The Molecular Biomarkers of Sperm Fitness

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Declaration

I, Jayme Leah Cohen, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Sperm are among the most specialised and variable cell types in the animal body, differing not only across species but also between and within individuals. This molecular and phenotypic diversity has important implications for fertility, yet the mechanisms generating variation among sperm and their evolutionary and clinical consequences remain poorly understood. This thesis combines quantitative proteomics, bioinformatics, and single-cell analysis to investigate how environmental and physiological stress shape sperm phenotype and to test whether molecular heterogeneity within ejaculates provides a substrate for haploid selection. In Chapter 1, tandem mass tag proteomics revealed that acute heat stress in zebrafish (*Danio rerio*) testes induces a coordinated shift in the proteome, with depletion of proteins linked to spermatogenesis and motility and enrichment of chaperones and stress-response factors, demonstrating dynamic germline remodelling under environmental challenge. Chapter 2 presents a comprehensive zebrafish sperm proteome that establishes a baseline for functional and comparative work and shows that stress-induced testicular changes translate into altered sperm protein composition. In Chapter 3, fractionation of human ejaculates by swim-up and methyl-cellulose migration revealed consistent proteomic differences between higher- and lower-fitness sperm subpopulations, identifying candidate surface biomarkers of sperm quality. Chapter 4 validated two of these candidates, OLFM4 and CTNG, as accessible on the sperm surface and inversely associated with sperm longevity using spectral imaging flow cytometry. Together, these studies demonstrate that sperm phenotypes reflect underlying molecular differences that can be detected, quantified, and linked to fitness-related traits. They provide molecular evidence for haploid selection acting within ejaculates and establish a translational foundation for biomarker-based sperm assessment in assisted reproduction.

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Abbreviations

ABWT – AB Wild-Type (zebrafish strain)

ACN – Acetonitrile

AIC – Akaike Information Criterion

ALH – Amplitude of Lateral Head Displacement

AOC3 – Amine Oxidase, Copper Containing 3 (Vascular Adhesion Protein-1)

ART – Assisted Reproductive Technology / Technologies

ATP – Adenosine Triphosphate

BD – Becton Dickinson (BD Biosciences)

BH – Benjamini–Hochberg (method for multiple-testing correction)

BH-FDR – Benjamini–Hochberg False Discovery Rate

BCF – Beat Cross Frequency

BPA – Bisphenol A

CASA – Computer-Assisted Sperm Analysis

CE – Collision Energy

CHIMERYS – CHIMERYS Search Engine (MSAID, Munich)

CID – Collision-Induced Dissociation

CO_2 – Carbon Dioxide

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

CTNG – Gamma Catenin (Junction Plakoglobin / JUP)

CV – Compensation Voltage (FAIMS)

CytoNorm – Cytometry Normalization Algorithm (R package)

DAPI – 4′,6-Diamidino-2-Phenylindole

DDSi – DNA Degradation Index

DEFB126 – Beta-Defensin 126

DESeq2 – Differential Expression Sequencing 2 (R package)

DHARMa – Residual Diagnostics for Hierarchical Regression Models (R package)

DNA – Deoxyribonucleic Acid

DTT – Dithiothreitol

EPPS – N-(2-Hydroxyethyl)piperazine-N′-(3-propanesulfonic acid)

FACS – Fluorescence-Activated Cell Sorting

FAIMS – Field Asymmetric Ion Mobility Spectrometry

FDR – False Discovery Rate

FISH – Fluorescence In Situ Hybridisation

FMO – Fluorescence Minus One (control)

f/s – Frames per Second

GIM – Genoinformative Marker

GLM – Generalised Linear Model

GO – Gene Ontology

HCD – Higher-energy Collisional Dissociation

HFEA – Human Fertilisation and Embryology Authority

HPA – Hypothalamic–Pituitary–Adrenal (axis)

HPG – Hypothalamic–Pituitary–Gonadal (axis)

HPLC – High-Performance Liquid Chromatography

HSF1 – Heat Shock Factor 1

HSP – Heat Shock Protein

HSP47 – Heat Shock Protein 47 (Serpin H1)

HSP70 – Heat Shock Protein 70

Hsp90 – Heat Shock Protein 90

HTF – Human Tubal Fluid (medium)

