Genetic and environmental components of sperm function in *Drosophila melanogaster*

DISSERTATION

Zur Erlangung des akademischen Grades

Doktor rerum naturalium

(Dr. rer. nat.)

vorgelegt

dem Bereich Mathematik und Naturwissenschaften

der Technischen Universität Dresden

von

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geboren am 02.10.1989 in Shangdong, V. R. China

Tag der Verteidigung: 17.12.2019

Gutachter: Prof. Klaus Reinhardt

Die Dissertation wurde in der Zeit von Oktober/2015 bis Oktober /2019 im Technische Universität Dresden

Reviewers

1st Reviewer: Prof. Dr. Klaus Reinhardt

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General introduction

Sperm function

Sperm function is essential to male fertility and have been widely studied in multiple research fields (Reinhardt et al., 2015). For example, in the western world, sperm dysfunction is one of the most common reasons for human infertility (Pizzol et al., 2014) and essential to assisted reproduction technologies. Sperm function is also useful for animal breeders to improve the livestock's reproductive capacity (Froman et al., 2006; Billard & Cosson 1992) and for geneticists to design conservation programmes (Roldan & Gomendio 2009). Last but not least, sperm function is the main focus of evolutionary biologists because only the functioning, successful sperm can pass genetic information to the next generation.

Following the manufacture of a sperm cell through to fertilisation and effects on offspring, a large number of traits may be used to characterise the functionality of sperm. These include morphology, ATP content, single- or double strand breaks of the DNA, ROS production, mitochondrial function, chromatin condensation and so on. According to the World Health Organization, the most common aspects of sperm function examined are i) sperm viability ii) sperm motility and swimming speed, iii) fertilisation potential and iv) effects on offspring. In this thesis we mainly focus on sperm viability, sperm quality, sperm metabolism and the fertilization ability. These are briefly described below.

Sperm viability

Sperm viability is literally the proportion of live sperm in a sample, and considered to be a way to assess ejaculate quality, functionality and longevity (Holman 2009a). Sperm viability has been demonstrated to have evolutionary significance. First, sperm viability plays a key role in sperm competitions and thus male fertilization. For example, post-copulatory sexual selection selected for higher sperm viability in numerous species (Hunter & Birkhead 2002). Second, adaptation in male and female

have been found to maintain sperm viability. Empirical studies showed male seminal fluid and female secretory fluid had an effect on sperm viability (Holman 2009b; den Boer et al. 2009). Third, sperm viability itself might have a heritable component and provide genetic basis for evolution of sperm viability (Simmons & Roberts 2005; Moore et al., 2004; Civetta et al., 2008). In addition to genetic components, sperm viability also has been suggested to be phenotypically plastic and affected by various environments (Moore et al., 2004; Locatello et al., 2007).

Sperm quality

There are technical pitfalls in sperm viability assessment with the commercial live/dead kit staining methods (Holman 2009a). For example, true value of sperm viability is hard to know because the commercial live/dead kit used for sperm viability itself kills sperm. Second, the number of sperm being killed during staining assay and sperm membrane properties might depended on sources from which they are isolated, such as male and female sperm storage organs. Third, sperm viability might dependent on the total number of sperm (Holman 2009). More recently, more pitfalls are discovered in the staining procedures for sperm viability evaluation (Eckel et al. 2017). On the other hand, there are also conceptual concerns (Eckel et al. 2017). Specifically, sperm viability, which is defined as the proportion of live sperm in an ejaculate, is considered to be more a male parameter rather than the future performance of sperm. Therefore, the resistance of sperm to external osmatic stress was assessed instead as an aspect of sperm quality in study to better quantify sperm performance (Eckel et al. 2017).

Sperm motility

Sperm move as a result of movements that are generated by the flagellum, a cell component containing the axoneme whose microtubules are associated with large

ATPases. Sperm motility is considered to be one of the most determinants of male fertility (Gage et al., 2004) because only motile sperm can migrate to fertilise eggs and pass their genes onto next generation. In evolution and ecology, sperm motility is assessed by a variety of parameters, such as the proportion of motile sperm and sperm velocity. The assessment of sperm motility provides us some insight into its evolutionary implications. Specifically, sperm motility has been suggested to under post-copulatory sexual selection. For instance, studies of fishes (Gage et al., 2004) and birds (Birkhead et al., 1999) have reported associations between measures of sperm motility and competitive male fertilization success. Second, a positive response to selection on sperm motility requires there to be additive genetic variation, either for motility itself, or for the morphological traits that affect motility. Indeed, there are evidences for mitochondrial genetic effects on sperm motility in human beings and mice (Ruiz-Pesini et al., 2000a; Tourmente et al., 2017). Our knowledge of the quantitative genetics of sperm is generally poor so far. The genetic effects on sperm motility might be generally low, but there might be still sufficient variation in sperm motility on which selection can act.

Sperm metabolism

As we summarized above, sperm viability and motility are commonly measured in ecology and evolution as a male trait, and these traits are under selection to some extent and important for evaluation of male fitness. However, the cellular mechanisms underlining these traits are still unclear. Sperm metabolism was suggested to be closely to those sperm traits. Specifically, sperm metabolism is critical for sperm survival, sperm aging and sperm viability (Reinhardt 2007; Ribou & Reinhardt 2012), especially when stored within female. Female of many species can store sperm within their sperm storage organs for a period of time before using them for fertilization. For example, mammals can store sperm for days or weeks while some social insects like ants and honey bees are able to store sperm for years (Holt

& Fazeli 2016; Orr & Brennan 2015). Sperm need to remain viable for a long time period during storage and afterwards fertilize eggs. Reactive oxygen species (ROS), which are produced as by-products of metabolism, can cause damages to a variety of biological molecules, for example DNA, proteins and membrane polyunsaturated fatty acids in sperm. The resulting damages can result in sperm aging and reduce sperm longevity (Sanocka & Kurpisz 2004). Therefore, alteration in metabolism in sperm during female storage might occur to retard the aging process and extend the longevity via directly by sperm itself or indirectly by females (Ribou & Reinhardt 2012). Moreover, sperm metabolism is essential to sperm motility as well. The direct energy used by sperm for motility is from ATP hydrolysis and accounts for about 70% of total ATP consumption (Bohnensack & Halangk 1986). Lastly, sperm metabolism has been demonstrated to play a key role in processes for sperm to acquire ability to fertilise eggs, like capacitation and acrosome reaction. For example, significant increase in oxygen consumption was found in rabbit and chicken sperm, which was associated with the process of capacitation (Hammer & Williams 1963). In addition, physiological level of ROS produced was suggested to be essential to sperm capacitation (Sanocka & Kurpisz 2004).

Metabolic pathways in sperm

Like somatic cells, sperm cells rely on two main metabolic pathways for ATP production, glycolysis and oxidative phosphorylation (OXPHOS). Briefly, glycolysis is the conversion of glucose to pyruvate first, and then to lactate. The process is known as aerobic glycolysis in the presence of O_2 and anaerobic glycolysis in the absence of O_2. During this process, two net molecules of ATP from one molecule of glucose are generated. In contrast, OXPHOS is a more complex process which occurs in mitochondria. Electrons from reducing equivalents (NADH or FADH) derived from glycolysis, tricarboxylic acid (TCA) cycle and fatty acid oxidation flow to O_2 through the electron transport chain (ETC). As a consequence, transmembrane proton

gradients are established and ATP is generated by ATPase when protons flow back to intermembrane matrix. OXPHOS is considered to be a more efficient energy pathways in comparison to glycolysis because the complete oxidation of one molecule of glucose through OXPHOS can yield 36 additional ATP (du Plessis et al. 2015).

Energy pathways possess several distinct features in sperm in comparison to somatic cells. First, the energy pathways within sperm are highly compartmentalized due to its highly specialized cell structure (Storey 2008). Specifically, glycolysis mainly takes place in principle piece of sperm. Principal piece acts as scaffold for the sequence of glycolytic enzymes, which received experimental validation by the presence of lactate dehydrogenase (LDH), fructose 1,6-bisphosphate aldolase (ALD), and pyruvate kinase (PK) on this scaffold (Krisfalusi et al. 2006). Moreover, the distribution of sperm-specific isoform GAPD (GAPD-S) along the entire principle piece indicated by the immunolocalization by its antibodies also provides evidence for the idea that glycolysis occurs in sperm principle piece (Buch et al. 1998). On the other hand, OXPHOS is believed to mainly occurs at midpiece of sperm where mitochondria are tightly packed into a helix structure. Advent of sonication and differential centrifugation make it possible to separate sperm midpiece (Mohri et al. 1965). Electronic microscopy further revealed a clear structure of midpiece where mitochondria wrapped around axoneme. As mitochondria is clearly established as the site for respiration and OXPHOS in somatic cells, the midpiece of sperm was therefore assigned the special role (Fawcett 1975). Moreover, there is no evidence for the presence of any respiratory enzymes in the principle piece and sperm head so far (Storey 2008).

Second, in term of enzymatic components of energy pathways, sperm show a variety of differences compared with somatic cells. In particular, sperm-specific forms of glycolytic enzymes exist, which are absent in somatic cells. For example, phosphoglycerate kinase-2 (PGK2), GPI, phosphofructose kinase-1 (PFK1) and

aldolase (EN) have been suggested to have isoforms that are exclusively present in sperm (Bunch et al. 1998; Vemugati et al. 2007) (see Vemugati et al. 2007 for listing and references). Compared with their isoforms in somatic cells, sperm-specific glycolytic enzymes usually get an extension at the N-terminal end of the amino acid sequence at molecular level. The sequence is proposed to enable them to anchor to the fibrous sheath of flagellum (Krisfalusi et al. 2006), but has not yet received experimental confirmation. Moreover, there is also a sperm-specific LDH isoform, designated as LDH-X (Blanco & Zinkham 1963; Goldberg 1963). In somatic tissues, LDH is absent in mitochondria. However, LDH-X was found to be present in the mitochondria of mammalian sperm. Further studies showed that the intramitochondrial LDH activity was involved in lactate and pyruvate metabolism in sperm (Storey 1978; Alvarez & Storey 1984).

Sperm metabolic traits most commonly measured

ATP content and ATP production

ATP, the direct energy source for sperm motility and other fundamental sperm functions, can be produced via both glycolysis and OXPHOS pathways within sperm. As a basic parameter of sperm energetics and an indicator of sperm energy status, ATP content is generally expressed as the amount of ATP present in a defined amount of sperm cells (most commonly in unit nmoles of ATP/10^8 sperm), and was mostly determined with luciferase-based ATP bioluminescent assay kit.

In contrast to ATP content, ATP production is referred to as the rate of ATP generated from glycolysis, OXPHOS or both pathways, which can be used as an indicator for the intensity of glycolysis, OXPHOS or the overall metabolism. Therefore, in practise, ATP production is generally determined indirectly from other metabolic parameters that can reflect the flux of glycolytic or oxidative metabolic pathway, like the simultaneous measurement of oxygen consumption and lactate production in rodents (Garrett et al. 2008). The unique calculation of ATP production allows researchers to estimate the relative contributions of glycolysis and OXPHOS pathways to the total ATP production, to identify the primary ATP-generation pathway and determine the metabolic status for the sperm of a given species (Tourmente et al. 2015; Mookerjee et al. 2017).

Lactate production

Pyruvate derived from metabolism of glucose or other glycolytic metabolites can be further reduced to lactate by LDH. At the same time, NADH is oxidized to NAD^+. The production of lactate from pyruvate is generally measured as an indicator of anaerobic glycolytic rate. In sperm, lactate production can be either evaluated directly by enzymatical (Umbreit et al., 1957; Friedland & Dietrich,1961) and

radiochemical methods (Hoskins & Patterson 1968), or estimated indirectly through measuring either NAD+ content fluorometrically (Garrett et al. 2008) and the acidification rate of the culture medium (Tourmente et al. 2015). The production of lactate is usually recorded as μmol/hr/10^8 sperm cells.

O_2 consumption

When oxidative phosphorylation occurs within sperm cells, O_2 is taken by mitochondria as the final receptor of electrons and reduced to water at complex 4 of the ETC. Mitochondria are responsible for the vast majority of O_2 consumed by sperm cells, O_2 consumption is therefore measured as respiration rate and considered to be a reliable indicator of OXPHOS intensity and mitochondrial functionality. In practice, O_2 consumption is generally expressed as ZO_2 (unit in (μL/10^8 sperm)/hr, i.e. the amount of O_2 consumed by 10^8 sperm per hour) and can be monitored manometrically with Warburg device, polarographically with O_2 electrode (Storey 2008; Clark et al. 1953) or fluorometrically with O_2 probe (Garrett et al. 2008).

Mitochondrial membrane potential (MMP)

Mitochondrial membrane potential is another parameter that is commonly determined in sperm to reflect mitochondrial activity or function. Fluorescent dyes like MitoTracker (Terrell et al. 2011a) and 5,59,6,69-tetrachloro-1,19,3,39-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) are commonly used in practice and fluorescent intensity or intensity ratio of 530 nm to 590 nm is calculated as an indicator of MMP (Mukai & Okuno 2004).

ROS production

In sperm, ROS are mainly generated within mitochondria as metabolic by-products through electron leakage from the ETC. To date, evidence for the generation of ROS

through cytoplasmic NAD(P)H oxidation in sperm cells is still weak in spite of its presence in mammalian sperm (Ford 2004). Studies suggest that ROS can be the indicators of both intense mitochondrial activity and mitochondrial dysfunction in sperm (Darr et al. 2016). Likewise, findings in sperm from two insect species revealed both positive and negative relationship between ROS production and metabolic rate (Ribou &Reinhardt 2012; Reinhardt & Ribou 2013). Interestingly, ROS play a dual role in sperm function as well. Excessive ROS result in oxidative stress which cause damage to sperm mainly through lipid peroxidation. On the other hand, at physiological levels, ROS act as signalling molecules which are essential to sperm capacitation (Ford 2004). In practise, a variety of methods are employed to measure ROS production. Specifically, ROS are generally measured as the production of their end product H_2O_2 with chemiluminescent probes like luminol and Mito B (Kobayashi et al. 2001; Cochemé et al. 2012), or in some cases as the production of superoxide with chemiluminescent probes, such as lucigenin and 1-pyrene butyric acid (Ford 2004; Reinhardt & Ribou 2013).

Interspecific variation in the presence of glycolysis and OXPHOS pathways in sperm cells

Even though the strength of empirical evidences for the presence of glycolysis differs considerably across studies, we found that sperm from species ranging from insects and decapods to fish and amphibians, to birds and mammals including human, appear to possess an active glycolytic pathway (Table G.1). In contrast, despite the fact that OXPHOS is a more efficient pathway for ATP production, the sperm from not all species investigated so far possess the OXPHOS pathway. For example, ultrastructure analyses revealed the absence of mitochondria, the organelle where OXPHOS occurs, in sperm from several insect species like beetles (Table G.1), rendering them not capable of producing ATP via OXPHOS. However, sperm from most other invertebrates like decapods and vertebrate species do have the ability to

generate ATP via OXPHOS. From an evolutionary point of view, our findings supported the idea that glycolysis might be a quite conservative energy pathway in sperm across species as all species investigated are capable of glycolysis. On the contrary, absence of mitochondria in sperm in several species indicates OXPHOS pathway might be not active in all species. In addition, most organisms tend to possess these two energy pathways to be energetically flexible and tenacious when they are exposed to various environments over lifetime.

Table G.1 Summary of the presence of energy pathways, primary energy pathway for motility and pathway switch in sperm across species.

Species	Sperm compartment	Glycolysis	OXPHOS	Primary pathway for motility	Metabolic pathways switch	Reference
Insects						
Drosophila hydei	Sperm from testes	Yes (glycolytic enzymes activity)	Yes (O_2 consumption)	-	-	Geer et al. 1975
Honeybee	Ejaculated/ stored sperm	Yes (acidification of the sperm media)	Yes (O_2 consumption)	-	from OXPHOS (ejaculate)to glycolysis	Paynter et al. 2014
Bombyx mori	Spermatophore sperm	Yes (glycolytic intermediates metabolised)	Yes (CO_2 output)	Both OXPHOS and glycolysis	-	Osanai et al. 1987
Aleochara bilineata	Stored sperm in spermathecae	Yes (glycolytic inhibitor used)	Yes (Cytochrome-c oxidase activity	Glycolysis	-	Werner et al. 1999
Red flavour beetle	Stored sperm	Yes (glycolytic inhibitor used)	Yes (oxidative inhibitor effect)	Glycolysis	-	Qazi et al. 1998
Cricket	Ejaculated/ stored sperm	-	Yes (ROS production)	-	Reduced ROS produced after storage in female	Ribou &Reinhardt 2012
Cimex Hemipterus	Sperm from seminal vesicle	Yes (mobility maintainence)	Yes (O_2-depedent motility)	-	-	Ruknudin & Raghavan 1988
Bacillus rossius	Sperm from seminal vesicle	Yes (Detected glycolytic enzymes' activity)	No (no mitochondria)	Glycolysis	-	Baccetti et al. 1973
Parlatoria oleae	Sperm from e testes/ spermathecae	Yes	No (no mitochondria)	Glycolysis	-	Robison 1966
Pseudococcus obscurus	Sperm from testes	Yes	No (no mitochondria)	Glycolysis	-	Ross & Robison 1969
Decopods						
Balanus Balanus	Semen	Yes (presence of glycogen)	Yes (O_2 consumption)	Glycolysis	-	Barnes 1962

Metopograpsus messor	Stored sperm in spermateca	Yes (lactate production)	Yes (O_2 consumption)	OXPHOS	-	Anilkumar et al. 1996
Paratelphusa hydrodromous	Spermatophore sperm	Yes (detection of LDH activity)	-	-	from glycolysis (spermatophore) to OXPHOS (stored)	Jeyalectumie & Subramoniam 1987
Scylla serrata	Spermatophore/ stored sperm	Yes (LDH activity determined)	Yes (SDH activity determined)	Glycolysis	-	Jeyalectumie & Subramoniam 1991
Fish						
Surfperch	Seminal/ ovarian sperm	Yes (lactate production)	Yes (CO_2 output)	Glycolysis	-	Gardiner 1978
Liza dumerili	Mature Sperm from sperm duct	-	Yes (O_2 consumption)	-	-	Van der Horst 1986
Leuciscus cephalus	Washed sperm	Yes (glycolytic enzymes activity determined)	Yes (presence of oxidative enzymes)	Glycolysis	-	Lahnsteiner et al. 1992
Psetta maxima	Washed sperm	-	Yes (oxidative inhibitors supress motility)	-	-	Dreanno et al. 1999
Chalcalburnus chalcoides	Pure semen	Yes (lactate production)	Yes (O_2 consumption)	-	-	Lahnsteiner et al. 1999
Clarias gariepinus	Testicular semen	Yes (glycolytic enzymes activity determined)	Yes (O_2 consumption)	-	-	Mansour et al. 2003
Oncorhynchus mykiss	Fresh semen	Yes (lactate production)	Yes (O_2 consumption)	-	-	Lahnsteiner et al. 1999; Ingermann et al. 2003
Amphibian						
Bufo arenarum	Washed sperm	Yes (lactate production)	Yes (O_2 consumption)	-	-	Del Río 1979
Xenopus laevis	-	-	Yes (O_2 consumption)	-	-	Van der Horst 1986

Leptodactylus chaquensis	-	-	Yes (O_2 consumption)	-	-	Del Río et al. 1975
Birds						
Rooster	Washed semen	-	Yes (electrons flow)	-	-	Kirby & Froman 1991
Rooster	Washed Semen	-	Yes (O_2 consumption)	-	-	Sexton 1974; Ashizawa et al. 1995
Turkey	Washed Semen	Yes (lactate production)	Yes (O_2 consumption)	-	-	Sexton 1974
Mammals						
Rabbit	Ejaculated sperm	-	Yes (O_2 consumption)	-	-	Hammer & Williams 1963
Ram	Epididymal sperm	Yes (lactate production)	Yes (O_2 consumption and CO_2 production)	-	-	Inskeep & Hammerstedt 1982
Tammar Wallaby	Epididymal sperm	Yes (lactate production)	Yes (CO_2 production and O2 consumption)	-	-	Murdoch & Jones 1998
Trichosurus Vulpecula	Ejaculated sperm	Yes (lactate production)	Yes (CO_2 production and O_2 consumption)	-	-	Rodger & Suter 1978
Bull	Ejaculated sperm	Yes (lactate production)	Yes (O_2 consumption)	-	-	Scott et al. 1962
Boar	Ejaculated sperm	Yes (lactate production)	Yes (O_2 consumption)	Glycolysis	-	Aalbers et al. 1961
Guinea pig	Cauda epididymal sperm	Yes (fructose consumption)	Yes (O_2 consumption)	-	-	Aonuma et al. 1974
Dog	Ejaculated sperm	Yes (lactate production)	Yes (MMP, CO_2 production)	Glycolysis	-	Nascimento et al. 2008;
Boar/pig hybrids	Ejaculated sperm	Yes (lactate production)	Yes (O_2 consumption)	-	-	Dziekońska 2015

Domestic cat	Ejaculated sperm	Yes (lactate production)	Yes (MMP)	OXPHOS	-	Terrel et al. 2011a; Terrell et al. 2011b
Cheetah	Ejaculated sperm	Yes (lactate production)	Yes (MMP)	OXPHOS	-	Terrel et al. 2011a; Terrell et al. 2011b
Stallion	Ejaculated sperm	-	Yes (O_2 consumption)	OXPHOS	-	Darr et al. 2016; Gibb et al. 2014
Bovine	Ejaculated sperm	Yes (lactate production)	Yes (O_2 consumption)	-	-	Garrett et al. 2008
Rat	Caudal epididymal sperm	Yes (activity of LDH-4 determined)	Yes (O_2 consumption)	-	-	Geer et al. 1975; Ferramosca et al. 2016
Mouse	Caudal epidydimal sperm	Yes (lactate production)	Yes (O_2 consumption)	Glycolysis	-	Tourmente et al. 2015; Mukai & Okuno 2004
Rhesus monkey	Ejaculated sperm	Yes (lactate production)	Yes (O_2 consumption)	Fructolysis	-	Hoskin & Patterson 1968; Hung et al. 2008
Human						
Human	Semen	Yes (oxidative inhibitors used)	Yes (glycolytic inhibitors used)	Glycolysis	-	Peterson & Freund 1969

"-", no information provided; LDH, Lactate dehydrogenase; MMP, mitochondrial membrane potential; ROS, reactive oxygen species.

Interspecific variation in rates of glycolysis or OXPHOS in sperm cells

Sperm cells are one of the most diverse cell types, for example in morphology. However, it remains unclear whether they exhibit considerable variation in terms of metabolism as well. In order to get a rough full picture of variation in sperm metabolism across species, here we summarized respiration rate (measured as O_2 consumption) and glycolytic rate (measured as lactate production) of almost all the species examined so far. As dramatic traits, sperm respiration and glycolytic rate are environment-dependent and measured under various physiological conditions across different studies. For example, respiration of steelhead trout was sensitive to PH and CO_2 (Ingermann 2003) and type and concentration of substrates influence glycolytic rate of human sperm (Peterson & Freund, 1963). Temperature also has an effect on sperm respiration (Kupriyanova & Havenhand, 2005). Therefore, we in principle picked the respiration and glycolytic rates that were measured under a proper defined physiological buffer saline (both glycolytic and oxidative substrate supplemented) at a close to *in vivo* temperature. Respiration values were summarized in the Table G.1 and shown in the Figure G.1. We found that there is a considerable variation in sperm respiration rates across taxonomic groups although the sample size varies a lot between groups (Figure G.1). In particular, mammalian sperm possess the highest respiratory rate, followed by bird, shellfish and amphibian sperm. In contrast, fish and decapod sperm exhibit relatively low respiration rate. On the other hand, within-group variation in respiration rates is also substantial. For example, the respiration rate of mammalian sperm ranges from a ZO_2 value of 2.5 to 39.7 (Table G.2). Among mammals, marsupial sperm consume oxygen in a much higher rate relative to eutherian sperm in general. Sperm respiration rates form most fish species are quite low, with a ZO_2 range from 0.08 to 3.43 (Table G.2). The variation within this fish is not large, except that *Psetta maxima* showed a high ZO_2 value range from 4.71 to 16.85. In terms of glycolysis, mammalian sperm differ a lot in lactate production among species. Specifically, the glycolytic rate of boar sperm is quite low while that of marsupial sperm is at the higher end of the range. Limited data is available for birds

and fishes, but their sperm undergo glycolysis at a much lower rate compared to that of mammals. Fishes, for instance, viviparous species have a negligible glycolytic rate, while the glycolytic rate in surfperch *(Cymatogaster aggregata*) is two orders of magnitude higher and hence comparable to certain mammalian species.

