring could have been altered by our treatments if the changes of methylation patterns accumulate that quickly.
– which remains to be tested. Either way, paper IV provides evidence that in zebrafish, short-term changes in the sperm competition intensity that a male experiences do not only affect the sperm traits of the male, but also the fitness and survival of his offspring and thus also female fitness.
Concluding remarks and future prospects

In summary, the work presented in this thesis demonstrates that offspring phenotype is affected by variation in sperm phenotype across males (paper IV) and also by intra-ejaculate variation of sperm (papers I, and III). Furthermore, we provide evidence for genetically caused variation in sperm phenotype within single ejaculates providing an opportunity for haploid selection (paper III).

In papers I, II, and III, we investigated the effects of intra-ejaculate sperm variation on offspring performance. In paper I, we selected different sperm longevity cohorts within ejaculates to perform IVFs and demonstrated that Atlantic salmon sperm of intermediate longevity sire faster hatching offspring than their sibling sperm with shorter or longer longevity. Our results indicate that there is a link between intra-ejaculate sperm longevity variation and offspring developmental time. But we could not determine whether the link between sperm phenotype and offspring phenotype we observed is based on genetics, epigenetics, or sperm ageing. In paper II, we found that intrinsic post-ejaculation sperm ageing does not affect offspring performance in the Atlantic salmon. Thus, we can conclude that intrinsic post-ejaculation sperm ageing does not appear to be the factor that led to the pattern observed in paper I. But this leaves us still with the question of which other factors are responsible for the effects observed in paper I. It would therefore be interesting to study whether the observed link between sperm longevity and offspring developmental time in Atlantic salmon is based on genetics, epigenetics, extrinsic post-ejaculation sperm ageing or pre-ejaculation sperm ageing.

In paper III, we went one step further in studying the trans-generational effects of selection on sperm phenotypes. This time, we studied the effects of intra-ejaculate sperm variation in another study system, the zebrafish. We performed similar IVF experiments as in paper I selecting for different sperm longevity cohorts and found that sperm longevity affects offspring fitness throughout life. We could further show that an effect on embryo survival was carried over into the F2 generation. Moreover, we found that neither pre- nor post-ejaculation sperm ageing were responsible for the observed pattern. In a different experiment, we separated sperm by the distance they covered during their active lifespan (which correlates with their longevity). We found that sperm that covered longer distances were genetically different from the sperm that swam for shorter distances. This result indicates that sperm phenotype reflects sperm genotype and thus that there is
potential for haploid selection in the sperm. The link between sperm longevity and embryo survival moreover suggests that haploid selection might help to eliminate deleterious mutations from the fertilizing sperm pool at fertilization. A next step would be to determine the exact genetic mechanism underlying the observed pattern. A current hypothesis is that varying epistatic interactions create variation among sperm and could affect offspring phenotype. Furthermore, testing for haploid selection in other animals with different fertilization strategies will be interesting.

In paper IV, we were interested in epigenetic sperm variation across males. We found that short-term changes in sperm competition do not only affect ejaculate traits but also offspring performance which confirms the importance of sperm-mediated epigenetic effects. In this study we did not focus on identifying the underlying mechanisms and it would therefore be interesting to study which epigenetic pathway is responsible for the observed trans-generational effect and whether paternal short-term environmental effects are more often inherited via RNAs, chromatin modifications or both of them.

In conclusion, we found that sperm phenotype affects offspring phenotype and that there is a link between sperm phenotype and sperm genotype, which shows that there is opportunity for haploid selection in sperm. Furthermore, we demonstrated that both sperm and offspring phenotype are affected by short-term variation in the social environment males are exposed to. I hope the work presented in this thesis will serve as the base for future research investigating trans-generational effects of sperm phenotype and haploid selection in animals.
Hos djur är den haploida fasen av livscykeln väldigt kort och reducerad till de encelliga gameterna (könscellerna). Därför fokuserar forskning som studerar naturligt urval (selektion) hos djur oftast på den diploida fasen av livscykeln, som är lång och består av de multicellulära organismerna. Följaktligen vet vi väldigt lite om selektion under den haploida fasen hos djur. Vi vet till exempel inte ens om fenotypen hos enskilda spermier reflekterar spermiens egna haploida genom, eller om spermernas fenotyp enbart påverkas av de diploida hanarna som de produceras av. Därför vet vi inte heller om det sker haploid selektion på spermier, och vilka konsekvenser den i så fall skulle ha. Under mina doktorandstudier har jag fokuserat just på dessa frågor. Jag studerade interaktionen mellan spermiers fenotyp och avkommans fenotyp och genom vilka mekanismer spermiens fenotyp påverkar avkomlingens fenotyp. Jag var särskilt intresserad av följande aspekter av spermernas biologi: (i) potentiella konsekvenser av haploid selektion hos spermier på avkommen, (ii) konsekvenserna av spermiers ålder på avkommen och (iii) konsekvenserna av spermieinducerade epigenetiska effekter på avkommen.