ICSI – Intracytoplasmic Sperm Injection

IHC – Immunohistochemistry

IQR – Interquartile Range

ISAS – Integrated Semen Analysis System

IZUMO1 – Izumo Sperm–Egg Fusion Protein 1

IVF – In Vitro Fertilisation

JUP – Junction Plakoglobin (γ-Catenin / CTNG)

JUNO – Oocyte Receptor for IZUMO1 (Folate Receptor 4)

KEGG – Kyoto Encyclopedia of Genes and Genomes

Kif – Kinesin-like Protein Family

LC–MS/MS – Liquid Chromatography–Tandem Mass Spectrometry

LFC – Log_2 Fold Change

LIN – Linearity (VSL/VCL × 100)

MC – Methyl Cellulose (assay)

MS – Mass Spectrometry

MS1 / MS2 / MS3 – Mass Spectrometry stages 1–3

NaCl – Sodium Chloride

NCBI – National Center for Biotechnology Information

NH_4HCO_3 – Ammonium Bicarbonate

OLFM4 – Olfactomedin 4

OT – Orbitrap Mass Spectrometer

P1 – Primary Gated Sperm Population (in Flow Cytometry)

PBS – Phosphate Buffered Saline

PDB – Protein Data Bank

PE – R-Phycoerythrin

pH – Potential of Hydrogen

PFA – Paraformaldehyde

PLCζ – Phospholipase C zeta

PyMOL – Python Molecular Graphics Viewer

R – R Statistical Programming Language

RNA – Ribonucleic Acid

rpm – Revolutions Per Minute

RTS – Real Time Search

SDS – Sodium Dodecyl Sulfate

SDC – Sodium Deoxycholate

SPE – Solid Phase Extraction

SPS – Synchronous Precursor Selection

SPAM1 – Sperm Adhesion Molecule 1

STR – Straightness (VSL/VAP × 100)

STRING – Search Tool for the Retrieval of Interacting Genes / Proteins

SW – Swim-Up (assay)

TFA – Trifluoroacetic Acid

TMT – Tandem Mass Tag

TMT10 – Tandem Mass Tag 10-plex kit

TMTpro – Tandem Mass Tag Pro (high-plex version)

UHPLC – Ultra-High-Performance Liquid Chromatography

UniProt – Universal Protein Knowledgebase

VAP – Average Path Velocity

VCL – Curvilinear Velocity

VSL – Straight-Line Velocity

WOB – Wobble (VAP/VCL × 100)

ZIRC – Zebrafish International Resource Center

ZMYND15 – Zinc Finger MYND-type Containing Protein 15

ZP – Zona Pellucida

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1 Introduction

1.1 Importance of Sexual Reproduction

Reproduction is the fundamental biological process by which life persists. Without this ability, no population can survive more than a single generation. Reproduction can broadly be categorised into sexual and asexual processes. Asexual reproduction involves the production of genetically identical offspring from a single parent, without the fusion of gametes, while sexual reproduction involves the combination of genetic material from two parents through the fusion of gametes, generating genetically diverse offspring. However, this is complicated as most eukaryotic life can be considered partially clonal and therefore undergo both asexual and sexual reproduction throughout their lifetime (Beukeboom & Perrin, 2014; Krueger-Hadfield, 2024).

Sexual reproduction is achieved in a variety of ways across taxa. Dioecious species have distinct male and female individuals with distinct gamete cells which undergo fusion to form an offspring, while hermaphroditic species possess both sets of reproductive organs and produce male and female gametes, either simultaneously or sequentially. Current scientific theory suggests that dioecious species evolved via an intermediate state of gynodioecy from hermaphroditic ancestors. The evolutionary trade-offs between these systems have previously been predicted to depend on mate availability, ecological stability, and the importance of outcrossing for maintaining genetic health (Otto & Lenormand, 2002).