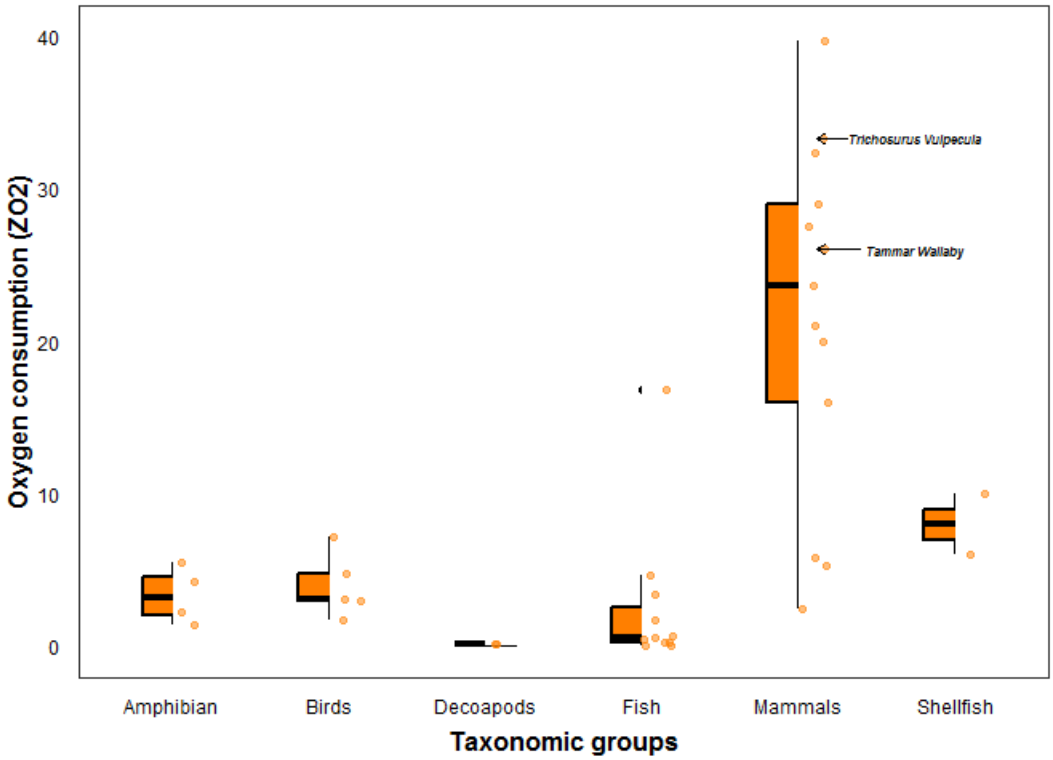


Figure G.1 Sperm oxygen consumption (ZO_2, (µL/10^8 sperm)/hr) across species grouped by taxonomy. Data were collected from literature and shown by taxonomic groups. Arrows indicated sperm respiration rates from two marsupial species.

Table G.2 Rate of lactate production and oxygen consumption rate in sperm across species.

Species	Compartment	Lactate production	O_2 consumption (ZO_2)	References
Mammals 30-37°C				
Human	Semen	-	4.3	Eliasson 1970
Rhesus monkey	Ejaculated sperm	2.81/ 2.06/ 4.38	16.0	Hoskins & Patterson, 1968
Rat	Caudal epididymis	-	20	Ferramosca et al. 2016
Bovine	Ejaculated sperm	1.44	32.3/ 29/ 39.7	Garrett et al. 2008
Pig hybrid	Ejaculated sperm	0.74/ 1.017	23.59/ 25.66	Dziekońska 2015
Boar	Ejaculated sperm	0.325/ 0.42	2.5	Aalbers et al. 1961
Trichosurus Vulpecula	Ejaculated sperm	2.81-4.38	33.3	Rodger & Suter, 1978
Ram	Ejaculated sperm	0.79	27.5	Inskeep & Hammerstedt,
Rabbit	Ejaculated sperm	-	5.3	Hamner & William, 1963
Tammar Wallaby	Ejaculated sperm	1.9-3.6	26/32.5	Murdoch & Jones, 1998
Bull		1.4	21	Scott et al. 1962
Birds 41 °C				
White Leghorn roosters	Washed semen	-	7.2	Ashizawa et al. 1995
Gallus domesticus	Washed semen	0.3889/ 0.3444	2.96/ 4.79	Sexton 1974
Meleagris gallopavo	Washed semen	0.0222/ 0.333	1.765/ 3.115	Sexton 1974
Amphibian 30 °C				
Bufo arenarum	Testicular mature sperm	-	1.4-4.2	van der Horst 1986; Del
Leptodactylus chaquensis	Testicular mature sperm	-	1.4-4.2	van der Horst 1986; Del
Xenopus laevis	Testicular mature sperm	-	2.2-5.5	van der Horst 1986
Fish 10-20°C				

Liza dumerili	Mature sperm from duct	-	0.08-0.7	van der Horst 1986
Oncorhynchus mykiss	Washed Semen	-	0.27 (conversed)	Ingermann et al. 2003
Salmo gairdnerii	Washed semen	-	0.081-0.27 (conversed)	Terner & Korsh 1963
Lepomis	Washed semen	-	1.18	Terner & Korsh 1963
Gadus morhua	Washed Semen	0.000254/ 0.000377	0.48	Mounib 1967
Salmo sala	Washed Semen	0.000182	0.52	Mounib 1967
Psetta maxima	Washed semen	-	4.71-16.84	Dreanno et al. 1999
Chalcalburnus chalcoides	Washed Semen	0.000135	1.69-3.43	Lahnsteiner et al. 1999
Decapods				
Balanus Balanus	Semen	-	0.18	Barnes 1962
Metopograpsus messor	Stored sperm in spermatheca	-	0.108	Anilkumar et al. 1996

"-", no information provided. Measured at body temperature in physiological buffers mostly supplemented with glucose or fructose. Lactate production for some species were converted into the same unit to make comparation between species possible.

We summarized and interpreted our data based on taxonomic classes to get a rough big picture of sperm metabolism. It does not mean that sperm metabolism is strictly restricted to their taxonomic classification. In the future, it is worthwhile to examine respiration or glycolytic rates in more species to gain more insight into evolution of sperm metabolism.

Variation in relative contribution of glycolysis and OXPHOS to sperm motility

As most species has suggested to be capable of generating energy via both glycolysis and OXPHOS, it raises an interesting question that whether these two pathways contribute equally to the energy supply of sperm. Sperm requires a substantial amount of energy to maintain its fundamental function, especially for sperm motility. If the sperm cell cannot swim, it cannot fertilize the egg. The studies of primary pathway by which this energy is generated in sperm has spanned a period of decades (Ford 2004; Storey 2008; du Plessis et al. 2015). OXPHOS was initially considered to be the dominant pathway as it is more efficient in ATP production. However, as restricted to midpiece of sperm, ATP produced by OXPHOS might not be able to diffuse as quickly as possible to the principle piece of sperm. Therefore, it might not supply the majority of energy to sperm flagellum for movement. Study in sea urchin sperm showed that an efficient shuttle system to transport ATP from midpiece to the flagellum exist, but not in sperm from other species (Tombes & Shapiro, 1985; Tombes et al. 1987). On the other hand, the local distribution of glycolytic enzymes along sperm principle piece enable it more convenient to provide ATP for sperm flagellum (Welch et al. 1992; Krisfalusi et al. 2006). In fact, contradictory results regarding the dominant energy pathways were obtained across species. For example, glycolysis was demonstrated to play a major role in sperm motility in human and mouse sperm, while OXPHOS was believed to be the predominant energy pathway in stallion sperm (Table G.1). Here we first defined sperm energetic phenotype with respiratory and glycolytic rates, and tried to get an overview of variation in sperm

energetic phenotype across species. There is a considerable variation in sperm energy phenotype among species (Figure G.2). For instance, mammalian sperm possess a phenotype with both high glycolytic and respiratory rates while birds and fishes showed another phenotype with relatively low glycolysis and OXPHOS.

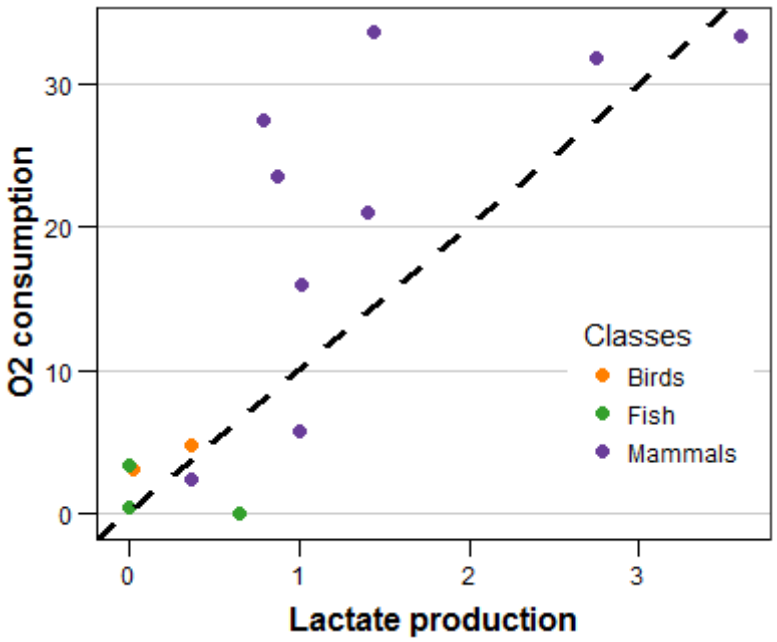


Figure G.2 Sperm energetic phenotype defined by O_2 consumption (ZO_2, (µL/10^8 sperm)/hr) and lactate production ((µmol/10^8 sperm)/hr) across species grouped by taxonomy. Data were collected from literature and transformed where necessary.

Absolute values might provide some information on sperm metabolic phenotype and interspecific variation, but the relative importance of these pathways for sperm function, sperm motility for example, are sometimes tell us more about the evolution of sperm metabolism. In order to identify preferred energy pathways for sperm function, with emphasis on motility, we reviewed most studied species based on following parameters: 1) preferred utilization of substrates supplemented

in the medium by sperm 2) dose-dependent effects of glycolytic or oxidative substrates on sperm motility 3) relative activity of glycolytic or respiration enzymes. 4) effects of either glycolytic or respiratory inhibitors on sperm motility. 5) effects of genetically knockout of enzymatic components of either glycolytic or oxidative pathways on ATP production or sperm motility (Table G.1). Mammalian species exhibited a considerable variation in reliance on either pathway for energy supply. In particular, human, rhesus macaque and mouse sperm were demonstrated to be largely dependent on glycolysis for ATP production to sustain motility (Table G.1). The lack of a relationship between MMP and sperm motility and the presence of glycogenesis-like metabolic pathway indicated a relatively more essential role of glycolysis in dog sperm (Rigau 2002). In contrast, OXHOS is the dominant energy pathway of stallion and bovine sperm (Terrell et al. 2011a). In addition, lack of transporter for glucose uptake and no intracellular glycogen deposits in felid sperm suggest a more important role for OXPHOS via fatty acids oxidation for energy supply (Terrell et al. 2011a; Terrell et al. 2011b). Insects also exhibited variation in their preference for energy pathways for ATP. For instance, glycolytic pathway was considered to be dominant in roove beetle and red flavour beetle, whereas bedbug (*Cimex lectularius*) sperm relies more on OXPHOS pathway (see more information in Table G.1). In summary, sperm vary considerably among species in the preference of energy pathway and do not necessarily show a pattern associated with the taxa classification. In general, there are mainly three types of sperm for those are capable of producing energy via both pathways: glycolytic dominant, OXPHOS dominant and both essential. The variation in energy pathways in sperm among species might be a result of post-copulatory sexual selection (i.e. sperm competition or female cryptic choice). Indeed, a recent study carried out on mouse sperm from three closely related species showed a difference in ATP production via glycolysis and OXPHOS (Tourmente et al. 2015). Species with higher level of sperm competition was associated with higher level of ATP production and a more usage of OXPHOS in relation to glycolysis. Considering the essential roles of metabolism in sperm function, it is reasonable to

assume that sperm metabolism is under sexual selection, but studies from more species are desirable for further confirmation.

Genetic and environmental components of sperm function

The vast evolutionary diversification is one of the most striking features of sperm. Variation in sperm function is believed to be driven by sexual selection and comes from three main sources as well as the interactions between them: the male nuclear genotype, the male mitochondrial haplotype and environments (Reinhardt el al. 2015; Figure G.3).

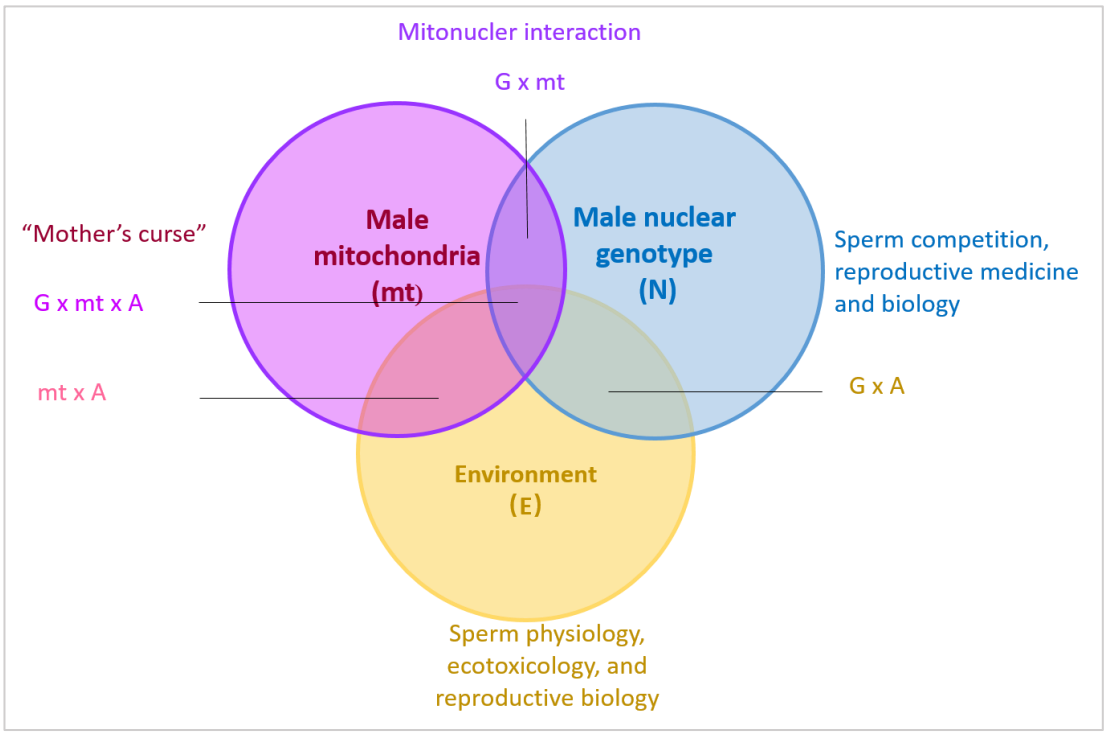


Figure G.3 Schematic graph showing the three main sources resulting in variation in sperm function and the corresponding research fields. The effects of mitochondrial genome (magenta) on sperm function are mainly studied in the context of "mother's curse" or the interaction with nuclear genome in the context of cytonuclear interaction. The effects of nuclear genome (blue) on sperm function are studied within the context of sperm competition in evolution or reproductive biology in andrology. Environmental influences on sperm (yellow) are investigated in the context of sperm physiology, ecotoxicology and reproductive biology. The most commonly studied environments are listed in the grey box at the bottom. Figure adapted from Reinhardt et al. 2015.

Male genotypic (G) effects on sperm function

Reproductive medicine, by seeking treatment for infertility conditions examines correlations between sperm function and a male's genotype (Aitken 2006). However, despite of intense research efforts in reproductive medicine, only about 10-15% of

infertility can currently be attributed to male genetic factors (Pizzol et al., 2014). Similarly, male G effects are the main focus and interest when it comes to long research tradition in sperm competition (Parker 1970; Bernasconi et al., 2004; Birkhead et al., 2009; Parker 2014). On the other hand, the nuclear genetic architecture of sperm traits like sperm morphology, viability and competitiveness have been characterised in previous studies (Dowling et al., 2007a; Dowling et al., 2007c; Kim et al., 2017). For example, most recent studies in zebra finch showed that an inversion polymorphism on Z chromosome explained almost all the genetic variation in sperm morphology (Kim et al., 2017) and they were found able to explain 41.9% and 45.5% of variance in length of midpiece and flagellum (Knief et al., 2017). However, there are also studies showed that male G effects might not be so evident and might be exceedingly low (see review of studies in Dobler & Reinhardt, 2016). For example, nonsignificant heritability for both standard components of sperm competition (i.e. sperm defence and sperm offence) were found across several offspring cohorts in *D. melanogaster* (Dobler & Reinhardt, 2016).

Mitochondrial effects (mt) on sperm function

An increasing number of studies show that mitochondrial genetic variance is functional and non-neutral. Indeed, in addition to aging, several degenerative diseases, and mitochondrial mutations can play a key role in reproduction as well. In particular, mitochondria affect many aspects of the sperm phenotype (Yee et al., 2013; Innocenti et al., 2013; Froman & Kirby 2005). For instance, using experimentally constructed mitonuclear introgression lines, naturally occurred mitochondrial haplotype are found to affect sperm length and sperm viability in seed beetle, sperm mentalism in rodent (Dowling et al. 2007c; Tourmente et al. 2017). The mt contribution to variation in sperm function can be substantial (but see Friberg & Dowling 2008). For example, 68% of the variation in sperm motility in humans was explained by variation in mitochondrial production of reactive oxygen species (ROS)

(Koppers et al., 2008). Similarly, the sperm competitive ability of fruit fly sperm differed by 30% between mitochondrial haplotypes (Yee et al., 2013). As the mitochondria are inherited only maternally, there is little scope for adaptive evolution of sperm function via mitochondria.

Mitonuclear interaction effects

In evolution, a close link between mitochondrial genetic variation and variation in phenotypes has been established in numerous species across a variety of phenotypic traits. However, more recently, an increasing number of studies further suggested that mitochondrial effects are more often expressed through its interaction with nuclear genome(Rand et al. 2006; Dowling et al. 2007b; Dobler et al. 2014; Wolff et al. 2014; Immonen et al. 2016; Mossman et al. 2016; Đorđević et al. 2017). Below we would like to briefly summarize the potential arenas where mitochondrial genome can interact with nuclear genome.

There are multiple ways in which mitochondrial and nuclear genomes can interact with each other as summarized before (Hill 2019). First, the enzyme complexes of electron transport chain, which are essential to oxidative phosphorylation (OXPHOS), are encoded by both nuclear and mitochondrial genomes (Taanman 1999; Phillip et al. 2010). This protein-protein interaction defines the electron flow and the OXPHOS efficiency, therefore both genomes might be under selection to reach the optimal OXPHOS function. Second, some proteins encoded by nuclear genome are transferred into mitochondria and play a key role in replication, transcription and translation of mitochondrial genome(Clayton, 2000; Levin et al. 2014; D'Souza & Minczuk, 2018). Genetic mutations in mitochondrial genome are expected to select for proper corresponding nuclear mutations during these protein-DNA and protein-RNA interactions due to the high mutation rate of mitochondrial genome compare to that of nuclear genome. Lastly, even though a broad

communication via signally pathways is present between nuclear and mitochondrial genomes (Poyton & McEwen 1996; Muir et al. 2016), its significance in the mitonuclear interaction has not been properly appreciated. Retrograde signalling (from mitochondrion to nucleus) can deliver the functional state of mitochondrion to the nucleus, which is mediated via OXPHOS associated molecules like ATP, ROS or NAD(P)H. In contrast, anterograde signalling is from nucleus to mitochondrion via nuclear encoded proteins. After transferred into mitochondrion, these proteins get involved in multiple processes, such as replication, transcription and translation of mitochondrial genome, and OXPHOS.

Considering the coordinate interaction between mitochondrial and nuclear genomes, mitonuclear interaction effects on phenotypes are expected and disruption of coevolved mitonuclear combination (well-known as mitonuclear coevolution hypothesis) will result in function loss (Rand et al. 2004). Indeed, several lines of evidence for mitonuclear interaction effects have been demonstrated over multiple biological scales (Wolff et al. 2014; Hill 2019). In biomedical studies in cybrid cell lines, in which nuclear genes from one population/species are paired with mitochondrial genes from another population/species, various degree of function loss of mitochondria has been demonstrated (Kenyon & Moraes, 1997). For example, a decrease in respiration by 20, 34 and 27 percent have been revealed in cybrid lines formed from human and chimpanzee, bonobo and gorilla (Barrientos et al. 1998). In addition to the mitonuclear interaction effects at cellular level, hybridization experiments also shown a fitness loss in the F2 generation. The findings are mainly from studies in marine copepod, *Tigriopus californicus* (Dey et al. 2000; Yamaoka et al. 2000). Defects in mitochondrial function like decreased cyclooxygenase (COX) activity and ATP production and fitness traits like lower survival rate of larvae and slower development were exhibited. Third, mitonuclear introgression lines, in which different mitochondrial haplotypes were introduced into various nuclear genetic backgrounds, were constructed via backcrossing in fruit fly, seed beetle and yeast as

well to investigate the mitonuclear interaction effects at interpopulation or interspecies levels (Wolff et al. 2014; Hill 2019).

Environmental effects (E) on sperm function

Environmental effects on sperm function are well known by some but neglected by other research fields. To date, a lot of attention considering environmental effects has been paid on 'lifestyle effects' on sperm function and sexually transmitted diseases, ecotoxicology of environmental pollutants and endocrine disruptors on sperm and sperm aging (Aiken et al., 2014; Yauk et al., 2008; Fraga et al., 1996; Tarín et al., 2000; Reinhardt 2007; Reinhardt et al. 2015). In addition, cumulative E effects (offspring variation based on environmental alterations of sperm cells) has also attracted more interest from researchers in sperm epigenetics (Marshall 2015; Jenkins & Carell 2012; Dada et al., 2012; Johnson et al., 2011), including several adaptive examples (Marshall 2015). However, in the field of ecology and evolution, there are few studies on environmental effects on sperm function. Among the various environments that affect sperm function, diets were one of the most representative factors that have been shown to influence sperm function. For example, antioxidant supplements in diets can improve sperm motility in boar and chicken and sperm competitiveness in crickets (Marin-Guzman et al. 2000; Eid et al. 2006). In addition, a specific case of E effects are female effects on sperm function, where females manipulate the sperm function itself, or the environment of sperm (Reinhardt & Ribou 2013, Ribou & Reinhardt 2012)

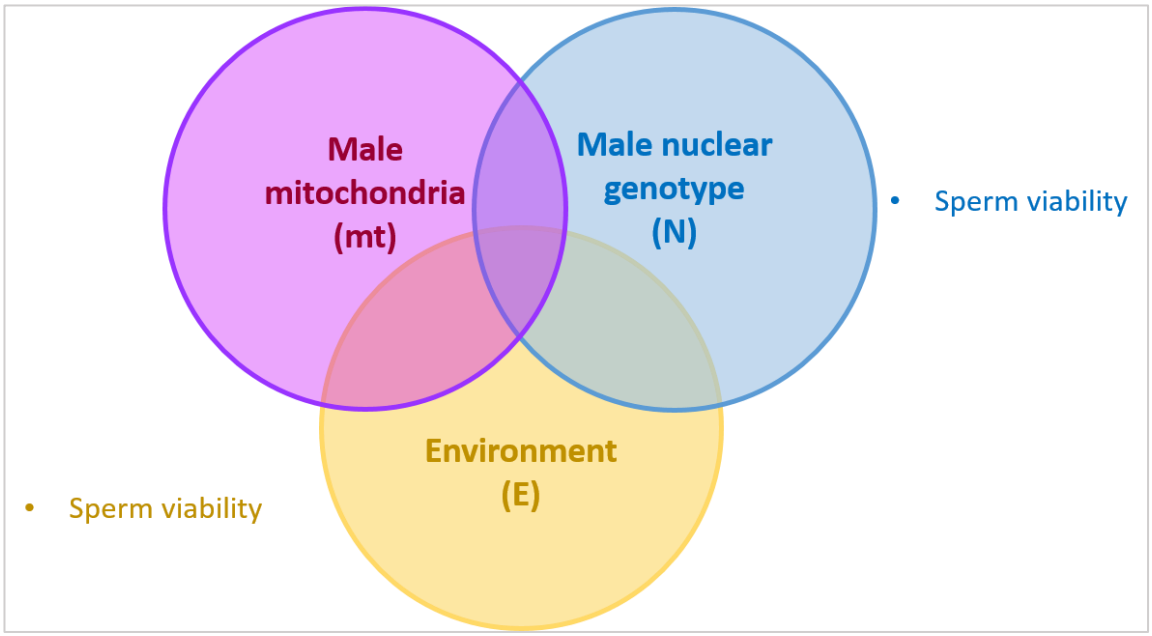
Conclusion

In conclusion, as we review in the general introduction and this chapter, sperm function including sperm metabolism can be affected by both genetic and environmental factors. In addition to nuclear effects, an increasing number of studies have suggested that mitochondrial genome influence sperm function as well.