För att undersöka om variationen i spermernas fenotyper inom ett ejakulat från en och samma hane påverkar avkommans fenotyp, undersökte vi om artificiellt urval av spermier inom ejakulaten av atlantlax (Salmo salar) och zebrafisk (Danio rerio) har en effekt på de avkommor som produceras. Vi selekterade spermier efter deras aktivitetslängd (längden av deras aktiva livsfas). Vi fann att variationen av spermernas aktivitetslängd inom ejakulaten påverkar avkommans utvecklingstid hos atlantlax och avkommans överlevnadschans och reproduktiva framgång. Avkomma som var ett resultat av befruktning från laxpermier med mellanlång aktivitetslängd kläcktes snabbare än de som resulterade från en befruktning från spermier som var aktiva under en kortare eller längre tid. Hos zebrafisk hade avkommen från spermier med en längre aktiv livsfas högre överlevnadschanser och en högre reproduktiv framgång än avkomman från spermier med en kortare aktiv livsfas. Vi kan därför konstatera att spermernas fenotyp påverkar de framtida avkomlingarnas egenskaper. Vi studerade också vilka mekanismer låg bakom dessa obseraverade samband mellan spermernas och avkommans fenotyp. Vi testade exempelvis om sambanden kunde förklaras med spermernas ålder. Men varken spermernas pre-ejakulationsålder (undersökt enbart hos zebrafisk) eller post-ejakulationsålder (undersökt både hos zebrafisk och...


För att sammanfatta, visar denna avhandling att spermiers fenotyp påverkar avkommans fenotyp, samt att både epigenetiska skillnader och haploida genetiska skillnader påverkar spermiernas egenskaper.
8 Acknowledgements

My special gratitude goes to Simone, my main supervisor. Thank you for the opportunity to do a PhD in your lab and for all your support and guidance during my PhD studies! Further, I want to thank you for your encouraging optimism that you have always kept during the past few years – even in difficult times. I also want to thank Göran, my second supervisor. Your supervision during my Master thesis provided me with a great base for my postgraduate studies! Thank you for many fruitful discussions during my PhD studies and your inspiring enthusiasm.

Next, I want to thank all the current and previous lab members of the Immler lab. Susi, Ghazal, Sandra, Bart, Berrit, Marta, Willian, Sergio, Roy, Krhiezha, Hwe-Yen, Cécile, Maria, Tuuli, Bao, Constance, Joaquin, Jessica, Yuki, Julia, Andrea, Aivars, João, Dovilė, it has been a pleasure to have you as colleagues and to work with you! Thank you for interesting discussions, help in the lab, entertaining group meetings and many delicious cakes. I enjoyed in particular our trips to Älvkarleby for various salmon experiments and of course our lab outings. A special thanks to my co-authors Ghazal, Krhiezha, Berrit, Roy, Bao, Tuuli, Sandra and Susi.

I want to thank Tracey, Janet and Wayne for a great collaboration (on a project that is not included in this thesis) and for introducing me to yet another model system: Drosophila melanogaster. I enjoyed my stay in Norwich very much and it has been fun to work together with you. Regarding the Drosophila project I also want to thank Maja for introducing me to the fly lab in Uppsala, and Anna, Andrea, Cécile, and Zuzana for helping me with experimental work. It has been fun to work with you in the fly lab!

Doing a PhD without the support of friends would of course not be very fun. I want to thank Hwei-yen for being not only a supporting colleague, but also a friend I could always count on. Hwei-yen & Ding thank you for your friendship and many delightful dinners, fikas and lunches.

Marta & Reto, and Lucie & Johan, I want to thank you for being great friends. What a blessing that Annika, Maël and Jakob were all born within a few weeks. I really enjoyed spending time with you, Marta and Lucie, during our maternal leave and the play dates that followed later were and are also very precious to me. I am very happy that Jakob has Annika and Maël as friends :-) Burris, I (we) miss you!

Mi, it has been a pleasure to share office with you and to chat about work and parent-related things in breaks. Thank you!
Giulia & Rado you have been superb neighbours. Thank you for babysitting Jakob during the last phase of my PhD!

Lisa, Sophie, Ioana & Björn, Josefin, Anna, you have been outstanding friends. Thank you for being there for me!


Jakob, mein kleiner Sonnenschein! Ich bin unendlich dankbar, dass es dich gibt und dass ich deine Mama sein darf. Deine Neugierde aufs Leben und die Welt und deine Lebensfreude miterleben zu können, ist das schönste Geschenk!
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Acta Universitatis Upsaliensis

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