Sexual reproduction is energetically expensive and inefficient because it requires the production of specialised gametes, the maintenance of complex reproductive systems, and the coordination of mating behaviours that often involve significant energy expenditure and risk (Wootton, 1985).Sexual reproduction results in only half of an individual's genes being passed on to offspring, the so-called "two-fold cost of sex" (Gibson et al., 2017). Despite these costs, sexual reproduction remains nearly universal among eukaryotes, suggesting that that there are long-

term evolutionary advantages that outweigh its short-term inefficiencies. These advantages, including the generation of genetic diversity and the removal of deleterious mutations, underpin the evolution of the biphasic lifecycle.

1.2 The Biphasic Lifecycle

Amongst sexually reproducing eukaryotic organisms, the alternation between haploid and diploid genomes, known as the biphasic lifecycle, is ubiquitous (Strasburger, 1905), where the haploid phase has one set of chromosomes, and the diploid phase has two copies of each chromosome (Immler, 2019; Otto & Goldstein, 1992). These phases are separated by several processes, including meiosis, fertilisation, syngamy, and zygote formation (Purchase et al., 2021). There are three main categories of biphasic life cycles: diplontic, haplontic, and haplo-diplontic (Mable & Otto, 1998).

In diplontic organisms, such as higher animals and some land plants, the diploid phase is dominant, and only a short part of the lifecycle occurs in the haploid phase. Conversely, in haplontic organisms, such as green algae, it is the haploid phase in which the majority of development occurs. Many types of moss, ferns, and fungi are considered to have a haploid-diplontic lifecycle, as development occurs substantially within both diploid and haploid phases (Hughes & Otto, 1999). Each lifecycle has advantages and disadvantages, primarily due to the differences in how natural selection can act to induce genetic variation depending on the ploidy of the genome.

Selection operates differently on haploid and diploid genomes because alleles in diploids can be masked by their homologous partners. Many deleterious mutations are known to be at least partly recessive (Caballero & García-Dorado, 2013; C. D. Jenkins & Kirkpatrick, 1995), meaning that their negative effects are expressed only when both alleles at a locus have the mutation. In diploid organisms, the presence of a non-mutated allele can therefore conceal (“mask”) the effect of a harmful mutation, reducing its exposure to purifying selection (Mable & Otto, 1998; Michod & Gayley, 1992). This masking effect allows deleterious alleles to persist

at low frequencies in populations, increasing the overall genetic load (Mable & Otto, 1998; Michod & Gayley, 1992).

In phenotypes expressed by haploid genomes, each locus carries a single allele, so every mutation, beneficial or deleterious, is directly exposed to selection if expressed (Mable & Otto, 1998, 2001) . Consequently, population genetic models have shown that purifying selection acts more efficiently on phenotypes expressed by haploid rather than by diploid genomes, resulting in faster removal of recessive deleterious mutations (Immler & Otto, 2018; Mable & Otto, 1998; Michod & Gayley, 1992). While this direct exposure increases the risk that an individual haploid will suffer the immediate effects of harmful mutations, it also accelerates the elimination of such alleles from the population as a whole.

Because of masking, when directly compared, individuals with diploid genomes are often shown to have a higher fitness (Perrot et al., 1991). However, due to the increased effects of purifying selection, overall populations with haploid genomes may be considered to have a higher fitness than diplontic populations (Mable & Otto, 1998). Higher fitness effects of masking were observed when the initial responses of both haploid and diploid *Saccharomyces cerevisiae* yeast to a mutagenic environment were compared, although both ploidy samples recovered fitness at similar rates (Mable & Otto, 2001). This suggests that, while masking is effective in preventing a rapid loss of fitness in response to mutation, the increased selective pressure experienced by haploid genomes enables faster recovery of fitness in response to a mutagenic event.

Overall, a predominantly diploid phase may be evolutionarily favoured in environments with weak selection against deleterious mutations and strong dominance of wild-type alleles, while haploid phases are favoured when selection is strong and mutation rates are high (C. D. Jenkins & Kirkpatrick, 1995; Mable & Otto, 2001; Otto & Goldstein, 1992; Otto & Marks, 1996; Perrot et al., 1991). These contrasting outcomes underscore how the balance between exposure and masking of genetic variation shapes the evolution of the biphasic lifecycle.

In diplontic species, the haploid phase is restricted to the gametes and, in some lineages, to mitochondrial genomes. Consequently, gametes represent a key stage where selection acts directly on haploid phenotypes before fertilisation, with direct effect on the evolution of the offspring produced (Immler, 2019).