Overwhelming evidence exists for environments to influence sperm function but it is neglected in ecology and evolution. There are also evidences for potential interaction effects on sperm function. For example, the interaction effects between mitochondrial and nuclear genomes, which were mostly tested for mitonuclear hypothesis in medicine and evolution, and the nuclear by environment interaction, which could indicate local adaptation in sperm function. There is also possibility for mito x nuclear x environment interaction effects on sperm as this three-way interaction effects have been found to influence development and lifespan.

The most common aspects of sperm function examined are sperm viability, sperm motility, fertilisation potential and effects on offspring as we summarized in general introduction. In this chapter, we reviewed previous studies to explore the evolution of sperm metabolism, with particular focus on the metabolic pathways in sperm. We found sperm metabolism is an important aspect of sperm function and might be under selection as well. Therefore, in our studies, we examined multiple aspects of sperm function, i.e. sperm viability, quality, metabolic rate, ROS production, male fertility and fertilization success. Our aim in this thesis is to disentangle the genetic and environmental components of sperm function in *D. melanogaster* at multiple levels. Specifically, in chapter 1, we established a method to assess sperm quality as the resistance of sperm to external stress, and examined sperm viability in three different sperm incubation medium for three *D. melanogaster* lines. In chapter 2, we disentangle the mitochondrial and nuclear effects on sperm metabolic rate, sperm viability, quality, male fertility and fertilization success in aging male. At last, we investigated the dietary PUFA effects on sperm volume and ROS production in *D. melanogaster* in chapter 3.

Chapter 1 Sperm quality evaluated by a novel external stress test across buffers and *Drosophila* lines



In this chapter, male genotype (N) and incubation buffer (E) were found to have an effect on sperm viability in *Drosophila melanogaster*.

Part of this part of work, method establish, was published in Frontiers in Ecology and Evolution (doi: 10.3389/fevo.2017.00165).

Abstract

Sperm quality is influenced by both genetic and environmental factors but few systematic screenings exist that examine these factors separately in Drosophila. Here, we test the effect of the most commonly used buffers on sperm viability (SV), the proportion of live sperm, and on sperm osmotic stress resistance (SOSR) in the three most commonly used Drosophila wildtype genotypes. SV differed in different buffers (phosphate buffered saline (PBS), Grace's Medium and Drosophila Ringer solution) and across genotypes (Oregon-R, Dahomey and Canton-S) but showed no interaction effect. However, for SOSR, the different genotypes dif-fered across buffers. These results suggest limited comparability between studies unless both buffer and genotype are the same. Our results also suggest that in order to maintain highest sperm quality in vitro, Orgeon R or Dahomey should be used.

1.1 Introduction

Sperm viability has been routinely measured as an assessment of sperm quality in multiple research fields (Holman 2009a; Morrell 2009). In ecology and evolution, sperm viability is increasingly assessed as a proxy of sperm quality for two main interesting questions. First, post-copulatory sexual selection, including both sperm competition and female cryptic choice, is predicted to produce adaptations in both male and female (Holman 2009a). Empirical studies have suggested that post-copulatory sexual selection affects sperm number (Gage & Morrow 2003; Alan 1993), quality (Miller & Pitnick 2002; Snook 2005) and seminal quality (Crudgington et al. 2009; Linklater et al. 2007). Second, the evolutionary causes and consequences behind sperm aging and survival has attracted increasing attention as it may play a role in sexual selection (Reinhardt 2007; Holman & Snook 2006).

Previous studies in numerous species have indicated that sperm viability is a male trait and might be affected by the genotype of the male. The key role sperm

viability play in sperm competition indicates that it can be under selection. For example, difference in the viability of sperm from male's seminal vesicle has been found between promiscuous and monogamous species, indicating sperm viability is under selection (Hunter & Birkhead 2002). Similarly, some of the factors affecting ejaculated sperm from *Teleogryllus oceanicus* are genetically variable and responsive to selection (Simmons & Roberts 2005). In *Drosophila*, male genotype has been found to have an effect on the viability of sperm, which was sampled from female after 4 days of storage (Civetta et al. 2008). Furthermore, a study on mitonuclear introgression line of seed beetle suggested that sperm viability varied significantly across mitochondrial haplotypes but not across nuclear genotype, which further indicated that the presence of the maternal genetic component of sperm viability (Dowling et al. 2007c).

On other hand, sperm viability can also be influenced by a variety of environments as well. Specifically, as a special case of environment, male and female sperm storage organs are believed to influence sperm viability. For example, seminal fluid from males has been suggested to positively affect the viability of its own sperm (den Boer et al. 2009) but might be sometimes harmful to sperm from its rivals (Holman 2009a). Female of internally inseminated species usually can store sperm in the reproductive tract for a period of time prior to fertilization, during storage it is important for sperm to remain viable, which is important for male fertility. In vitro study in *Apis mellifera* has provided evidence for female secretory fluid to improve sperm viability (den Boer et al. 2009).

Sperm viability has been widely measured in numerous studies especially after a commercial fluorescent live/dead kit (SYBR14/ propidium iodide) has become available. However, this fluorescent staining method has been found to use with pitfalls. For example, the assay itself kills sperm and the number of sperm being killed during staining assay might vary between different organs where they are extracted (Holman, 2009a; Eckel et al, 2017). Besides, there are also conceptual concerns (Eckel

et al, 2017) that proportion of live sperm right after sample collection may not be informative about the future t performance of those sperm. To address these issues, a new method was recently established based on the decrease of sperm viability over time-course in an external osmotic stress solution to assess sperm quality (Eckel et al, 2017).

Another practical problem regarding sperm viability measurement is that it is generally measured in various types of incubation buffers across studies, even for the same species (see e.g., Holman et al. 2009a, Eckel et al. 2017). For example, Drosophila Ringer's solution and HEPES-buffered saline have been used for sperm viability measurement in *D. melanogaster* (Radhakrishnan & Fedorka, 2011; Paz et al., 2013). In addition, there were more kinds of diluents used in *A. mellifera* for sperm viability measurement (Rzymski et al., 2012; Paynter et al., 2014; Gençer et al., 2014; den Boer 2015). The usage of different diluents across studies hampers the comparability between studies, especially for the same species or closely-related species. However, there is few studies exploring the effect of various incubation buffers on sperm viability of closely-related species so far, i.e. no systematic screenings of sperm viability in a range of genotype and under different environments exist. Therefore, in this experiment, with the established new method of sperm staining, we would like to investigate in three populations of *Drosophila melanogaster* 1) whether male genotype have an effect on sperm viability and quality 2) how incubation buffer, which differs in components and physiological parameters, affect sperm viability and quality. 3) whether there is an interaction effect between male genotypes and incubation buffer on sperm viability and quality.

1.2 Materials and methods

1.2.1 Fly stocks and incubation buffers

Wildtype *D. melanogaster* (Oregon-R strain) were used in this study for the development of new viability staining methods. They were maintained on standard yeast food at 25◦C and 65% RH on a 12:12 h L:D cycle. All males used in the experiment were virgins.

Three most commonly used *D. melanogaster* lines in the lab (Dahomey, Oregon R and Canton S) were used for sperm quality assay. Dahomey was collected from Benin (corresponding to BB lines of mitonuclear introgression lines), Oregon R was obtained from Dr. Bernard Moussian (University of Tuebingen) and Canton S from Dr. Marko Brankatschk (BIOTEC, TU Dresden). These lines were maintained in our laboratory for three years on a standard yeast food (corn 90 g/l, yeast 40 g/l, sugar 7164 100 g/l, agar 12 g/l, Nipagin 20 ml/l, propionic acid 3 ml/l). They were maintained as a group of 10 males in each 20 ml vial with 7 ml normal yeast food at 25◦C and 65% RH on a 12:12 h L:D cycle during experiment. All the males used were virgins aged 10 or 11 days old.

Three types of medium were used as the incubation buffer in sperm quality assay. They are specifically Grace's Medium (GM), phosphate buffered saline (PBS) and Drosophila ringer solution (DRS). The detailed composition of the buffers can be found in Table 1.1.

1.2.2 Sperm quality measurement according protocol 1

In order to find a better way to assess sperm quality, we measured the decrease in sperm viability in two different protocols. In protocol 1, we examined the proportion of live sperm in two treatment groups immediately after staining. In protocol 2, the proportion of live sperm from the same sample was measured at four successive time points after staining.

Fifteen 14-day old virgin males were taken for dissection. Both seminal vesicles from the same male were dissected in a drop of 10 µl of Grace's medium. One seminal vesicle was transferred to another drop of 10 µl of Grace's medium to release sperm out by puncturing the seminal vesicle with a fine insect pin. Almost all sperm, together with 2 µl of Grace's medium were transferred into a drop of 10 µl ddH_2O with pipette tips. Sperm sample was properly mixed via pipetting it six to seven times before stained immediately with live-dead kit (Invitrogen), i.e. 0.5 µl SYBR14® and 1 µl PI (LIVE/DEAD® Sperm Viability Kit, ThermoFisher Scientific) were added, termed as control group. After staining, the sperm sample was covered with a 22 × 22 cover slip and four different areas were haphazardly selected and images were taken at 400 × magnification with Leica Dmi8 fluorescent microscope. The same procedures were applied to the second seminal vesicle except its incubation in ddH_2O for 30 min before taking images with fluorescent microscope (Treatment group). The images were taken blinded by a colleague who was unaware of the treatment group assignment. Images were imported into imageJ (Schneider et al 2012) to count the sperm. sperm was counted manually by eye. Sperm with green head was counted as live while sperm head stained with red or partially red as dead.

1.2.3 Sperm quality measurement with protocol 2

Ten 14-day old virgin males were dissected in the same way as described in the above paragraph. Sperm from one seminal vesicle was stained immediately with live/dead kit before covered with 22 x 22 mm cover slip. One area of the sperm sample was haphazardly selected for image taken. Four images of the same area were subsequently taken immediately (t_0), 5 (t_5), 15 (t_{15}) and 30 (t_{30}) minutes later after staining. The excitation fluorescence was on for ~30 s during taking images and stayed off in between. The area of the sperm sample was selected and the images taken blind by a colleague who was unaware of the research question. Images were imported into imageJ (Schneider et al 2012) to count the sperm. sperm was counted

manually by eye. Sperm with green head was counted as live while sperm head stained with red or partially red as dead.

1.2.4 Sperm quality assay across lines and buffers

Males from three *D. melanogaster* lines were assigned into three buffer groups. We totally got 9 line x buffer groups. Males from each group were randomly picked for sperm dissection. In detail, a male was placed in a drop of 10 µl specific buffer on a glass slide and dissected for seminal vesicle. One seminal vesicle was removed and transferred to another drop of 10 µl of the same buffer on the same slide. The sperm were released out by puncturing seminal vesicle with an insect pin. Sperm were immediately stained with 1µl of Propodium iodide and 0.5 µl of SYBR 14 (1:50 dilution; ThermoFisher Scientific). A 22x22 mm cover slip was added. A 22 x 22 mm cover slip was placed on the sample before taking images on an epifluorescence microscope (Leica DMi8, Germany). One area of the sample showing at least approximately 20 sperm cells was haphazardly chosen. Four images of the same area were taken immediately (t_0), 5 (t_5), 15 (t_{15}) and 30 (t_{30}) minutes after staining. The excitation light was switched off between each time point. Images were imported into imageJ (Schneider et al 2012) to count the sperm. sperm was counted manually by eye. Sperm with green head was counted as live while sperm head stained with red or partially red as dead. In this study, the proportion of live sperm at t_0 was assessed as sperm viability, and the alteration in sperm viability over 30 minutes was analysed as sperm quality. At last, around 12-16 males per line x buffer group were dissected, and in total 118 individuals were dissected.

Table 1.1 Composition and pH of three different incubation buffers used in this experiment.

Composition	**Grace's medium (GM)**	**Phosphate buffer saline (PBS)**	**Drosophila ringer solution (DRS)**
Product number	G8142-500ML (Sigma-Aldrich®)	8561.0005 (Chemsolute®)	(Cold Spring Harbor Protocols)
NaCl		81.81	2.7
CaCl2 (anhydrous)	1.0		0.33
MgCl2 (anhydrous)	1.068189		
MgSO4 (anhydrous)	1.357585		
KCl	2.24	2.013	13.6
NaHCO3	0.35		
NaH2PO4	0.876923		
Tris-base- Alanine	0.2		1.21
L-Alanine	0.225		
L-Arginine HCl	0.7		
L-Asparagine	0.35		
L-Aspartic Acid	0.35		
L-Cystine 2HCl	0.025		
L-Glutamic Acid	0.6		
L-Glutamine	0.6		
Glycine	0.65		
L-Histidine	2.5		
L-Isoleucine	0.05		
L-Leucine	0.075		
L-Lysine HCl	0.625		
L-Methionine	0.05		
L-Phenylalanine	0.15		
L-Proline	0.35		
L-Serine	0.550		
L-Threonine	0.175		
L-Tryptophan	0.1		
L-Tyrosine□2Na	0.720		
L-Valine	0.1		
D-Biotin	0.00001		
Choline Chloride	0.0002		
Folic Acid	0.00002		

myo-Inositol	0.00002		
Niacin	0.00002		
D-Pantothenic Acid	0.00002		
p-Aminobenzoic Acid	0.00002		
Pyridoxine□HCl	0.00002		
Riboflavin	0.00002		
Thiamine	0.7		
Dextrose anhydrous	0.4		
D-(-)-Fructose	0.055		
Fumaric acid	0.37		
Ketoglutaric acid	0.67		
L-(-)-Malic acid	0.06		
Sucrose	26.68		
pH	6.2	7.4	7.2

1.2.5 Statistical analysis

All the data analysis was performed in R, version 3.3.3 (R Development Core Team, 2016). For sperm quality assays using two different protocols. Data were fitted into a GLMM (generalised linear mixed model) with "binomial" error distribution using lme4 package (Bates et al. 2014). The proportion of live sperm were treated as response variable with cbind function (number alive| number dead). For data collected with protocol 1, treatment was treated as fixed factor while male ID as random factor. Image ID was further added as another random factor for overdispersion correction (Browne et al. 2005). For sperm viability data collected with protocol 2, time was treated as an ordinal fixed factor while male ID and image ID as random factors.

For sperm assays across lines and buffers, sperm viability data (t_0) was fitted into a GLMM (generalised linear mixed model) model with "binomial" error distribution using lme4 package (Bates et al. 2014), in which buffer, lines and the interactions between them were treated as fixed factors and male id as random factor. Image ID was added as another random factor for overdispersion correction (Browne et al.

2005). For sperm quality, all data (t_0, t_5, t_{15}, t_{30}) were used and fitted into a GLMM model with "binomial" error distribution. Buffer, lines, time and the interactions between them were treated as fixed factors and male id as random factor. Image ID was added as another random factor for overdispersion correction. The fitness of goodness of the all the models was examined visually with graphs. Step-wise model reduction was performed backwards to get the best fitting model. Significance of terms in the models was examined with type 3 ANOVA in car package (Fox & Weisberg 2011). The fitted estimates and the standard errors predicted from the model were obtained with effects package (García-González & Simmons 2005).

If the three buffers or three genotypes overlap in SV or SOSR values, the results might not be significantly different. However, concluding that no significant differences exist between buffers or genotypes could be false, if only two buffers or two genotypes are being used (a likely case if one lab uses one buffer and one genotype, another lab uses another buffer and genotype). To uncover such potential effects, we provide a simple robustness analysis, akin to a lottery what could happen if one draws a combination of two buffers and two genotypes and analysed the data then for these nine combinations.

1.3 Results

1.3.1 Sperm viability decreased when exposed to external stress (protocol 1) and over a time series (protocol 2)

Sperm viability significantly decreased when sperm were exposed to external stress for 30 minutes compared to control group (Figure 1.1). There was also a significant decrease in sperm viability over a time course of 30 minutes (Figure 1.2).

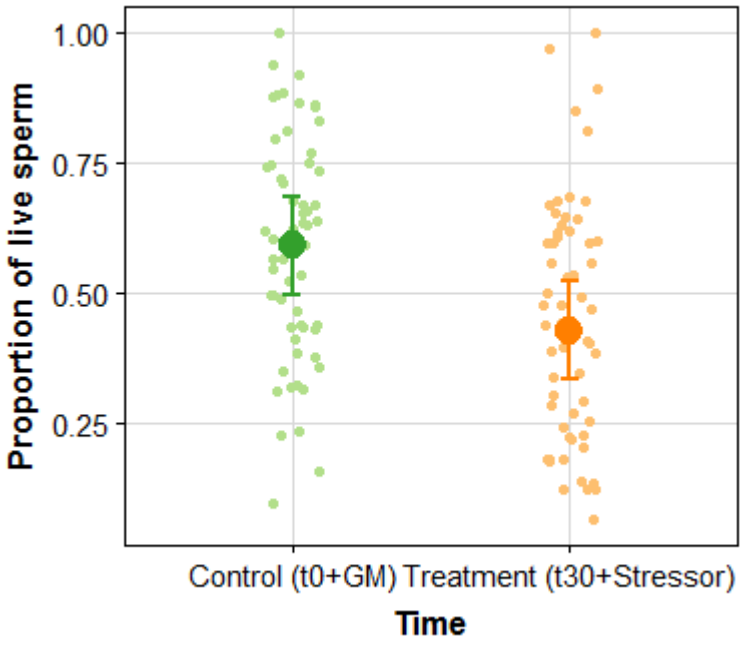


Figure 1.1 Sperm viability of two treatment groups according to protocol 1 in *D. melanogaster*. Viability of sperm from two seminal vesicle of the same male was examined at different buffers (PBS and stress solution buffer) at different time (0 and 30 mins after staining). Data were shown as mean ± SE, with lighter symbols showing individual data points.

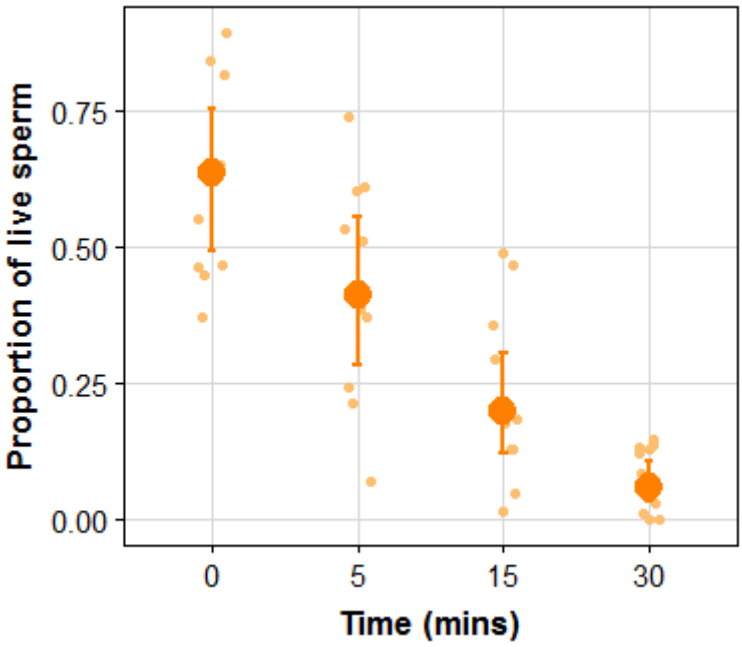


Figure 1.2 Sperm viability of the same chosen area examined at 4 different time points (0, 5, 15 and 30 mins) according to protocol 2 in *D. melanogaster*. Data were shown as mean ± SE, with lighter symbols showing individual data points.

1.3.2 Male genotype and incubation buffer affect sperm viability

Sperm viability differed significantly across *Drosophila* lines (Table 1.2). Specifically, sperm viability was the highest for Oregon R (mean 88.5% across the three buffers). In contrast, Canton S showed the lowest SV, with a pooled mean of 72.2% across buffers. The incubation buffer had an effect on sperm viability as well (Table 1.2). Sperm viability was the highest when incubated in Grace's medium regardless of line, followed by PBS buffer. The viability of sperm incubated in DRS was the lowest among all three incubation buffers. We found there was no genotype x buffer interaction effects on sperm viability (Table 1.2, Figure 1.3).

Table 1.2 Effects of male genotype (line), incubation buffer (buffer) and the interaction between them on sperm viability.

	Sperm viability		
Fixed effects	**Df**	**χ2**	**P value**
Line	2	9.671	**0.008**
Buffer	2	15.276	**<0.001**
Line: buffer	4	4.396	0.355

The significant effects are indicated in bold.

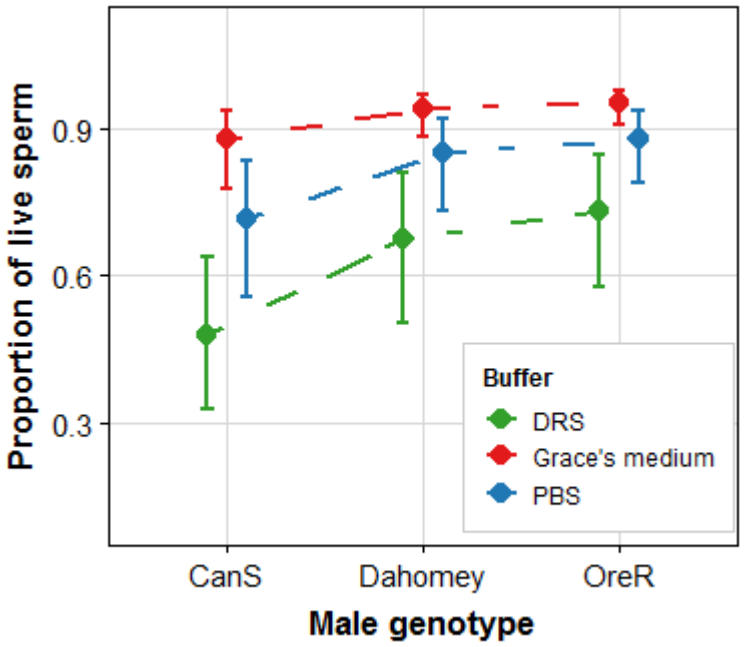


Figure 1.3 No interaction effect between male genotype and buffer on sperm viability (t0). Predicted values from model were shown as mean± SE.

1.3.3 Genotype and buffer did not influence sperm quality

Sperm viability declined significantly over 30 minutes across all lines and incubation buffers (sperm quality, time, $p<0.001$). SOSR showed no significant effect (buffer x line x time, $p = 0.376$, Table 3, and no other interaction effects (Figure 1.4, Table 1.3). Our robustness analysis additionally showed in 1 out of 9 buffers-genotype pairings significant buffer x lines effects (Table 1.4).

Table 1.3 Effects of male genotype (line), incubation buffer (buffer) and the interaction between them on sperm quality.

	Sperm quality (Decrease in SV over time)		
Fixed effects	**Df**	**χ2**	**P value**
Line	2	5.454	0.065
Buffer	2	22.997	**< 0.001**
Time	3	93.132	**< 0.001**
Line: buffer	4	3.436	0.488
Line: time	6	6.440	0.376
Buffer: time	6	8.795	0.185
Line: buffer: time	12	12.912	0.375

The significant effects are indicated in bold.

Table 1.4 The significance of G x E effects on sperm quality for all nine combinations of 2 lines x 2 buffers (after removal of combinations of a specific incubation buffer and a specific line)

	DRS	Grace's medium	PBS
Can S	p= 0.487	p= 0.757	**p=0.0427**
Dahomey	p= 0.644	p= 0.381	p= 0.750
Ore R	p= 0.266	p= 0.411	p= 0.182

The significant effects are indicated in bold and grey shaded.

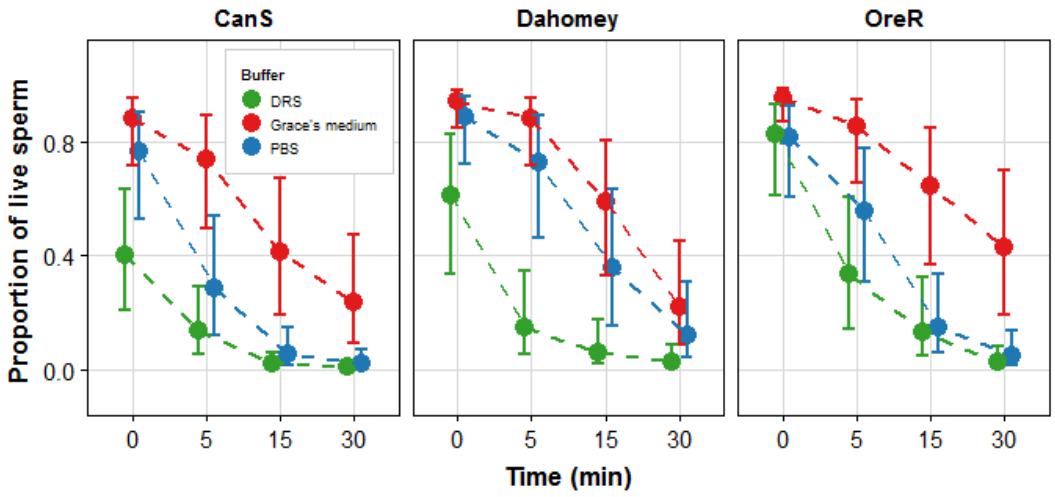


Figure 1.4 No buffer by male genotype (Canton S, Dahomey and Oregon R) interaction effects on sperm quality. Predicted values from model were shown as mean± SE.

1.4 Discussion

In this study, first we characterized the quality of sperm based on the performance with two protocols, one was examined with the external stress protocol, another with a time series protocol. We found both methods were able to detected the change in sperm viability. However, we chosen time-series method for sperm quality assessment across buffers and genotypes as it is more suitable for assessment of sperm future performance. For sperm quality assessment, we found a significant

effect of buffers on sperm viability in *Drosophila*, and among the buffers used, GM produced the best results. It also produced the smallest differences between lines. Hence, we can generally recommend GM for those protocols that require *D. melanogaster* sperm to be alive as long as possible. We also found sperm viability to vary across genotypes, with Oregon R males having the highest, and Canton S male the lowest values. These data suggest that care should be taken when generalising sperm viability results from one genotype to another. Moreover, we found there were no effects of male genotype, incubation buffer and their interaction on sperm quality. Below we will discuss our findings in detail.

1.4.1 Genotype and sperm function

Genetic components, both nuclear and mitochondrial components, of sperm viability has been demonstrated in numerous species, for example in *T. oceanicus* (García-González & Simmons 2005). In our study, we found that sperm viability varied across males of different genotypes, with Oregon R line had the highest sperm viability and Canton S the lowest. Our finding is in line with a previous study on *D. melanogaster*, which showed that male genotype had an effect on the viability of sperm sampled from female 4 days later after mating (Civetta et al. 2008). There is another study in *D. melanogaster* showing sperm viability within female reproductive tract mirrored male genotype (Civetta et al. 2008). The reasons behind the variation in sperm viability is still unclear. A possible explanation is that variation in sperm viability is a response to post-copulatory sexual selection as sperm viability play a role in sperm competition (Hunter & Birkhead 2002; García-González & Simmons 2005). Surprisingly, we found that male genotype did not influence the decrease of sperm viability over time when exposed to external stress, and the reason behind is unclear.

1.4.2 Buffer chemistry and sperm function

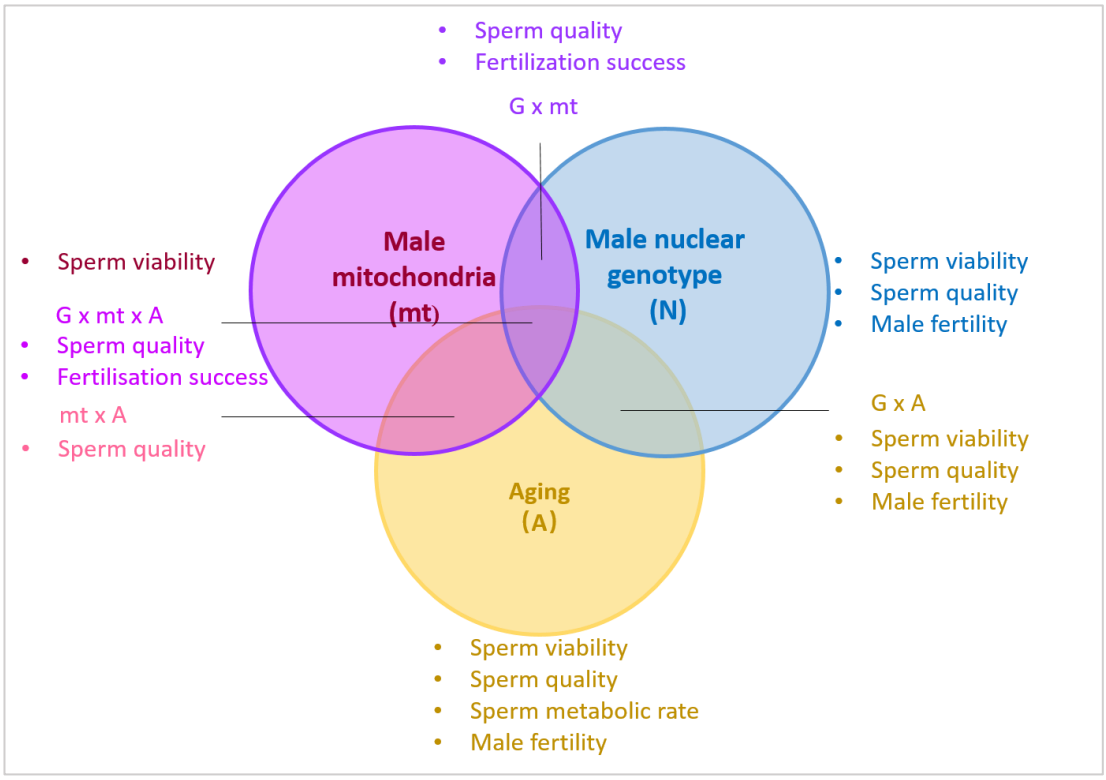
Our study may also shed some light on the specific differences between buffers. For example, GM, which awarded the highest sperm viability, contains free amino acids and carbohydrates, whereas DRS generating the lowest sperm viability is lacking both. The reason why GM was much better than the other buffers is seemingly not related to the presence of Na+, K+, PO43- or Cl- ions as these are found in all three buffers. The superiority of GM is, therefore, related to either the presence of certain sugars or certain amino acids. One hand, seminal fluid and female secretory fluids have been suggested to enhanced sperm viability (den Boer et al. 2008; den Boer et al. 2009), and free amino acids and carbohydrates have been revealed to be present (Sexton et al. 1971; Johnson et al. 1972; Størset et al. 1978; Rodger & White 1975). On the other hand, supplementation of carbohydrates in extender for sperm cryopreservation and storage have been found to increase sperm quality, for example membrane integrity, viability, motility and metabolism (Størset et al. 1978; Rodger & White 1975; Bucak & Tekin 2007; Aboagla & Terada 2003; Hu et al. 2010; Gómez-Fernández et al. 2012). The effects of carbohydrates and amino acids components on sperm viability might be due to the potential roles in sperm metabolism. Both sugars and amino acids (Arginine) have previously been invoked in contributing to *Drosophila* sperm metabolism (Geer et al., 1975; Osanai et al., 1993) and future experiments may be useful to disentangle their effects.

1.4.3 Male genotype by incubation buffer interaction effects on sperm function

The sperm traits that are mostly commonly examined for G x E effects are sperm length, sperm motility, sperm metabolism and sperm defence ability (P1) (Bucak & Tekin 2007; Aboagla & Terada 2003; Hu et al. 2010; Gómez-Fernández et al. 2012). However, few studies investigated G x E effects on sperm viability. In our study, we found that males of different genotypes did not vary in sperm viability and quality

when the data from all line x buffer combination were taken. However, our robustness analysis showed that the lack of a statistical interaction effect may have to do with the fact that we used three lines and buffers and further increased variation within lines by examining SV over time. Our simple robustness analysis, akin to a lottery what could have happened if other researchers had drawn other combinations than we had, showed that 1 out of 9 genotype x buffer combinations would result in noncomparable results (i.e., genotype x buffer effects). Specifically, genotype x buffer interaction effect has been found on sperm quality after removal of data from Canton-S line and PBS incubation buffer.

Chapter 2 Mitonuclear epistasis modifies sperm function in aging male in *Drosophila melanogaster*



In this chapter, multiple aspects of sperm function were found to be affected by both genetic factors (mt, N and mt x N), male age (A) and interactions between them.

Abstract

Nuclear and mitochondrial genomes affect health and aging, including in male reproduction. Evolutionary theory makes the medically important prediction that mt mutations can accumulate in a population if they only affect males. We disentangled mt and nuclear effects on male reproductive aging by analysing five fertility and sperm metabolic traits in in Drosophila melanogaster introgression lines whose nuclear and mt genomes were mix-and-matched. We i) quantify the dramatic effects of male reproductive aging and ii) demonstrate for the first time that, in addition to the nuclear genome, male reproductive aging was affected mitonuclear epistasis. We iii) show that mitonuclear coevolution did not rescue reproductive aging confirming that evolution cannot target male fertility via population structure and iv) not all male reproductive traits were affected by mitonuclear epistasis in the same way. Our results showing that mitonuclear effects can modulate male reproductive aging have clinical and evolutionary implications.

2.1 Introduction

The mitochondrial genome (mtDNA) was traditionally considered to be a bystander and selectively neutral in adaptive evolution. However, numerous studies across species suggested that mtDNA might be another source of variation of health and other phenotypes, including morphological, metabolic, fitness and life-history traits (Ballard et al. 2007; Camus et al. 2012; Dobler et al. 2014; Patel et al. 2016). This notion applies to both sexes but recently male reproductive traits like sperm have attracted particular attention and are believed to be more suitable for examination of mtDNA effects for two main reasons. First, maternal transmission of mitochondria facilitates the accumulation of mtDNA mutations that are deleterious to males but beneficial, neutral or only slightly deleterious to females. Therefore, the sex-biased mutation load, also known as “mother’s curse”, is predicted to be more severely expressed in traits that only males possess (Camus et al. 2012; Gemmell et al. 2004;

Camus et al. 2015). Second, reproductive traits like sperm, are believed to be susceptible to mtDNA genetic variance as they are highly energetically reliant (Ruiz-Pesini et al. 1998; Ruiz-Pesini et al. 2000b; Turnbull et al. 2002). Empirical studies were conducted either in natural population or experimentally constructed lines and mitochondrial effects on male reproduction, with particular focus on sperm traits, have been found in some (Dowling et al. 2007c; Yee et al. 2013; Tourmente et al. 2017) but not in other studies (Dowling et al. 2007a; Mossman et al. 2010).

As a primary site of both ATP but also reactive oxygen species (ROS) generation, mitochondria play a central role in affecting and regulating critical phenotypic traits such as aging (Guarente 2008). However, the function of mitochondria requires for cooperation between mitochondrial and nuclear genomes. For instance, four multisubunit complexes in the electron transport chain, which are essential for oxidative phosphorylation, are jointly encoded by both genomes (Taanman 1999). Second, some mitochondrial translational machineries that are critical to mitochondrial DNA replication and mitochondrial RNA transcription contain both nuclear and mitochondrial components (Levin et al. 2014). Lastly, a variety of signalling pathways between these two genomes exist to manipulate intergenomic communication (Poyton & McEwen 1996). Therefore, variation in phenotypic traits is caused by the interaction between mitochondrial and nuclear genetic variance. As predicted, mitochondria have been often found to affect aging, fitness and development via its interaction with nuclear genome, sometime exhibited sex-specific patterns (Rand et al. 2006; Dowling et al. 2007b; Immonen et al. 2016; Mossman et al. 2016; Đorđević et al. 2017).

High energetic dependence of eukaryotes on mitochondrial respiration requires for optimization of mitochondrial function, which could result in coevolution of mitochondrial and nuclear genome. There is compelling evidence across species for mitonuclear coevolution over evolutionary time, ranging from fungi and plant to animals, including humans (Blier et al. 2001; Dowling et al. 2008; Reinhardt et al. 2013;

Dobler et al. 2018). The mitonuclear coevolution model predicts that mutations are more likely to occur within mitochondrial genome and so select for compensatory nuclear mutations; incompatibilities between genomes may then lead to defected performance (Rand et al. 2004). Empirical support for this idea comes from reduced respiratory function in nuclear cytoplasmic hybrids (cybrids) (Dey et al. 2000; Yamaoka et al. 2000), as well as their altered survival, viability, development and fitness in incompatible mitonuclear combinations (Reinhardt et al. 2013; Dobler et al. 2018; Wolff et al. 2014) but idiosyncratic mitonuclear effects were also found in some studies, especially in male-specific traits (Dobler et al. 2014; Immonen et al. 2016; Reinhardt et al. 2013).

A final complicating issue to predict the phenotype from mitonuclear combinations is that mitochonrial and nuclear genome might have independent, and different, but also epistatic effects on reproduction in aging males. This is true for both the accumulation of deleterious mutations as well as for any possible trade-offs between reproduction and health (or lifespan). For example, nucleus and mitonuclear interaction but not mitochondria alone were found to affect longevity in *Drosophila* (Rand et al. 2006). A second effect related to aging could pertain to what Immonen et al. (2016·) call indirect genetic effects - male mitochondrial effects that translate into fitness variation in females (Immonen et al. 2016). For example, the age-related change in reproductive tissues, sperm and seminal fluid quantity and quality may differ between mitochondrial haplotypes and so caused fitness effects. Immonen et al. (2016) found large effects of mitonuclear interaction of male senescence upon reproduction in their mates in seed beetle (Immonen et al. 2016). We, therefore, i) undertook to test for the presence of mitonuclear epistasis, ii) to separate these from nuclear and mitochondrial effects *per se*, iii) to test for mitonuclear coevolution, while iv) simultaneously accounting for male age. By using several male reproductive parameters, that may be influenced to a varying degree by the mitochondrial and the nuclear genome, we are able to provide a comprehensive

picture of mitonuclear interaction effects on male reproduction in *Drosophila melanogaster*.

Sperm traits including sperm viability, sperm quality and sperm metabolism are important proxies for male fertility in medicine and biology. Specifically, sperm viability plays a key role in sperm competition (Hunter and Birkhead 2002), which determines fertilization outcome in polygamous species. Further studies suggested that the resistance of sperm to external stress (what we call sperm quality) are more suitable and precise than sperm viability *per se* to represent sperm quality and therefore male fertility (Eckel et al. 2017). Sperm metabolism is believed to undermine certain sperm traits and closely linked to male fertility. One hand, ATP produced by metabolic pathways in sperm is the direct major energy source for sperm motility (Bohnensack and Halangk 1986). On the other hand, reactive oxygen species (ROS) generated as by-product can both positively and negatively affect sperm function. Therefore, in this study, we examined sperm viability, sperm quality and sperm metabolism as reproductive traits at the sperm cell and ejaculate levels to test their relatively distance towards male fertility.

2.2 Materials and methods

2.2.1 Fly stock and maintenance

Outbred stocks of three distinct *D. melanogaster* populations were used to create nine mitonuclear genotype combinations (mitonuclear introgression lines, Figure 2.1). These three source populations were originally and separately collected around Coffs Harbour, Australia (Williams et al. 2012; Dowling et al. 2014), in Benin (former Dahomey, Africa) (Clancy 2008) and around Dundas (near Hamilton), Canada (MacLellan et al. 2009). Each population was cultured as large populations (NE > 1500) to maintain the genetic variation. Based on geography, the mitonuclear genotype of

Australian stocks was assigned as AA (mitochondrial haplotype A with nuclear genotype A), the same to Benin (BB) and Canada (CC) stocks (Figure 2.1). The isogenic reference female stock was used in male fertility and fertilization success to reduce effects of genetic variation in females (derived from a bw-LHM population; Rice et al. 2005). The outbred but genetically identical tester females were generated by crossing virgin females from isogenic line one with males from isogenic line two. The tester females were all 5-day old virgins and used to mate with introgression males. All the effects examined were supposed to arise from introgression males because there was no genetic variation in females. During our experiments, all the stocks used were cultured in artificial incubators under conditions of 25°C and 65% RH on a night: day cycle of 12:12 hours standard corn-yeast-sugar medium (corn 90 g/l, yeast 40 g/l, sugar 7164 100 g/l, agar 12 g/l, Nipagin 20 ml/l, propionic acid 3 ml/l) with additional live dry yeast.

2.2.2 Construction of mitonuclear introgression lines

The mitonuclear introgression lines we used in the study was constructed with the three source populations (Figure 2.1). Briefly, 45 of the virgin females with desired mitochondrial haplotype were crossed with 45 of males that harbour the desired nuclear genotype. Each line was created three times independently to avoid non-random sampling effects in the initial 45 females (which harbour the whole variation in their mitochondrial genomes for each introgression line), ending up with 27 lines in total. Gene flow was avoided as well upon creation via keeping the lines separated from each other. For subsequent generations, 45 of virgin females from each mitonuclear introgression line were backcrossed with 45 males from each of the source population with desired nuclear genotype. More than 99.99% of the initial female nuclear genome were replaced and the desired mitochondrial genomes were paired with a complete new nuclear background. The backcrossing was continued after 17 generations to avoid potential processes that could result in coadaptation

between mitochondrial and nuclear genomes. All lines were treated with tetracycline added to the food (0.2 g/l solved in 40 ml Ethanol) for three generations to remove *Wolbachia*. Elimination of *Wolbachia* was further confirmed by PCR-tests after the treatment (Richardson et al. 2012).

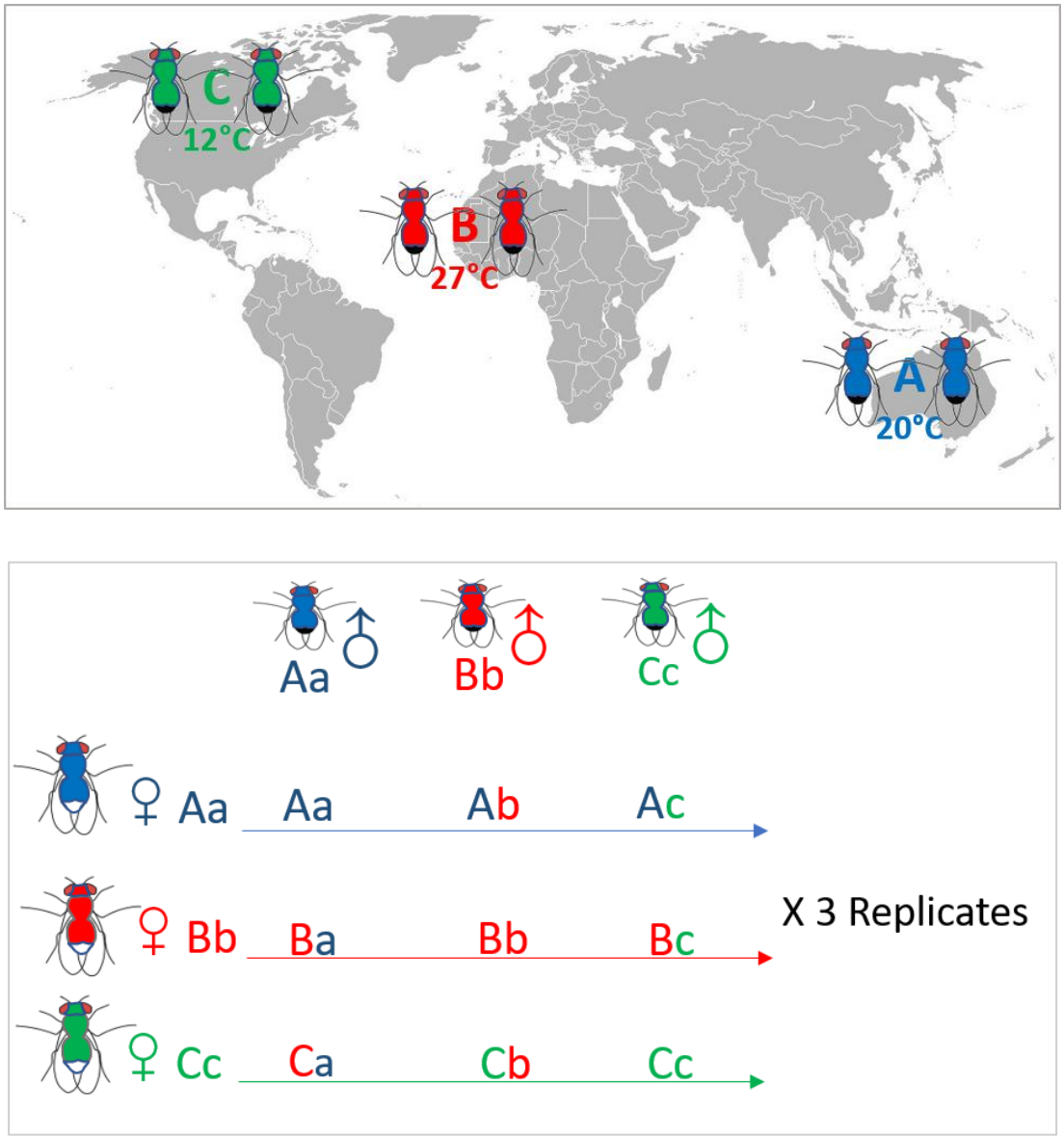


Figure 2.1 The origin of three source populations of *D. melanogaster* (upper), and mitonuclear introgression lines (lower). The source population were separately collected from Australia (A), Benin (B) and Canada (C), where the local temperature is different. The constructed mitonuclear introgression lines were maintained in 3 replicates in the lab. Capital letters denote mitochondrial haplotypes while small letters denote nuclear backgrounds where mitochondria were placed in.

2.2.3 Experimental design and procedure

In order to examine the separate genetic effects of the mitochondrial and the nuclear genome on male reproductive aging, we assayed the fertility of males aged 5 days or 6 weeks from each of the 27 mitonuclear introgression lines.

2.2.3.1 Sperm viability (SV) and quality (SQ) assays

Fifteen males per line were examined in two blocks per age group. In detail, 15 males of appropriate age from all 27 lines were picked at random to examine sperm viability (SV), the proportion of live sperm per sample and sperm quality (SQ) *sensu* Eckel et al. (2017), the temporal change in SV in an osmotic stressor medium.

Each male has two seminal vesicles where mature sperm are stored. Both seminal vesicles were dissected in 10 µl of phosphate buffer saline (PBS) on a glass slide. To measure SV, one vesicle was transferred to a new slide and the sperm released into a drop of 10 µl PBS buffer and then immediately stained with live-dead kit (Invitrogen), i.e. 0.5 µl SYBR14® and 1 µl PI were added, termed as t_0 group. After staining, the sperm sample was covered with a 22 × 22 cover slip and incubated in the dark for 5 minutes before taking pictures on an epifluorescence microscope (Leica DMi8, Germany). For each sample, five different areas were haphazardly selected and images were taken at 400 × magnification (a highly repeatable procedure resulting in sufficient precision in *D. melanogaster*, Eckel et al. 2017). Live sperm stain green, dead sperm stain red and the number of red and green sperm were manually counting on photographs using ImageJ V. 1.34 software (Schneider et al 2012). A complete or largely green head was counted as live, the rest as dead. Later we corrected the counting method by considering double stained sperm as dead for 80 randomly selected pictures, due to the strong correlation of results between the two counting methods (Figure S1), we believe that the counting methods did not influence our final results.

To assess SQ we modified the protocol in (36) and only analysed SV after 30 minutes in a stressor medium. Briefly, the other vesicle was transferred into to a drop of 10 µl of mixed stress buffer (PBS: distilled H_2O 1:1.5) on a new glass slide. Sperm were released by puncturing the vesicle with a fine insect pin and spread evenly by shaking the slide gently and horizontally. The sperm sample were left in the stress buffer in a moisture chamber for 30 minutes and stained thereafter, termed as t_{30} group. SQ is the resistant ability of sperm to osmotic stress. In our design it is simply analysed as the time treatment effect.

Depending on mortality of males during dissection and or the successful dissection of both vesicles simultaneously we reached sample sizes of ca. 15 young and 10 old males. Due to the time-consuming dissection procedures, the SV measures of 5-day young males extended for 14 days, that of 6-weeks old males for 8 days. To account for the potential variance caused by different days, we used dissection day as a random factor in our model.

2.2.3.2 Sperm metabolic rate (SMR) assay

We analysed the metabolism of sperm from male seminal vesicle was characterized using NAD(P)H autofluorescent lifetime imaging technique (FLIM) as described previously (Reinhardt et al. 2017). Based on the observation that NAD(P)H is i) autofluorescent (as opposed to its oxidised form NAD^+), ii) in cells found in either free form or bound to proteins involved in glycolysis and iii) that during metabolism (total NAD(P)H reduction) free NAD(P)H will be used up before bound NAD(P)H (Blinova et al. 2005), we used the redox ratio, the proportion of protein-bound over total NAD(P)H as an expression of metabolic state of cells (see Ribou & Reinhardt 2012, Reinhardt & Ribou 2013 for previous applications).

In total, five males per line were used for sperm metabolic assay. The entire reproductive tract including seminal vesicles of each male was dissected in 20 µL of PBS buffer on a glass slide. The dissection was done gently to ensure the integrity of the reproductive tract system. The reproductive tract was transferred to a new drop of 20 µL of PBS buffer and covered with 22 x 22 mm cover slip. A slight amount of clay was added to the four corners of cover slip as stands to avoid the rupture of the seminal vesicle sample. At last, the cover slip was sealed with nail polish and examined with FLIM.

Our FLIM setup consists of a Zeiss Axio Examiner Z microscope (Zeiss, Germany) and a 450/30nm dichroic filters with two-photon excitation. The sample is placed under the microscope and a wavelength of 740 nm excites NAD(P)H in a two-photon setup. Whole sperm vesicle was imaged using the following fixed settings: 13.2 mV laser power, under 40x magnification with 1 X zoom. Each image taken as a cumulative stack of 70 frameworks. The fluorescence lifetime was obtained for every pixel with an in-built single-photon counting correlation device. Data files were imported into SPCimage software (Becker & Hickl GmbH, Berlin, Germany) and fit to a bi-exponential decay which provided the proportional contribution of each of the two lifetimes to the total decay. A pixel with a larger number of photons within the seminal vesicle was chosen to get a fixed shift value. The shift value was obtained by setting the bin number of the chosen pixel to 5 and further used for matrix decay calculation of all the pixels in the image. A region of interest (ROI) was manually defined to include pixels exclusively from sperm samples. The mean proportion of free NAD(P)H in the ROI was derived and used as estimate of sperm metabolic rate. An alternative approach of using phasor plots (Ranjit et al. 2018) to select pixels exclusively from sperm sample was also used to define ROI. A strong positive relationship was found between these two different approaches (Figure S2).

2.2.3.3 Male fertility (MF) and fertilization success (FS) assay

The effects of introgression males were characterised as well on male fertility and fertilization succession indirectly expressed by their reference female mating partner. Male fertility (F) is the number of offspring that a male induces in these isogenic females (N=601 males). FS is the proportion of fertilised eggs from the total (N=601 males). Briefly, fifteen focal, virgin males from each introgression lines were collected and kept sexually isolated until being mated individually with isogenic reference females. Because of escape, mortality or a total failure to produce offspring, we ended up with ca. 15 young and 7 old males for each line. Eggs were counted blind with respect to each line. In detail, when focal males were 5 days or 6 weeks old, they were paired individually with 5-day old reference females in separate vials at day 1 and left to stay together for 24 hours. At day 2, males were discarded and female were transferred to a new vial for egg laying, and at day 3 females were transferred to a third vial. The number of eggs for three days were recorded with a binocular microscope and a counter. After egg counting, vials with eggs inside were maintained at an artificial incubator until offspring hatched from eggs were counted a week later.

2.2.4 Mitochondrial haplotype reconstruction

The initial classification of mitochondrial haplotype was simply based on geography (geography-based classification). However, in order to confirm the grouping at molecular level, mitochondrial genomes of all 27 introgression lines were sequenced (Genbank NO. PRJNA532313, Voigt et al. unpublished data). Thirty-four single nucleotides polymorphisms (SNPs) in total in mitochondrial genomes were discovered across these lines. To subdivide the 27 introgression lines based on variance in SNPs, which was particularly reflected by each reproductive parameter measured in our study, principal component analyses (PCAs) were conducted first to reduce the dimension of variance between SNPs. Based on PCAs results, all 27

introgression lines were then clustered into three new mitochondrial haplotype groups and the new classification was consistent for all the reproductive parameters examined (SNPs-based, Figure S3). Specifically, the main differences were that in SNPs-based classification AA3 line was much closer to mitochondrial haplotype B while CA1 and CA3 line more likely to be mitochondrial haplotype B rather than C (Figure 2.2). Data in our experiment were further analysed for both classifications of mitochondrial haplotypes. Results were present mainly for the SNPs-based classification but comparison was performed where different results were obtained from both classifications.

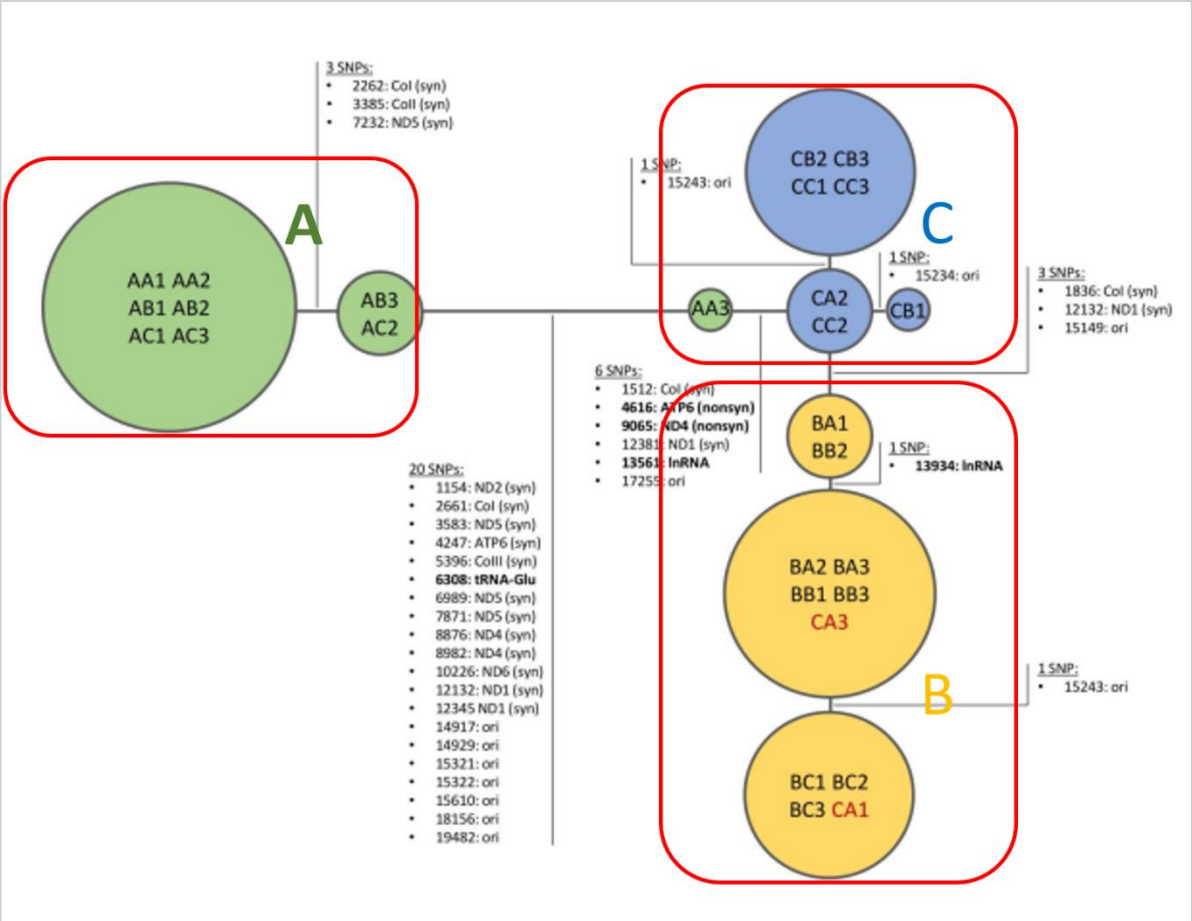


Figure 2.2 The reconstruction of mitochondrial haplotypes of the mitonuclear introgression lines based on PCAs analysis (image from Voigt et al. unpublished data). The 34 single nucleotides polymorphisms (SNPs) that are different in frequency between 27 introgression lines were listed. The original geography-based grouping was indicated by circles of different colours: Males from Australia with A mitochondrial haplotype was in green circles while males from Benin with B mitochondrial haplotype in yellow circles and male with C haplotype in blue circles. The new SNP-based grouping was circled with red rectangle boxes and indicated with letters A, B and C separately.

2.2.5 Statistical analysis

The crossed experimental design enables us to analyse our data with linear (LMM) or generalised linear mixed models (GLMM) where mitochondrial haplotype (mito), nuclear genotype (nuc), male age (age), stress treatment where necessary (treatment), and their interaction were fitted as fixed effects. A random structure was also included in these models. Specifically, sperm viability and sperm quality data were fitted into GLMM model with cbind function (Binomial error distribution), where introgression replication lines (line) blocks (block), repeated males (male ID) and repeated measurements (obs ID) were treated as random factors. Sperm metabolism data was fitted into LMM model (Gaussian error distribution), where line was treated as random factor. Male fertility data was modelled using GLMM model (Poisson error distribution), in which line and male ID as random factors. Fertilization success data were analysed with GLMM model with cbind function (Binomial error distribution), in which line and male ID as random factors. All of our models were implemented in R version 3.3.3 (R Development Core Team, 2016) with lmer4 package (Bates et al. 2014). For the binomial and poisson models, overdispersion was estimated as the sum of squared Pearson's residuals divided by the residual degrees of freedom. If overdispersed, individual observation-level random effect (i.e. male ID or obs ID) were included into the model to account for that (Browne et al. 2005). The goodness of fit of models was validated by visual inspection. The significance of terms

in the models was assessed with type III Anova incorporated in car package (Fox & Weisberg 2011) which analysed the deviance based on the χ2 distribution. Because we are interested in mito x nuc x age three-way interaction, model reduction was performed backwards only when the three-way interaction was not significant. Based on the findings of these models, further analyses were performed separately for young and old males with GLMM/LMM models to inspect possible genetic effects within age group. Moreover, we also fitted same type of models to examine the mitonuclear coevolution hypothesis, in which a two-level factor called coevolutionary history, male age and their interaction were fitted as factors. The estimates and its standard error predicted from models were obtained with effects package (García-González & Simmons 2005).

2.3 Results

2.3.1 Mitochondrial haplotype influences sperm viability, nuclear genotype influences aging in sperm viability

Sperm viability was significantly lower in old than young males (Table 2.1). The effect of age on sperm viability was also contingent on the nuclear genotypes as the magnitude of decrease differed across nuclear genotypes (Figure 2.3). Mitochondrial effect but not its interaction effect with nuclear background on sperm viability were detected (Figure 2.3). However, geography-based classification of mitochondrial genomes revealed no mitochondrial and its interaction effects on sperm viability. The different results between the two classification indicate that the 20 SNPs separating AA1 from mitochondrial haplotype A and those 4 SNPs separating CA1 and CA2 from haplotype C might play a role. Sperm viability did not differ significantly between males with a coevolved compared to mismatched mitonuclear genotype (Table 2.2).

Table 2.1 Male age, mitochondrial, nuclear genome and their interaction effects on sperm viability.

Sperm viability (629 males)					
SNPs-based				**Geography-based**	
Fixed effects	**Df**	**χ2**	**P value**	**χ2**	**P value**
Mito	2	6.616	**0.037**	2.621	0.270
Nuc	2	18.561	**< 0.001**	16.623	**< 0.001**
Male age	1	27.866	**< 0.001**	29.406	**< 0.001**
Mito: nuc	4	3.770	0.440	2.417	0.660
Mito: male age	2	1.860	0.395	0.097	0.953
Nuc: male age	2	11.786	**0.0027**	12.652	**0.002**

The significant effects are indicated in bold.

Table 2.2 Male age, evolutionary history and the interaction effects on sperm viability.

Sperm viability			
Fixed effects	**Df**	**χ2**	**P value**
Evolutionary history	1	0.197	0.657
Age	1	29.003	**< 0.001**
Evolutionary history: age	1	0.020	0.887

The significant effects are indicated in bold.

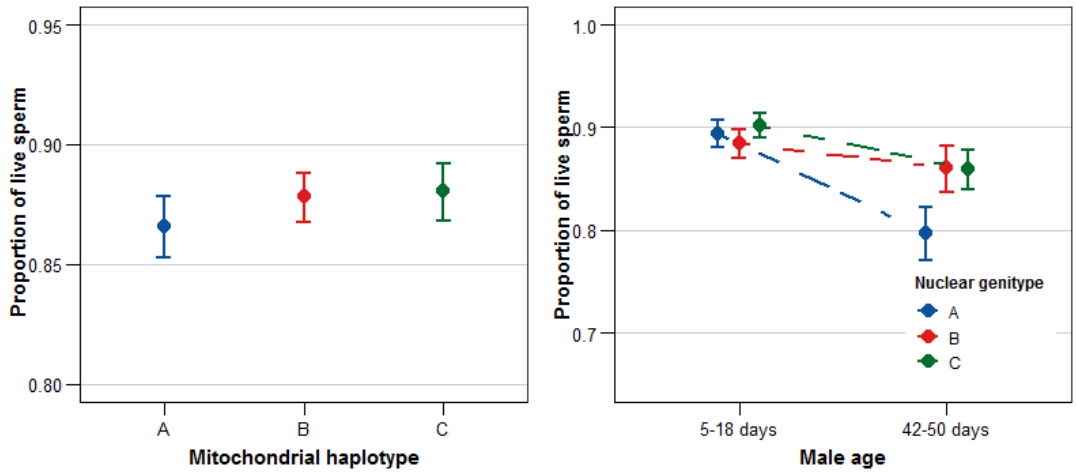


Figure 2.3 Mitochondrial effects (left) and male age by nuclear genotype interaction effects (right) on sperm viability. Data were shown as mean ± SE predicted from model.

2.3.2 Age-dependent mitonuclear interaction effects on sperm quality

Sperm viability decreased significantly after stress treatment in all males. The decrease, termed sperm quality, was affected by mitochondrial, nuclear genomes and their interaction (Table 2.3). There was no effect of male age *per se* on sperm quality, but the mitonuclear interaction effect on sperm quality was age-dependent, reflected by the differences in sperm quality between young and old males across mitonuclear genotypes (Table 2.3). However, the age-dependent mitonuclear effect on sperm quality was unlikely to arise from mitonuclear coadaptation because sperm quality did not differ significantly between males with a coevolved compared to mismatched mitonuclear genotype (coevolution history, p=0.139; coevolution history: age, p=0.600)

Table 2.3 Male age, mitochondrial, nuclear genome and their interaction effects on sperm quality.

Sperm quality (1258 males)					
		SNPs-based		Geography-based	
Fixed effects	**Df**	**χ2**	**P value**	**χ2**	**P value**
Treatment	1	66.085	**< 0.001**	160.463	**< 0.001**
Mito	2	5.978	0.050	0.682	0.711
Nuc	2	11.913	**0.002**	7.500	**0.024**
Male age	1	15.194	**< 0.001**	14.849	**< 0.001**
Mito: nuc	4	5.062	0.281	2.170	0.705
Mito: male age	2	2.227	0.328	0.786	0.675
Nuc: male age	2	4.418	0.110	2.944	0.229
Mito: nuc: male age	4	4.366	0.358	4.847	0.303
Mito: treatment	2	22.704	**< 0.001**	11.676	0.002
Nuc: treatment	2	18.504	**< 0.001**	10.312	**< 0.005**
Male age: treatment	1	0.171	0.679	3.643	0.056
Mito: nuc: treatment	4	34.720	**<0.001**	20.336	**<0.001**
Mito: age: treatment	2	19.981	**<0.001**	7.960	**0.019**
Nuc: age: treatment	2	9.486	**0.008**	10.809	**0.004**
Nuc: mito: age: treatment	4	21.264	**<0.001**	14.757	**0.005**

The significant effects are indicated in bold.

2.3.3 Age but not genetic factors affect sperm metabolic rate

Sperm metabolic rate increased significantly with age (Table 2.4). However, as shown in Figure 2.4 (upper left), we found that the age-dependant increase in sperm metabolic rate between young and old males was not dependent on mitonuclear genotypes. In addition, we found no overall mitonuclear interaction effect on sperm

metabolic rate (Table 2.4). A closer look at the sperm metabolic rate in young and old males separately revealed no mitonuclear interaction effects in both groups as well (Figure 2.4). However, there was a tendency of nuclear genotype in sperm metabolic rate in old males, with males of nuclear genotype B exhibiting the lowest sperm metabolic rate (Figure 2.4).

Co-evolved males did not show significant difference in sperm metabolic rate from non-coevolved males (Figure 2.4, Table 2.5). Furthermore, they did not differ significantly in age-dependent increase in sperm metabolic rate either (Figure 2.4, Table 2.5).

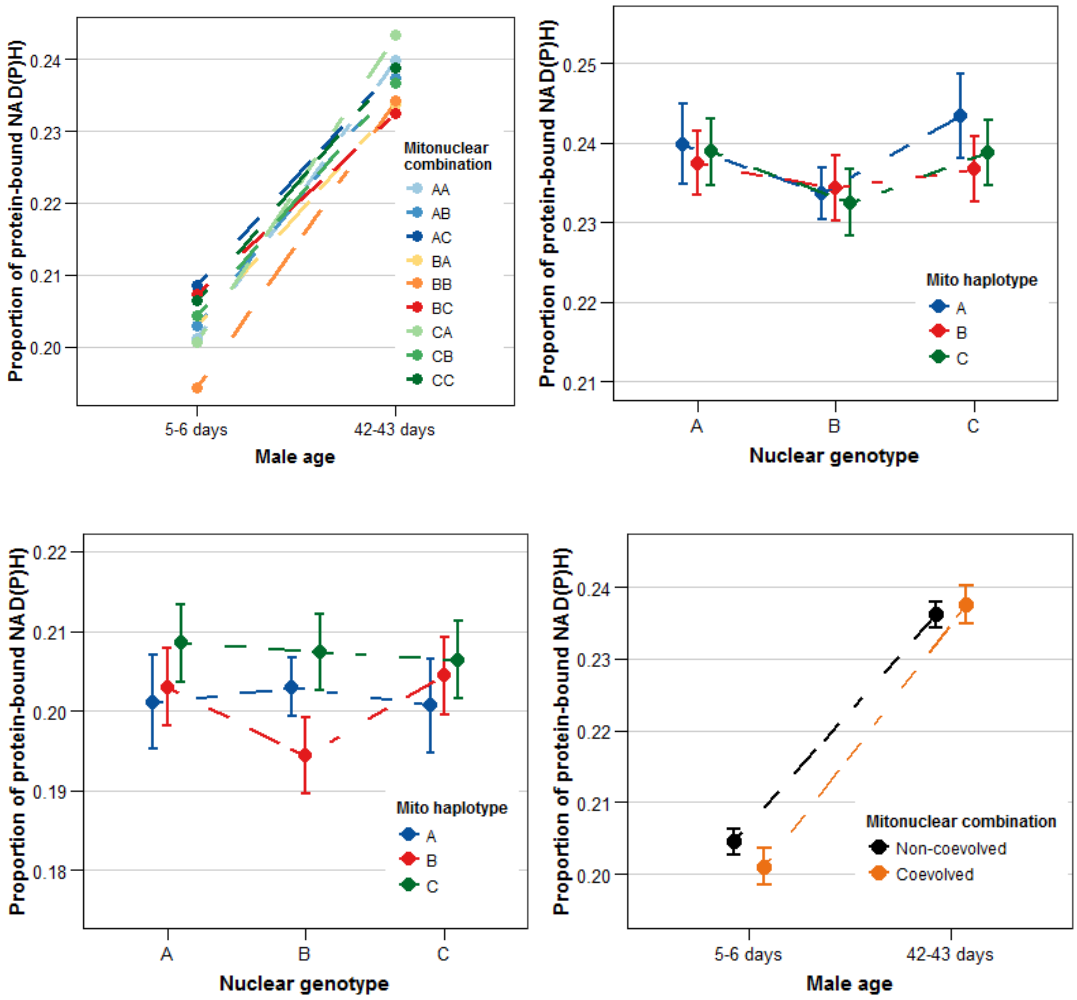


Figure 2.4 Age-dependent mitonuclear effect (upper left) in males but no mitonuclear effects on sperm metabolic rate separately for young (upper right) and old males (lower left). No difference in sperm metabolic rate between coevolved and non-coevolved lines (lower right). Data were shown as mean ± SE predicted from model.

Table 2.4 Male age, mitochondrial, nuclear genome and their interaction effects on sperm metabolic rate.

	Sperm metabolic rate				
SNPs-based				**Geography-based**	
Fixed effects	**Df**	**χ2**	**P value**	**χ2**	**P value**
Mito	2	2.381	0.304	0.780	0.677
Nuclear	2	0.120	0.942	0.148	0.929
Male age	1	24.882	**< 0.001**	34.166	**< 0.001**
Mito: nuclear	4	0.643	0.958	0.2692	0.992
Mito: male age	2	1.941	0.379	0.9483	0.662
Nuclear: male age	2	0.705	0.703	0.6760	0.713
Mito: nuclear: male age	4	2.738	0.603	2.0799	0.721

The significant effects are indicated in bold.

Table 2.5 Male age, evolutionary history and its interaction effects on sperm metabolic rate.

	Sperm metabolic rate		
Fixed effects	**Df**	**χ2**	**P value**
Evolutionary history	1	0.191	0.663
Age	1	152.595	**< 0.001**
Evolutionary history: age	1	1.230	0.268

The significant effects are indicated in bold.

2.3.4 Mitonuclear interaction epistasis in MF was age-dependent

Old males had a significantly lower fertility than young males (Table 2.6) but the age difference showed different magnitudes across nuclear genotypes (Figure 2.5). We found no mitochondrial nor a mitonuclear epistatic effects on male fertility. However, further analyses of male fertility performed separately for young and old males suggested that the a marginally significant mitonuclear interaction effect was present in younger, but absent in older males (Figure 2.5, upper right). However, geography-based classification of mitochondrial genomes revealed both mitochondrial and nuclear additive effects on male fertility, but the mitochondrial effects were not contingent on nuclear background where it expressed. Old male had a significantly lower offspring number, and the age effect was further dependent on mitochondrial haplotype and nuclear genotype. The reduction of offspring in old males relative to young males differed across mitonuclear genotypes (Table 2.6). These two distinct results revealed the existence of potential mitochondrial SNPs that might play a key role in male fertility. To further find out those SNPs, we constructed a new model in which line AA3 were classified into mitochondrial haplotype A while lines CA1and CA3 into mitochondrial haplotype B. We found the regrouping of lines CA1 and CA3 caused the main differences. Therefore, the results indicate that 4 mitochondrial SNPs separating lines CA1 and CA3 from mitochondrial haplotype C might be essential to male fertility.

We found that coevolved lines and non-coevolved lines dis not differ significantly in male fertility. Moreover, there was no difference in age-dependant decrease in male fertility between co-evolved and non-coevolved lines (Figure 2.5).

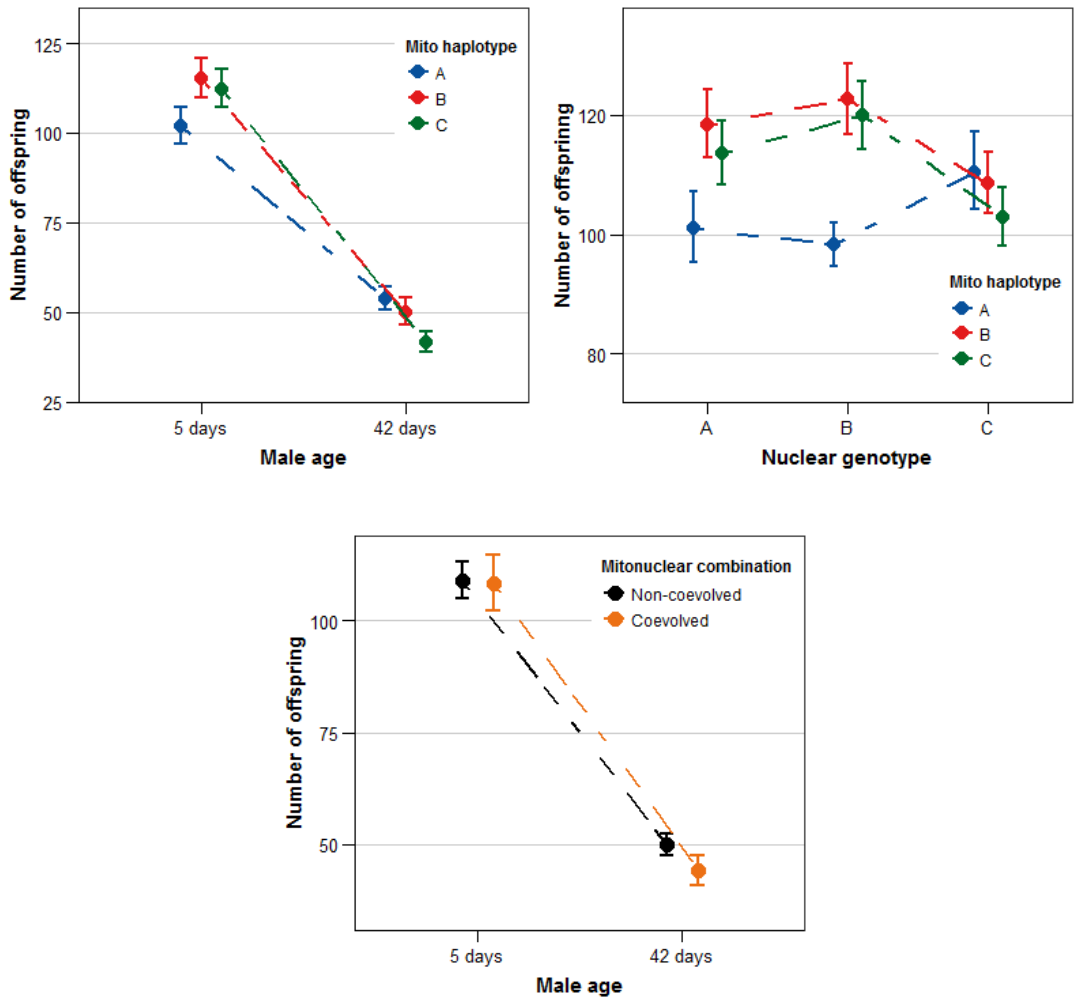


Figure 2.5 Male age by nuclear genotype interaction effects (upper left), mitochondrial haplotype by nuclear genotype interaction effects on male fertility (upper right). No difference in male fertility between coevolved and non-coevolved lines (lower). Data were shown as means ± SE predicted from model.

Table 2.6 Male age, mitochondrial, nuclear genome and their interaction effects on male fertility reflected by reference female.

	Male fertility (SNPs-based)			**Male fertility (Geography-based)**			**Male fertility (AA3->A, CA1 & CA3-> B)**		
Fixed effects	**Df**	**χ2**	**P value**	**Df**	**χ2**	**P value**	**Df**	**χ2**	**P value**
Mito	2	1.0959	0.578	2	6.012	**0.049**	2	0.902	0.637
Nuclear	2	6.4638	**0.040**	2	6.286	**0.043**	2	6.409	**0.041**
Male age	1	18.8465	**< 0.001**	1	25.124	**< 0.001**	1	24.756	**<0.001**
Mito: nuclear	4	8.7500	0.068	4	8.462	0.076	4	8.507	0.075
Mito: male age	2	0.9431	0.624	2	8.403	**0.015**	2	1.176	0.555
Nuclear: male age	2	7.6045	**0.022**	2	7.942	**0.019**	2	7.986	**0.018**
Mito: nuclear: male age	4	6.0316	0.197	4	10.579	**0.032**	4	6.261	0.181

The significant effects were indicated in bold. AA3->A, line AA3 was classified into mitochondrial haplotype A; CA1 & CA3->B, CA1 & CA3 were classified into mitochondrial haplotype B.

2.3.5 Mitonuclear interaction epistasis in FS was age-dependent

We found that the mitochondrial genetic effect on male fertilization success depended on the nuclear background in which it was expressed (Table 2.7), and that this mitonuclear epistasis effect was age dependent as revealed by the significant mito × nuclear × age three-way interaction (Figure 2.6). Separate analyses for young or old males revealed that the three-way interaction was not derived from the presence of mitonuclear interaction in one age group but absence in the other age group as it was not found in both groups. Instead, various crossing-overs in reaction norms between age groups suggested it is more likely that certain mitonuclear genotypes that had a relatively low fertilization success in one age group showed a relatively high fertilization success in the other age group (Figure 2.6). Again, mitonuclear effects were not due to mitonuclear coevolution because neither main evolution nor its interaction effects with age were found (Figure 2.6). We found no qualitative difference in results between geography-based and mitochondrial SNPs-based classifications.

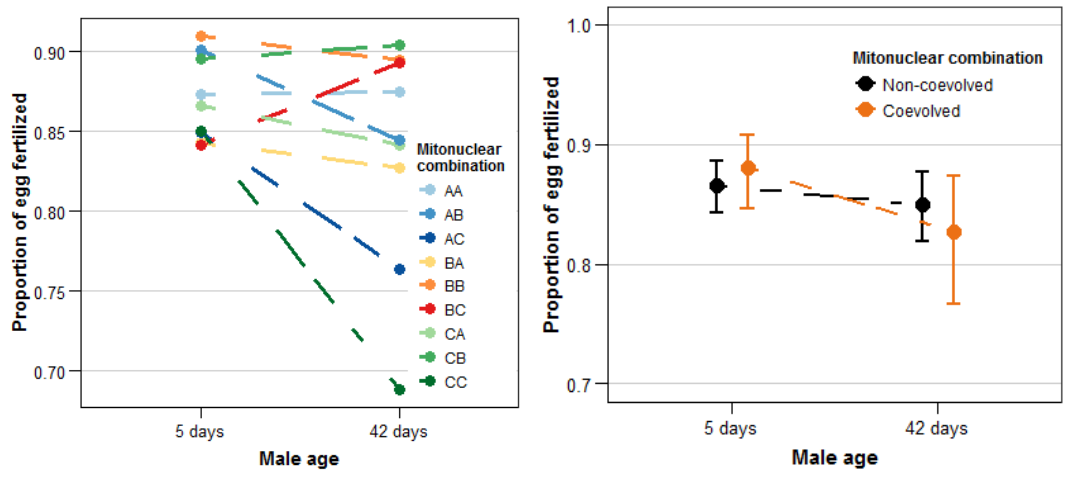


Figure 2.6 Age-dependant mitonuclear interaction effects on fertilization success (left) and no mitonuclear adaptation in fertilization success (lower). Data were shown as mean ± SE predicted from model.

Table 2.7 Male age, mitochondrial, nuclear genome and their interaction effects on fertilization success reflected by reference female.

	Fertilization success					
	SNPs-based			**Geography-based**		
Fixed effects	**Df**	**χ2**	**P value**	**Df**	**χ2**	**P value**
Mito	2	1.429	0.490	2	1.602	0.449
Nuclear	2	4,578	0.101	2	3.695	0.158
Male age	1	0.001	0.971	1	0.053	0.818
Mito: nuclear	4	15.847	**0.003**	4	11.006	**0.027**
Mito: male age	2	0.238	0.888	2	2.859	0.239
Nuclear: male age	2	2.369	0.306	2	2.314	0.314
Mito: nuclear: male age	4	12.127	**0.016**	4	9.683	**0.046**

The significant effects were indicated in bold.

2.3.6 Sperm viability, sperm quality and sperm metabolic rate do not predict male fertility

In order to explore to relationship between all the sperm parameters examined, we did correlation analysis for all the 27 mitonuclear introgression lines. As age had an effect on all sperm parameters, we analysed the data separately for young and old males. Fertilization success was positively associated with male fertility in both young and old male (Figure 2.7, Table 2.8). Surprisingly, sperm quality was negatively associated with male fertility in young but not old males. Sperm viability and metabolic rate were not related to male fertility in both age groups (Table 2.8). In addition, there were not relationship between sperm viability and quality sperm metabolic rate in both age groups (Table 2.8).

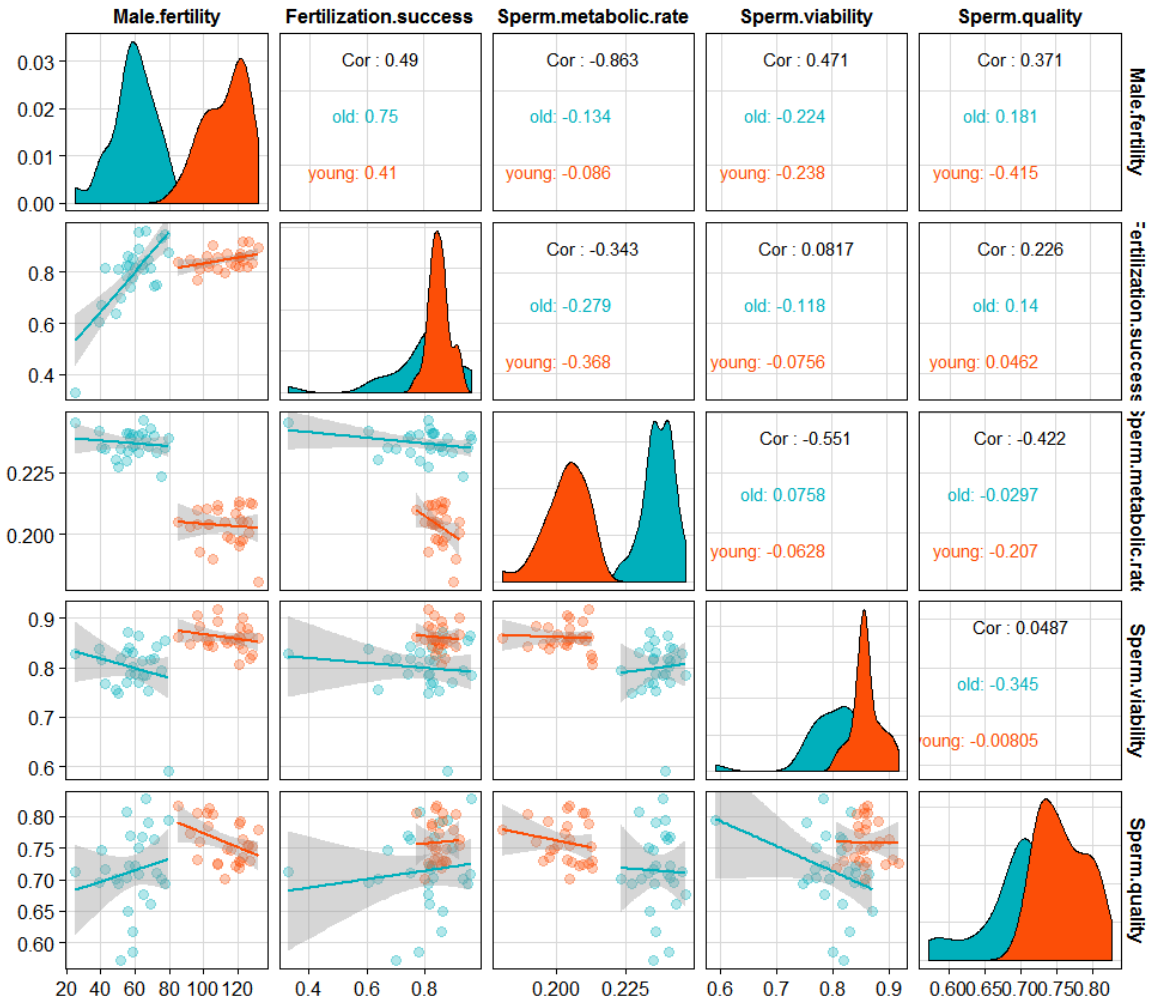


Figure 2.7 Correlation matrix between all sperm parameters examined for young and old males separately**.** The upper part above diagonal showed the "Pearson" correlation coefficients: the black numbers are correlation coefficients for data from both young and old male, blue numbers for data from old males only while orange numbers for data from young males only. The lower part below diagonal showed the associated correlation plots among these sperm function parameters.

Table 2.8 Correlation matrix between all sperm function parameters based on data including both age groups (a), young male only (b) and old male only (c).

(a)

	Male fertility	Fertilization success	Sperm metabolic rate	Sperm viability
Fertilization success	0.49			
Sperm metabolic rate	-0.86	-0.34		
Sperm viability	0.47	0.08	-0.55	
Sperm quality	0.37	0.23	-0.42	0.05

(b)

	Male fertility	Fertilization success	Sperm metabolic rate	Sperm viability
Fertilization success	0.41			
Sperm metabolic rate	-0.09	-0.37		
Sperm viability	-0.24	-0.08	-0.06	
Sperm quality	-0.42	0.05	-0.21	-0.01

(c)

	Male fertility	Fertilization success	Sperm metabolic rate	Sperm viability
Fertilization success	0.75			
Sperm metabolic	-0.13	-0.28		
Sperm viability	-0.22	-0.12	0.08	
Sperm quality	0.18	0.14	-0.03	-0.34

The numbers in the table were correlation coefficients, and significance was indicated by grey shades at the level of $p < 0.05$.

2.4 Discussion

By disentangling mitochondrial and nuclear from mitonuclear interaction effects on sperm physiology and male reproductive aging in *D. melanogaster,* we demonstrate that 1) mitochondrial effects in male reproduction were moderate relative to larger effects of age and nuclear genome, 2) mitochondrial effects on male reproduction were mostly expressed through its age-associated interaction with nuclear genome, 3) mitonuclear interaction or age-dependant mitonuclear interaction were not shaped by mitonuclear coadaptation and that 4) age and mitonuclear epistasis affected most sperm and fertility traits differently. Finally, our design allowed us 5) to suggest some SNPs in the mitochondrial genome that may play an important role in shaping male fertility. Below we will discuss these those points in details.

2.4.1 Age-dependent mitonuclear effects on male reproduction

Unsurprisingly, our research confirmed that the male nuclear genome affects reproductive traits. This notion is the basis of both diagnostic reproductive medicine (andrology) as well as evolutionary biology predicting reproductive success by post-copulatory processes such as sperm competition. We quantified the dramatic age-associated decreases in male reproductive function at three levels: sperm cell, ejaculate and offspring production. Generally, age-related decreases likely represent a mixture of an accumulation of damage caused by extrinsic sources (e.g., lifestyle effects, or sperm ecology (Reinhardt et al. 2015) and intrinsic or genetic sources of individual aging. Given the standardised rearing in our study, the large age-related effects, with old males sometimes having only 50% of the reproductive outcome of young males, was more likely caused by intrinsic aging properties than extrinsic sources of damage accumulation. Specifically, old male had lower sperm viability, a tendency for lower sperm quality, higher sperm metabolic rate and reduced fertility. While our findings were consistent with numerous studies on male reproductive aging showing reduced sperm counts, sperm function and fertility (Kühnert &

Nieschlag 2004; Sharma et al. 2015), our study was able to further dissect the source of this aging in terms of the contribution by the mitochondrial and the nuclear genomes.

There is ample evidence that male reproduction can be affected by mitochondrial genomes, especially those that are considered to be male-specific harmful(Patel et al. 2016; Immonen et al. 2016; Clancy et al. 2011; Yee et al. 2015). Some of these studies were able to disentangle mitochondrial from nuclear effects experimentally and showed mitochondrial effects on reproduction to be expressed as mitonuclear interaction, i.e., the effect of the mitochondrial genome was dependent on the nuclear background it was placed in. For example, interaction between mitochondrial and Y-linked gene was found to shape male fertilization success in *D. melanogaster* (Yee et al. 2015). Because both mitochondrial as well as nuclear genomes are known to affect male aging, our study went further and examined their effects on reproductive aging. Clearly, the data show that both genomes affect aging of the male reproductive system, usually in an interactive manner, i.e. the mitonuclear genotype affected reproductive aging. Although this has been revealed before in seed beetle (Immonen et al. 2016), our analysis shows the direction that age modifies mitonuclear epistasis might not be necessarily be decrease with age (reproductive aging). For example, aging in sperm quality and fertilization success was not affected by mitonuclear epistasis because there were not age effects on these two parameters. The mito x nucl x age three-way interaction in sperm quality and fertilization success means age manipulates these two parameters in multiple direction based on mitonuclear genotype. In addition, our finding further shows that mitonuclear epistasis effects on reproductive traits are not modified by age in the same way. For example, age manipulation effects are more dependent on certain mitonuclear genotype as a unit for male fertilization because there was only mitonuclear effects found, while the manipulation of mitonuclear epistasis by age might be more complicated for sperm quality as mitochondrial

haplotype and nuclear genotype found to affect sperm quality, and their further interaction with age.

The complicated three-way interaction effects on reproduction revealed the complex architecture of male reproductive traits in *D. melanogaster* and these findings have both clinical as well as evolutionary relevance. For example, the mitonuclear interactions are not simply pair-wise as they are modified by age. Previous studies suggested mitochondrial mutations can accumulate over age and lead to age-related alterations in phenotypes. In medicine, several mitochondrial degenerative diseases were demonstrated to be later-onset, which means the severity of phenotype might be more pronounced at late age (Taylor & Turnbull, 2005). However, our results indicated that the age-related alteration in male reproduction might be more complicated, as it might further interact with nuclear genome. This interaction might be even more prominent than mitochondrial genome *per se* in age-related phenotypes or diseases. For example, mitochondria x nuclear x age effects on male fertilization success was found rather than mitochondria x age effect.

In addition, we rarely found mito x age two interaction effects on male reproduction except for sperm quality. This finding indicates that mitochondrial genetic variance may not result in difference in age-related reproductive consequence.

2.4.2 No evidence for mitonuclear coadaptation in male reproduction

Mitonuclear coadaptation hypothesis predicts defected mitochondrial performance for non-coevolved mitonuclear genotype relative to coevolved combinations. However, an increasing number of studies showed sex-specific patterns in mitonuclear coadaptation across phenotypic traits, including lifespan and reproduction (Camus et al. 2012; Frank 2012; Maklakov & Lummaa 2013; Immonen et al. 2016; Dobler et al. 2014). Specifically, female traits are more likely to

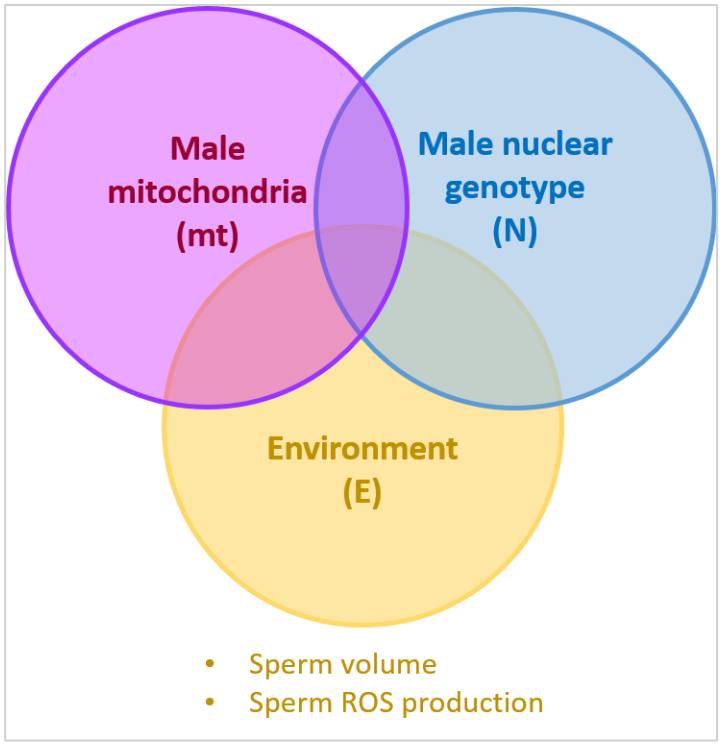
substantiate mitonuclear coadaptation prediction while male traits tend to reflect genotypic idiosyncrasies (Dobler et al. 2014; Immonen et al. 2016). The irregular mitonuclear epistasis patterns in male traits might be due to the maternal inheritance of mitochondria and sex-specific selection (Rand et al. 2001), which make mitonuclear coadaptation less efficient in males. Therefore, compensatory mutation occurred in nuclear genome might not be able offset the negative effects by mitochondrial mutations. Indeed, there is study showing the estimated rate of nuclear compensatory evolution is less than one-fourth that of male-harming mitochondrial mutations (Wade 2014). Our study in *D. melanogaster* showed that there was no significant difference between coevolved males and non-coevolved lines in male reproductive performance at sperm, ejaculate and offspring levels. Instead, irregular mitonuclear epistasis is always found true for male reproductive performance. Take sperm metabolic rate as an example, males with mitonuclear genotype “BB” had relatively low sperm metabolic rate in both age group. However, high sperm metabolic rate was achieved when mitochondrial haplotype “A” was paired with nuclear genotype “B”. Our findings are in line with numerous studies, which found irregular mitonuclear epistasis pattern across traits (Camus et al. 2012; Frank 2012; Maklakov and Lummaa 2013; Immonen et al. 2016; Dobler et al. 2014). Furthermore, mitochondrial mutations accumulate over time and some of the mutations have late-onset effects, therefore the mitonuclear coadaptation might change over age. However, we found this pattern in male reproductive traits in *D. melanogaster* did not change over time. In other words, alteration in reproductive fitness over age show no difference between coevolved and non-coevolved lines.

2.4.3 Sperm viability, quality and metabolic rate did not predict male fertility

Sperm function was suggested to play an essential role in male fertility. Various sperm traits were examined to understand which sperm trait determines male

fertility. However, most of the studies from livestock failed to find the association between specific sperm traits and male fertility, and the results are sometimes contradictory or inconclusive (Colenbrander et al. 2003, Foote 2003, Rodriguez-Martinez 2003). On the other hand, sperm concentration in ejaculates and sperm motility was found to be associated with male fertility in subfertile men while it was unclear in healthy subpopulations (Drobnis & Overstreet 1992; Tesarik 1994). In our study, we found that sperm viability and metabolic rate were not associated with male fertility in *D. melanogaster* in both age groups. Our results indicated that sperm traits itself might not be suitable determinants of male fertility, at least in *D. melanogaster*. This might be due to the potential interaction between sperm and its surrounding environments before fertilization. For example, seminal fluids and female secretory fluids were suggested to influence sperm viability (Holman 2009b; den Boer et al. 2008; den Boer et al. 2009). In addition, there were also studies suggested sperm metabolism differed between ejaculated and stored sperm in insects (Reinhardt & Ribou 2013, Ribou & Reinhardt 2012). However, sperm quality was found to be negatively associated with male fertility in young males only, which was surprising. We do not know the reasons behind, but this indicates that the resistant capacity of sperm to external stress *in vitro* might not be equivalent to that when sperm are in *in vivo* environments of female. The effect direction could even be the opposite as seminal fluid and female secretory fluids were more likely to contribute to sperm performance *in vivo* while osmatic stress could reflect sperm performance to some extent.

Chapter 3 Dietary polyunsaturated fatty acids affect sperm volume and metabolism in *Drosophila melanogaster*



In this chapter, Dietary PUFAs were found to have an effect on sperm volume and metabolism in *D. melanogaster*.

Abstract

Dietary fatty acids can accumulate in sperm and affect their function in vertebrates. As *Drosophila melanogaster* shares several pathways of lipid metabolism and shows similar lipid-dependent phenotypes but lacks some hormones that in vertebrates regulate lipid metabolism, there is currently no clear prediction as to how dietary fatty acids affect the sperm of D. melanogaster. We manipulated the amount and identity of dietary polyunsaturated fatty acids (PUFA) in the food of D. melanogaster males (a treatment known to affect membrane fluidity) and measured changes in sperm parameters. We found that i) males reared on food containing PUFA-rich, plant-derived lipids showed a slower increase in sperm volume over male age compared to males reared on yeast-derived lipid food which is richer in saturated fatty acids. ii) The resistance of sperm membrane integrity to osmotic stress was not altered by dietary lipid treatment but iii) food containing yeast-derived lipids induced a 46% higher in-situ rate of production of reactive oxygen species in sperm cells. These findings show that dietary lipids have similar effects on sperm parameters in Drosophila as in vertebrates, affect some, but not all, sperm parameters, and modulate male reproductive aging. In concert with recent findings of sex-specific seasonal variation of diet choice in the wild, our results suggest a substantial dietary impact on the dynamics of male reproduction in the wild.

3.1 Introduction

Sperm function is essential to male reproduction as only normal and functional sperm can successfully compete with rival sperm and pass on their genes to the next generation. Sperm function is not only influenced by genetic factors, but also by lifestyle and various other environmental factors (Reinhardt et al. 2015), notably diet. For example, the dietary ratio of proteins to carbohydrates affects male fertility (Bunning et al. 2015), while vitamin supplementation has been shown to increase

sperm motility in boar and chicken (Marin-Guzman et al. 2000; Eid et al. 2006) and sperm competitiveness in crickets (Almbro et al. 2011). Fatty acids are carbon chains with a carboxyl group (COOH) at one end and a methyl group (CH3) at the other. The carbon chain can be saturated or unsaturated with one (MUFA) or more double bonds (PUFA). PUFA are further classified into omega-3 (n-3), omega-6 (n-6), and omega-9 (n-9) PUFA based on the distance of the closest double bond to the first methyl group, which is called omega. Some studies show that dietary fatty acids affect fatty acids profiles in sperm (Safarinejad 2011; Comhaire et al. 2000) and the effect of dietary fatty acids on sperm function attracted much recent attention, particularly in humans. Examples include reduced sperm concentration and reduced total sperm count in humans (Jensen et al. 2013) or lower sperm motility in rats (Fernandez et al 2011; Ferramosca et al. 2016) after consumption of saturated fat, increased sperm motility in sheep (Samadian et al. 2010) after increased uptake of n-3 PUFA, or lower sperm motility in rainbow trout (Vassallo-Agius et al. 2001) or lower sperm competitiveness in guppies (Rahman et al. 2014) when fed a n-3 PUFA-deficient diet. To date, most studies linking lipids and fertility were carried out in vertebrates and the extent to which their findings apply to invertebrates is unclear. Given similar lipid metabolism and similar metabolic phenotypes in flies and vertebrates(Gáliková & Klepsatel 2018; Carvalho et al. 2012), lipids may be expected to affect male fertility also in invertebrates. Indeed, one recent study demonstrated that *Drosophila melanogaster* males that lack an enzyme of lipid metabolism are sterile (Laurinyecz et al. 2016).

The cellular composition of lipids, particularly PUFA, is expected to play an essential role in sperm function for several reasons. First, PUFA are required for spermatogenesis and for the acrosome reaction that makes sperm capable of fertilizing eggs (Roqueta-Rivera et al. 2011; Laurinyecz et al. 2016). Second, lipids are also important signalling molecules in basic metabolic processes and are therefore also likely acting in sperm (Fernandis & Wenk 2007; Masoodia et al. 2015). Third, PUFA are a structural component of the plasma membrane and shape membrane

fluidity and flexibility (Connor et al 1998; Alvarez & Storey 1995), with potential consequences for sperm performance like sperm-egg fusion. Forth, high levels of PUFA make sperm susceptible to lipid peroxidation by reactive oxygen species (ROS), which may cause sperm dysfunction and male infertility (Lenzi et al 1996). Finally, sperm can convert endogenous and exogenous lipids into energy (Lardy & Phillips 1941; Mita & Yasumasu 1983).

While some of the mechanisms demonstrated in vertebrates through which dietary fatty acids influence sperm parameters may also apply to invertebrates (e.g. improved membrane fluidity after PUFA consumption), others may not (e.g., hormone-based effects on spermatogenesis). Moreover, in many studies it is not clear to what extent PUFA represents a part of the natural diet of the study organism. In the present study, we rear males of the genetic model *D. melanogaster* on two diets that differ in their composition of PUFA and other fatty acids and that mimic a natural dietary range in this species. We examine the effects of these diets on sperm volume, sperm membrane integrity, and sperm ROS production.

3.2 Methods

3.2.1 Diets and fly stock

Two diets (plant food, PF and yeast food, YF) that differed in lipid and fatty acid composition were used (Carvalho et al. 2012), which are thought to reflect differences between growth on fresh fruit in the early, and rotten fruit in later season (Brankatschk et al. 2018). Four times more lipids are contained in PF than YF, and the fatty acids are longer and more unsaturated in PF (Table 3.1). The diets are nearly isocaloric at 800 kcal/L (Table 3.1).

Table 3.1 Recipe (a) and differences in lipids composition (b) of yeast and plant food.

a)

Fly foods	Components	Gram	Protein (%)	Sugar (%)	Lipids (%)	Calories (kcal/l)	Calories (%)
Yeast food	Yeast extract	20	23.8	18.7	0.9	43.5	5.37
	Pepton	20	34	2.5	36.9	73.4	9.07
	Sucrose	30	0	116.1	0	116.1	14.36
	Glucose	60	0	240	0	240	29.68
	Brewers yeast	80	169.6	137.3	28.8	335.7	41.51
	Total/L		28.1	63.6	8.2	808.62	100
Plant food	Sugar beet	22	2	60.7	0	62.7	7.96
	Malt	80	256	22.4	10.1	288.5	36.62
	Oil sunflower	2	0	0	17.7	17.7	2.24
	Pepton	38	64.6	4.7	70.1	139.4	17.7
	Cornmeal	80	26.6	232.6	20.3	279.5	35.48
	Total/L		44.3	40.7	15	787.83	100

b)

Fly Food	Sum FA carbons/species			Sum FA double bonds/species		
	Triglycerides	Diglycerides	Phospholipids	Triglycerides	Diglycerides	Phospholipids
Plant Food	50-60	34-36	26-36	2-8	1-5	2-5
Yeast Food	40-54	26-36	34-40	0-6	0-4	0-2

D. melanogaster were collected in Australia in 2010 (Dowling et al. 2014). The fly lines were obtained in 2013 (from D. Dowling, Monash University) and kept in our laboratory since then in large outbred populations. A continuous supply of virgin males reared on PF and YF from the egg stage was generated as follows. Fifteen females and fifteen males, placed in a 20 ml vial filled with 7 ml PF or YF were allowed

to mate for eight hours. After which the males and female were transferred to a new 20 ml vial to lay eggs for one day before being discarded. Virgin males were continuously collected for four days and maintained in groups of 20 individuals with PF or YF at 25 °C and 65 % relative humidity on a 12:12 h L:D cycle.

3.2.2 Sperm volume

Eight virgin males each kept in sexual isolation for 5, 7, 10, 15, 25 or 45 days in pre-assigned cohorts were dissected in 10 µl of phosphate-buffered saline (PBS). One of the two sperm-containing seminal vesicles was transferred to 2 µl of ddH_2O on a new glass slide with two transparent tape bands attached to provide a bridge of defined height for the cover slip. An image of the sperm vesicle was first taken under 35x magnification with Leica 2000. After that a cover slip that touched the vesicle was added to guarantee the defined height and squeezed the sperm out. A second image was taken after sperm had evacuated from the vesicle for 300 seconds because the released sperm mass did not become larger after that time according to observation. The area of the sperm vesicle and sperm mass after evacuation were measured with ImageJ (Schneider et al. 2012). Both indicators of sperm volume were highly correlated (Figure 3.1).

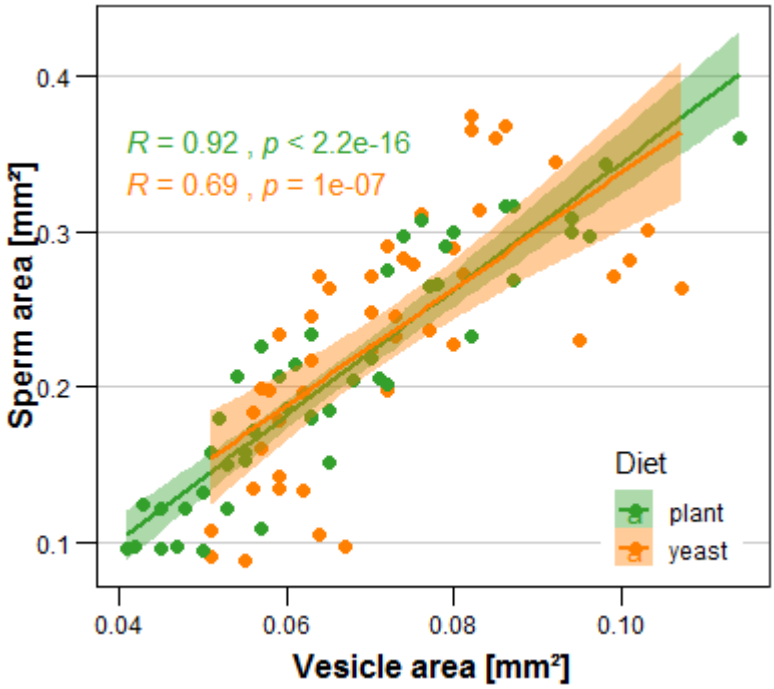


Figure 3.1 Correlation between sperm area and vesicle area for PF and YF males. The fitted regression lines and 95% confidence intervals were shown. Scatter points represented each measurement. R denotes "Pearson" correlation coefficient.

3.2.3 Sperm quality

Following (Eckel et al. 2017), 15 28-day old virgin males of either treatment were dissected in 10 µL of ice-cold Grace's medium. One of the two seminal vesicles was transferred to 12 µL of stress buffer (Grace's medium diluted 1:5 in ddH_2O). Sperm was stained immediately with 0.5 µL of 2 µM SYBR14 (1:50 in DMSO) and 1 µL of 2.4 mM Propidium Iodide (LIVE/DEAD Sperm Viability Kit, ThermoFisher Scientific), covered with a cover slip and an image of one haphazardly selected area of the sperm sample was taken at 400x magnification (Leica DMi8 fluorescence microscope, Leica, Germany) immediately, 5, 15 and 30 minutes later. The numbers of live sperm (with green heads) and dead sperm (with red or partly red heads) in each image were counted by eye. We used the change in the proportion of live sperm in the stress buffer over time as a measure of sperm quality (Eckel et al. 2017).

3.2.4 Sperm ROS production

In order to measure the production of reactive oxygen species in the sperm, we used time-resolved microfluometry. This method has several advantages over other probe-based measures of ROS concentration, including being independent of cell number and of intracellular cellular probe density (Oter & Ribou 2009). It is based on the fluorescence lifetime of the probe 1-pyrene butyric acid (PBA) (Berezin & Achilefu 2010; Ribou 2016), which decreases as the cellular concentration of oxygen radicals increases (Oter & Ribou 2009). Because the probe lifetime is affected by small, radical ROS species such as superoxide (O^{2-}) but not by the hydrogen peroxide (H_2O_2) to which these radicals are rapidly converted, this method provides a measure of *in-situ* (i.e., less than 20 min half life) ROS production (Ribou & Reinhardt 2012).

Ten 35-day old males from each treatment were dissected in 20 µL of PBS. One seminal vesicle was transferred to another drop of 20 µL of PBS punctured with a fine insect pin to release the sperm. The PBS was carefully removed and 20 µL of PBA diluted to 2.5x 10^{-6} mM was added. After eight minutes of incubation, sperm were washed 5-10 times with 1x PBS before being assayed as previously described (Ribou & Reinhardt 2012). Briefly, six readings per sample of the lifetime decay of PBA fluorescence were taken. For each sample, an area of the slide containing PBS but no sperm was measured before the first reading and another after the final reading. This provided a measure of PBA lifetime in the experimental extracellular environment, which was later subtracted from the lifetime measures taken of the sperm samples. The relative rate of ROS production across samples was calculated using the Stern–Volmer equation (Ribou & Reinhardt 2012).

3.2.5 Effect of treatment on body size

We measured wing size (Reeve 2000) and thorax length (Wilkinson et al. 1990) as indicators of adult body size. Wings were dissected from 13-day old males (N=15

each), placed in tubes with 70% ethanol and, to remove stains, washed four times with 70% ethanol. The fifth wash was with 50% glycerol, whereafter the wings were mounted on glass slides and fixed on one end with nail varnish. Images of wings were taken in black and white mode under 50x magnification using a DMi8 fluorescent microscope (Leica, Germany). The area where veins intersected with the boundary was measured using ImageJ (Schneider et al 2012) (Figure 3.2). Thorax length of a different set of 13-day old males (N=16 for PF and N=15 for YF) was measured by anesthetising males with CO_2 and taking images of males that were placed fully parallel to the white pad plane (Figure 3.2). The distance between the base of the most anterior humeral bristle and the tip of the scutellum was measured (Figure 3.2).

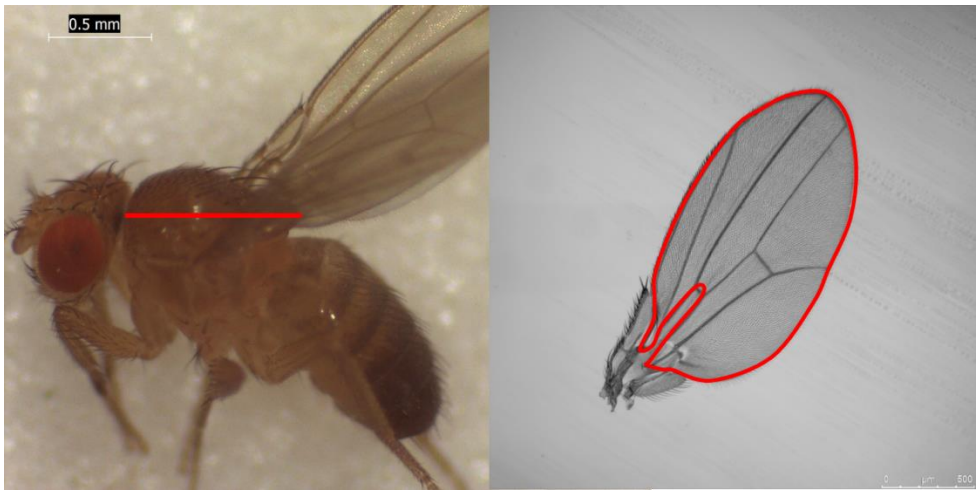


Figure 3.2 Thorax length (left) and wing area (right) measured for PF and YF males. The thorax length and wing area measured are outlined by red line.

3.2.6 Statistical analysis

Data analyses were performed in R (version 3.3.3, R Development Core Team) using linear mixed model approaches containing diet, male age (ordinal factor) and its interaction as fixed factors (sperm volume) or diet only (ROS production), and male

ID as random factor. Sperm quality data was modelled using a generalized linear mixed model (GLMM; binomial distribution) with the lme4 package (Bates et al. 2014). Response variable of proportion of live sperm was created with cbind function (number alive| number dead). Diet, time (ordinal factor) and its interaction were fixed factors, and male id as random factor. After examination of model dispersion, image id was further added as random factor for overdispersion correction (Variance partitioning in multilevel logistic models that exhibit overdispersion). Significance of factors and interaction terms was evaluated with type 3 ANOVA function in the car package (Fox & Weisberg 2011). For wing size and thorax length, Welch's two sample t-tests was performed to examine significance of difference.

3.3 Results

Sperm volume significantly differed between PF and YF males ($p<0.001$) and the increase in sperm volume with male age was faster in the YF than in PF treatment (diet x age interaction $p=0.018$) (Figure 3.3). Sperm quality did not differ between treatments (decline slope: diet x time interaction, $p=0.208$) (Table 3.2). The PBA fluorescence lifetime was 9.76 ns shorter in the sperm of YF males than in the sperm of PF males ($p<0.001$; Figure 3.4), corresponding to a 46.27% higher rate of *in-situ* ROS production. None of the differences were likely caused by body size differences because body size did not differ significantly between treatments (thorax length PF: 0.837±0.008 mm, YF: 0.835±0.008 mm, $t_{24.469} =0.200$, $p =0.843$; wing size PF: 7453.350±54.810 μm^2, YF: 7441.580±54.810 μm^2, $t_{28.992} =0.152$, $p =0.881$).

Table 3.2 Effects of diet, time and their interaction on sperm viability.

Fixed effects	Degree of freedom	χ2	p value
Diet	1	3.380	0.066

Time	3	1140.709	**<0.001**
Diet: time	3	4.547	0.208

The significant effects were indicated in bold.

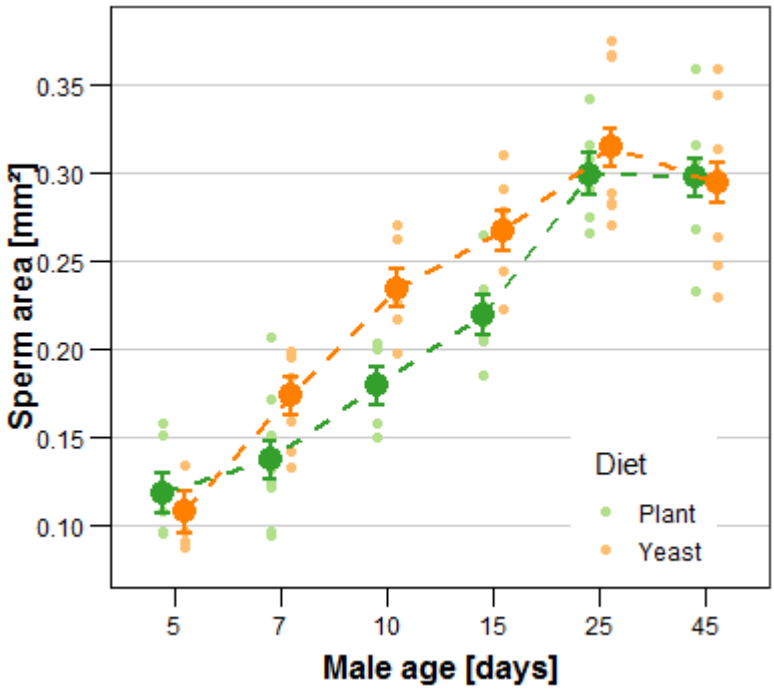


Figure 3.3 Sperm area change over 45 days in response to dietary lipids. Data were shown as mean ± SE, with lighter symbols showing individual data points.

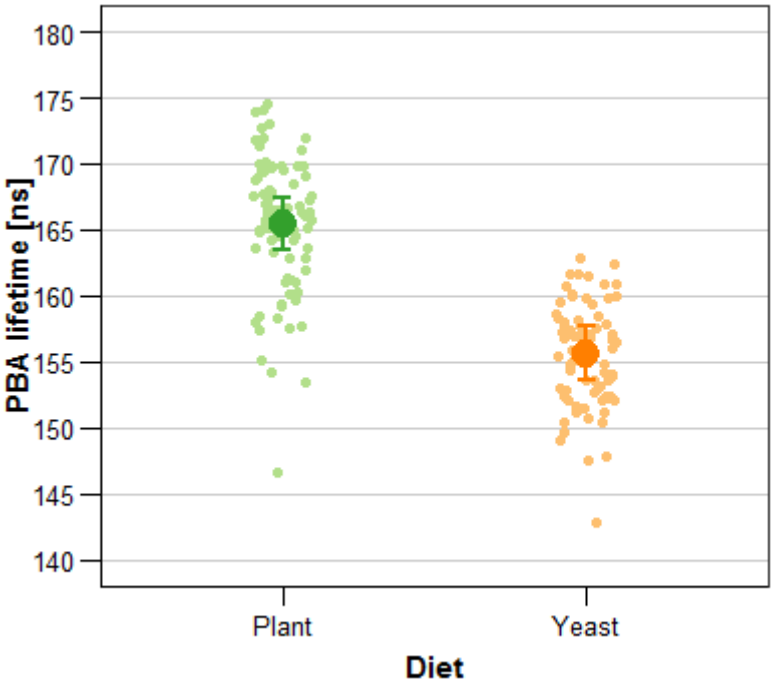


Figure 3.4 PBA lifetime differences in sperm of PF and YF males. Data were shown as mean ± SE, with lighter symbols showing individual data points. Note that larger lifetime values translate into lower ROS production rates.

3.4 Discussion

Dietary fatty acids clearly affected sperm production in *D. melanogaster*, mirroring previous results showing lower sperm count in humans that consume a diet rich in trans fatty acids (Chavarro et al. 2014), higher sperm counts in crayfish consuming a PUFA-rich diet (Harlioğlu et al. 2013) or boar with a enriched n-3 PUFA (Lin et al. 2016) (but see Byrne et al. 2017)). Dietary fatty acids play a key role in spermatogenesis (Moore 1998) and so may explain reduced sperm counts. In *D. melanogaster*, the lack of an enzyme in fat metabolism caused complete male sterility by disrupting spermatogenesis (Laurinyecz et al. 2016). More natural variation in dietary lipids used here caused a quantitative difference in the rate at which sperm was generated. Given these differences, studies comparing sperm counts after dietary intervention may benefit from also standardising their sampling time points.

At later ages (days 25 to 45) dietary lipids caused no effects on sperm volume within treatment. As few individuals will reach these later ages in the wild, males may be under selection to prefer foods high in saturated fatty acids provided the food availability. However, such a diet has also been shown to reduce survival under low temperatures in females (Brankatschk et al. 2018). If this effect holds true for males, they may face a trade-off between a diet maximizing reproduction and one maximizing survival. Given the age-dependence of the dietary effect we found on sperm volume, future studies in this area may benefit from taking age into account.

As PUFA contribute to membrane fluidity and flexibility (Meizel & Turner 1983; Fleming & Yanagimachi 1981) we predict that PF would make sperm membranes more resistant to osmotic stress than YF. It is unclear why we instead found no difference across diets in membrane integrity. While increased PUFA concentration may also increase the susceptibility to stress, for example after cryopreservation (sperm membrane property integrity), this argument does not apply to intracellular oxidative stress: we found YF, not PF, to cause a substantially higher ROS production in sperm. This is to our knowledge the first demonstration of dietary lipids affecting the rate of sperm ROS production (note that this result cannot be explained by a difference in the free radical scavenging activity of PUFA across the two diets because our method measures the amount of ROS being generated, not their net accumulation in the form of H_2O_2 after scavenging takes place).

In conclusion, we show that dietary lipids affect sperm function, especially sperm metabolism, in *D. melanogaster*, as in vertebrates (Ferramosca et al. 2017). Future studies will have to genetically target those enzymes in *Drosophila* that were activated in mice after PUFA supplementation (Ferramosca et al. 2017): sperm enzymes involved in glycolysis, tricarboxylic acid cycle and the respiration chain and that reduced oxidative damage.

General discussion

Sperm function and its components in *D. melanogaster*

As we outlined in general introduction part, sperm function has been studied in several research fields as it is essential to male fertility. In previous studies a variety of sperm traits have been examined as an assessment of sperm function. Among those traits, sperm viability, sperm motility and sperm metabolism are often commonly examined. However, sperm function can be influenced by various factors. Specifically, nuclear genome has been demonstrated to play a role in sperm function, especially in sperm competitive capacity (Bernasconi et al., 2004; Birkhead et al., 2009; Parker 2014). In addition to nuclear effects, there are increasing evidence for effects of mitochondrial genome on sperm function. Mitochondrial genetic variance has been suggested to affect sperm length and sperm viability in seed beetle and sperm metabolism in rodent (Dowling et al. 2007c; Tourmente et al. 2017). Given the coordinated collaborations between nuclear and mitochondrial genomes in OXPHOS, replication and transcription of mitochondrial genome as well as intergenomic signalling (Hill 2019), potential mitonuclear effects on sperm function are expected even though empirical evidence so far is still less. A recent review summarised all the previous work on environmental effects on sperm and found that various factors affects sperm function but largely neglected in ecology and evolution (Reinhardt et al. 2015). In my thesis, we used *D. melanogaster* as a model to disentangle both genetic and environmental components of sperm function at sperm cell, ejaculate and offspring levels.

Environmental effects: incubation buffer on sperm viability and dietary PUFA on sperm metabolism

Various environments have been suggested to affect sperm phenotype plasticity, including temperature, pH and diet (male, maternal, and paternal as well as its amount and composition) (Reinhardt et al. 2015). Sperm viability has been routinely

measured as a proxy of sperm quality to investigate ecological and evolutionary questions. Male seminal fluids and female secretory fluids have been shown to influence sperm viability positively or negatively (den Boer et al. 2009; Holman 2009b). However, it is not clear whether the effects on sperm viability is derived from the protein components in the fluid or the nutrients inside. In Chapter 1, we incubated sperm from three different *D. melanogaster* populations in three different incubation buffers. We found that incubation buffer had an effect on sperm and the sperm viability was highest when sperm was incubated in Grace's medium. Previous work have demonstrated that dietary fatty acids affect fatty acids profiles in sperm (Safarinejad 2011; Comhaire et al. 2000) and on sperm function. PUFA is a key class of saturated fatty acids and suggested to play a role in sperm function such as sperm motility. In Chapter 3, we fed *D. melanogaster* on two diets (i.e. YF and PF), and found a slower increase in sperm volume over age in PF than YF males and 46% fewer ROS production in sperm of PF males than YF males.

Mitochondrial effects: sperm viability and sperm quality

The mitochondrial genome was traditionally considered to be a bystander in adaptive evolution. However, numerous studies across species suggested that mtDNA might be another source of variation of health and other phenotypes(Ballard et al. 2007; Camus et al. 2012; Dobler et al. 2014; Patel et al. 2016). This notion might apply to sperm parameters for two main reasons: more pronounced mitochondrial effects in male traits (Camus et al. 2012; Gemmell et al. 2004; Camus et al. 2015) and sperm function is highly energy-consumed (Ruiz-Pesini et al. 1998; Ruiz-Pesini et al. 2000b). In Chapter 2, using mitonuclear introgression lines we found that mitochondrial haplotype affected sperm viability, with haplotype exhibiting the highest sperm viability. In addition, we further found sperm quality differed between mitochondrial haplotype as well.

Nuclear effects: sperm viability, sperm quality and male fertility

Nuclear genome has been found to affect sperm competitive ability (Parker 1970; Bernasconi et al., 2004; Birkhead et al., 2009; Parker 2014). In our study, we found that it influenced sperm viability as well. In specific, sperm viability was found to be highest in Oregon R male in Chapter 1 and in males with nuclear genotype C in Chapter 2. In Chapter 2, sperm quality and male fertility were found to be significantly different between nuclear genotypes. In addition, sperm viability and male fertility decreased with age, i.e. aging in sperm viability and male fertility. we found that nuclear genotype affected aging in sperm viability and male fertility nuclear by age as two-way interaction were found.

Mitonuclear interaction effects in aging male: sperm quality and fertilization success

Mitochondrial and nuclear genomes cooperate in multiple aspects (Hill 2019). A series of phenotypes were suggested to be affected by mitonuclear interaction, including mitochondrial function, metabolism and life-history traits (Dey et al. 2000; Yamaoka et al. 2000; Reinhardt et al. 2013; Wolff et al. 2014; Dobler et al. 2018). More recently, their potential effects have been explored in reproduction, especially in males (Immonen et al. 2016). In our study, we found mitonuclear genotype influenced sperm quality and fertilization success in aging males and the direction over age were dependent on the specific mitonuclear genotype. Surprisingly, the result is not in line with previous findings that the direction of changes in mitonuclear-dependent reproductive traits was the same, which showed decline over time (Immonen et al. 2016). Our finding implicated the complex of the genetic architecture of sperm function *in D. melanogaster*.

Perspective and outlook:

In our study, we clearly showed in Chapter 3 that mitonuclear effects on sperm function was present. In addition, environmental factor like dietary PUFA was demonstrated to affect sperm function i.e. ROS production as well. Therefore, it is worthwhile to explore whether sperm function will be affected by the three-way interaction of N x mtDNA x E. The three-way interacion on sperm function is expected for two main reasons. First, our study already showed that the genetic architecture of sperm function was complicated and could be interaction with other factors like male age. The effects of dietary PUFA on sperm function provided a good reason to explore the N x mtDNA x E interaction on sperm function. Second, an increasing number of evidences for N x mtDNA x E interaction on other phenotypes has been accumulated (Dowling et al. 2007; Zhu et al. 2014; Mossman et al. 2016). For example, N x mtDNA x temperature effects have been found to influence egg-to-adult development in seed beetle (Dowling et al. 2007) and N x mtDNA x diet effects on lifespan and development time in *Drosophila* (Zhu et al. 2014; Mossman et al. 2016).

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Appendix

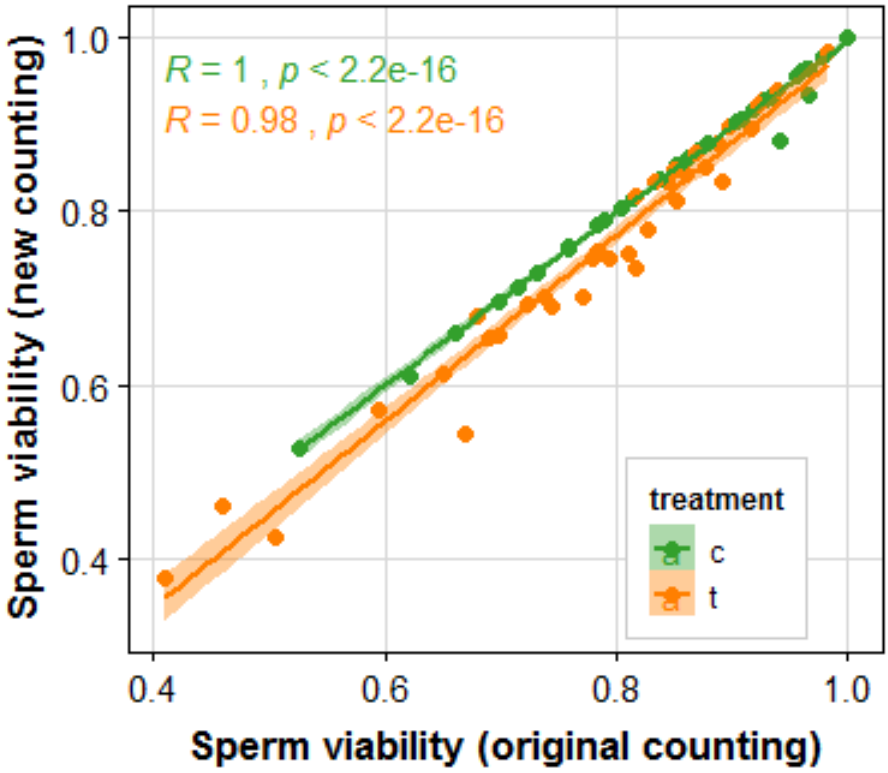


Figure S1 Correlation between the two methods of counting for sperm viability. A complete or largely green head was counted as live, the rest as dead by original counting method. Later double stained sperm was considered as dead by new counting method. The fitted regression lines and 95% confidence interval were shown. Scatter points represented each measurement. R denotes "Pearson" correlation coefficient. In the legend, "c" represented control group in which sperm viability was examined immediately and in PBS buffer. "t" denoted treatment group in which sperm viability was examined 30 minutes later after incubation in a stress solution.

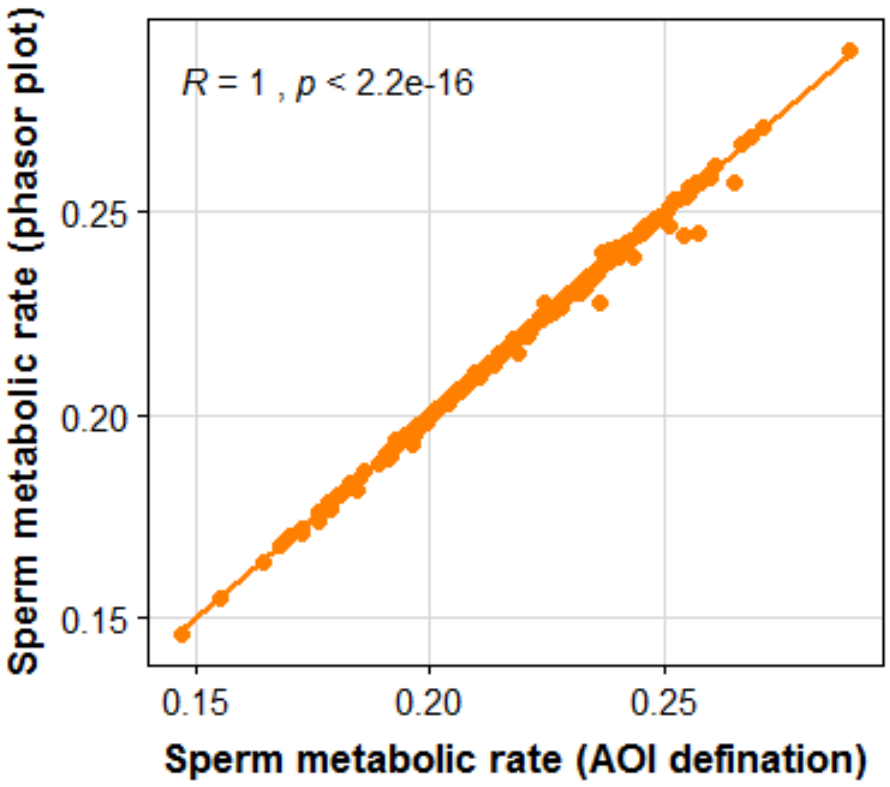
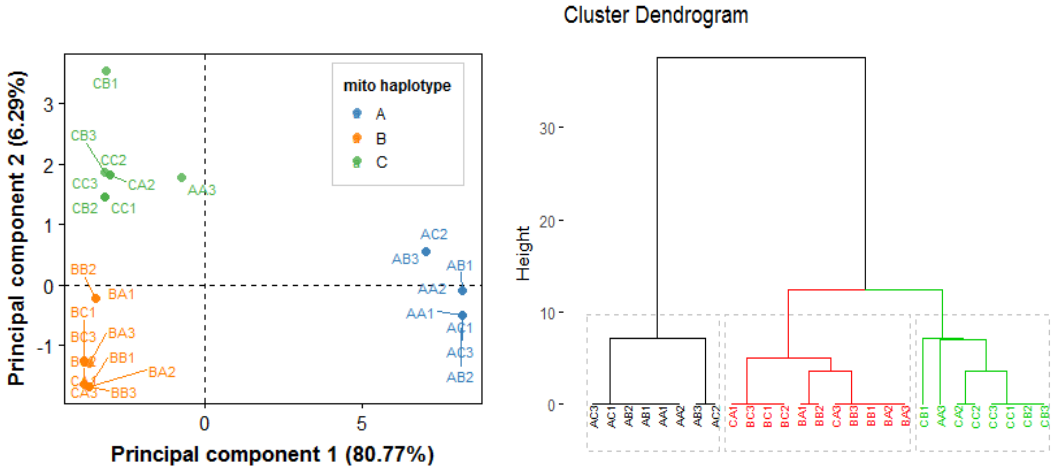
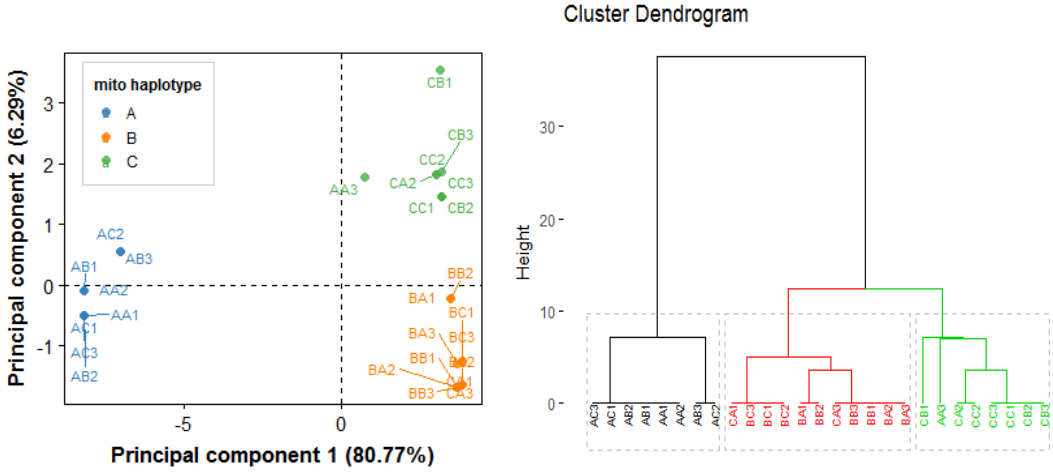


Figure S2 Correlation between the two approaches to extract sperm metabolic rate data from Area of Interest (AOI). AOI was defined manually in approach 1 (x axis) while AOI was defined with phasor plot in approach 2 (y axis). The fitted regression lines and 95% confidence intervals were shown. Scatter points represented each measurement. R denoted “Pearson” correlation coefficient.

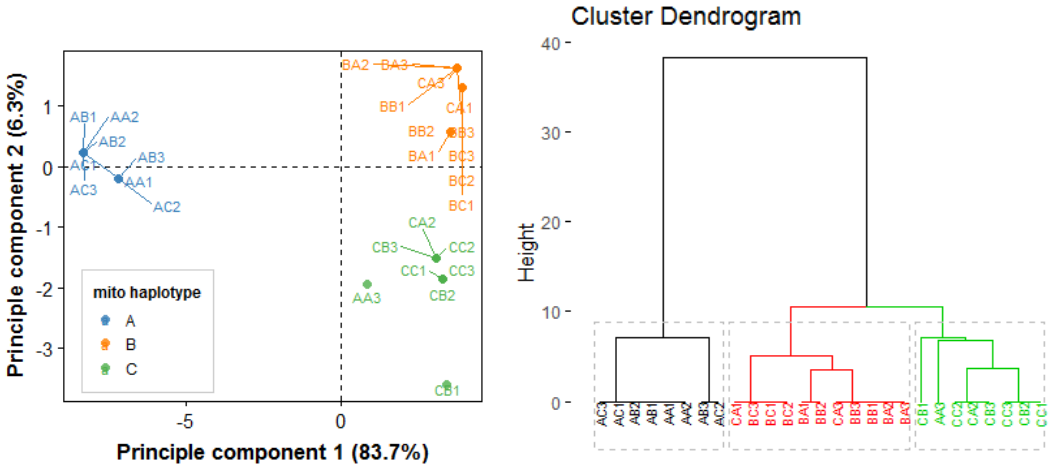
(a)


mito haplotype
A
B
C
Principal component 2 (6.29%)
Principal component 1 (80.77%)
Cluster Dendrogram
Height

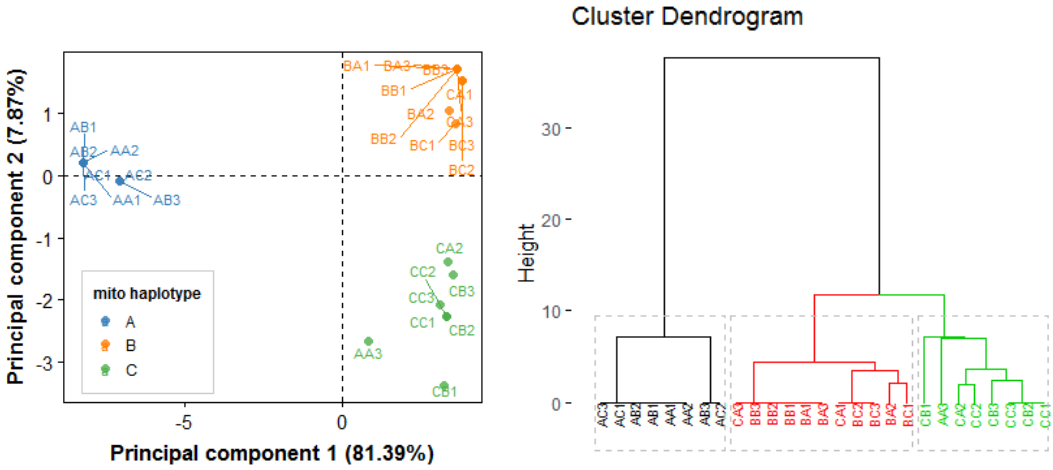
(b)


mito haplotype
A
B
C
Principal component 2 (6.29%)
Principal component 1 (80.77%)
Cluster Dendrogram
Height

(c)


Principle component 2 (6.3%)
Principle component 1 (83.7%)
mito haplotype
A
B
C
Cluster Dendrogram
Height

(d)


Principal component 2 (7.87%)
Principal component 1 (81.39%)
mito haplotype
A
B
C
Cluster Dendrogram
Height

(e)

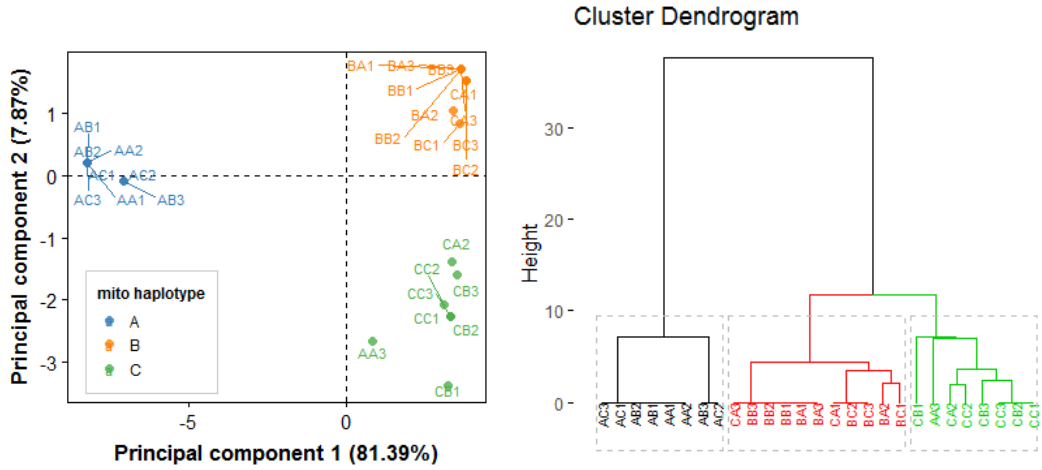


Figure S3 Mitochondrial haplotype reconstruction based on PCAs anlysis for (a, left) sperm viability, (b, left) sperm quality, (c, left) sperm metabolic rate, (d, left) male fertility and (e, left) fertilization success. X axis represents the principal component 1 and y axis is the principal component 2. The values in brackets indicate the percentage of variation in sperm function parameters explained by the associated components. Clustering analysis of the 27 mitonuclear introgression lines based on the PCA results for (a, right) sperm viability, (b, right) sperm quality, (c, right) sperm metabolic rate, (d, right) male fertility and (e, right) fertilization success with K-mean clustering method. The number of clusters was set to be 3.

Acknowledgements

When, in my high school days, I first read the excellent work that Morgan had done with fruitflies, I never imagined that one day I would work with them. Life is a box of chocolates, and so it turned out that I not only work with this tiny little organism in Dresden, but with something even smaller - its giant sperm. How interesting! In the past four years, with this project, I have metamorphosed/ mutated/transformed into a person who has some knowledge and experience about the fruit fly, starting as a beginner who had troubles in distinguishing females from males. I gained some techniques and expertise as well, especially those on microscopes and statistics, which hopefully will be helpful in the future. I also improved my English skills a lot, and will keep working on it. Altogether, I really appreciate all the benefits I obtained from my research project.

Working on a Ph.D project is a long journey. Luckily, I worked with some really kind people who helped me a lot to accomplish it. I would like to first thank my supervisor, Prof. Klaus Reinhardt. Thank you for the chance that you gave to me to work in the lab and study in Germany. You soon changed my impression that Germans are not humorous. You are indeed a person full of humorous bacteria and virus (that's a compliment in a Chinese way!). You provided me with constructive advices and enlightened me all the time during my work. You forced me to think and showed me how to do presentation in a clear and logical way. You helped me with my writing and I really learned a lot from you. You expressed your concerns about my life and encouraged me quite often when I felt down. You did much more than that to train me to be a researcher and to make my life easier. Thank you for all the efforts you made. I would like to thank Dr. Ralph Dobler. I am impressed by how organised you are. Thank you for showing me how to work with fruit flies. Thank you for the advices you gave to me to design and improve my experiments. You are also the one who guides me through the learning of statistics and make me realise its beauty afterwards. I appreciate your help and comments on my manuscript. I would also like

to thank Dr. Biz Turnell. I admire the clear way you do research and the way you give talks. I thank you for your help to improve my presentation slides and valuable advises for my speaking skills. Thank you for showing me how to use ROS machine and FLIM microscope. Thank you for your help with my manuscripts.

I would like to express my appreciation to all my colleagues. With the help from all of you, I have been able to finish my studies. Thank you Anja, Barbara, Conni, Cornelia, Christin, Christian M, Christian K, Ensi, Veronika, Wei, Renata, Reda, Steven. As a group, we spent time discussing scientific questions together. We also sat together having small talks, went out drinking some beer, or juice. On our group retreats, we even spent several evenings together, relaxing and having fun. All time we ever spent together and all the joy you brought to my life are so valuable - they will be a sweet memory for me. Thank you guys a lot for your help and kindness.

I made several friends out of the lab, they are another source for me to relax and re-energise. Here, I would like to specifically thank Yilong Li, Xueyan Zhao, Zhaochun Wang and Qiang Liu. We travelled together, had hotpot, made dumplings and celebrated Chinese new year. You guys are family for me in Dresden. I also would like to thank my parents and sisters for all your unconditional support. Family is always a warm harbour for me wherever I am. At last, I would like to thank my girlfriend Guanhua Wang for her understanding and support.

Ruijian Guo

30/9/2019, Dresden

List of publications

Eckel BA, Guo R, Reinhardt K. 2017 More Pitfalls with sperm viability staining and a viability-based stress test to characterize sperm quality. *Frontiers in Ecology and Evolution* **5**, 599. (doi: 10.3389/fevo.2017.00165)

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Publication

Eckel BA, Guo R, Reinhardt K. 2017 More Pitfalls with sperm viability staining and a viability-based stress test to characterize sperm quality. *Frontier in Ecology and Evolution* 5, 599. (doi: 10.3389/fevo.2017.00165)

Zhang Z, Guo R, Li M. 2017 Identification, characterization and expression analysis of superoxide dismutase genes in *Dastarcus helophoroides* (Coleoptera: Bothrideridae). *Gene* 606. (doi: 10.1016/j.gene.2017.01.003)

Guo R, Reinhardt K. Dietary polyunsaturated fatty acids affect volume and metabolism of *D. melanogaster* sperm. (accepted)

Guo R, Dobler R, Dowling DK, Wetzker C, Reinhardt K. Mitonuclear epistasis modifies male aging in fertility and sperm physiology. (in preparation)

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Dresden, den 30.9.2019

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