1.3 Sexual Dimorphism in Gametes

Most species that undergo sexual reproduction exhibit anisogamy, where males and females produce gametes of different sizes. Male gametes are by definition smaller and produced in larger quantities, whereas female gametes are larger but typically produced in smaller quantities. These differences arise from a fixed energy budget for gamete production, where the total reproductive investment per individual is constrained. Under such constraints, the number of gametes that can be produced is inversely related to their size. Because zygote size is positively correlated with zygote fitness, selection favours the evolution of one large, resource-rich gamete and one of many small, mobile gamete when fusion occurs between dissimilar gametes (Parker et al., 1972).

This pattern is explained by disruptive selection theory, which proposes that gamete size variation was driven by selection favouring two extremes under conditions of random gamete fusion, as originally modelled for external fertilisers (Parker et al., 1972) Large gametes have a higher probability of successful zygote development due to greater energetic reserves, while smaller gametes gain an advantage through increased numbers and encounter rates. Over evolutionary time, these contrasting selective pressures produced the differentiation between oocytes and sperm, an outcome that persists even in species with internal fertilisation and reduced sperm competition (Parker, 1982).

Because oocytes are relatively few, energetically costly to produce, and have a high probability of being fertilised, they experience limited opportunity for haploid selection (Parker et al., 1972). Sperm, by contrast, are numerous, less energetically costly, and subject to intense post-copulatory competition and selection pressures. This suggests that haploid selection will have less opportunity

to act on the oocytes than on the smaller, more numerous, less costly, and more varied sperm (Immler & Otto, 2018).

1.4 Haploid Selection in Gametes

In predominantly diploid organisms, such as many animals, the haploid phase is restricted to the gametes, providing a brief but potentially important window during which selection can act directly on haploid phenotypes before fertilisation (Mable & Otto, 1998; Perrot et al., 1991). The theoretical possibility of haploid selection has long been recognised, as alleles in haploid genomes are directly exposed to selection without the masking effects of dominance that occur in diploids (Immler, 2019; Immler & Otto, 2018).

For many years, however, it was assumed that selection during this stage was minimal, largely because gametes, particularly sperm, were thought to be transcriptionally inactive (Joseph & Kirkpatrick, 2004). This view stemmed from early observations that sperm lacking nuclei were still capable of fertilisation (Lindsley & Grell, 1969; Lyon et al., 1972; Muller & Settles, 1927) and from evidence of extensive cytoplasmic bridges between developing spermatids, which allow for the sharing of gene products and equalising of gene expression among cells (Greenbaum et al., 2011; Weber & Russell, 1987). These features were interpreted as mechanisms that reduce haploid individuality and therefore minimise the scope for selection to act at the gamete level.

More recent findings have challenged this assumption. Studies have shown that post-meiotic transcription occurs in spermatids from the fruitfly *Drosophila melanogaster* (Barreau et al., 2008), indicating that haploid genomes can exhibit gene expression activity. In addition, unequal sharing of transcripts and proteins among connected spermatids has been observed in mammals, including the mouse gene Spam1 (Zheng et al., 2001), suggesting that cytoplasmic sharing is incomplete and that individual sperm may retain haploid-specific expression patterns. Such evidence implies that haploid gene expression could contribute to

functional variation among sperm produced by the same individual, creating the opportunity for selection to act on the haploid genome.

Understanding the mechanisms and consequences of haploid selection in gametes is therefore essential to explaining how genetic variation is filtered before fertilisation and how this process might influence both reproductive success and the genetic quality of offspring.

1.5 Potential for Haploid Selection Within Ejaculates

Although variation in sperm phenotype alone is not direct evidence of haploid selection, it defines the phenotypic substrate upon which such selection could occur prior to fertilisation. Within-ejaculate variation in sperm morphology, physiology, and motility has been observed across a wide range of taxa, including mammals, birds, and fish (Firman & Simmons, 2015; Lüpold & Pitnick, 2018). This variation forms the foundation for potential haploid selection within the ejaculate.

1.5.1 Competition among sperm within an ejaculate

When multiple sperm from the same male compete to fertilise a single oocyte, even subtle performance differences can influence success. In externally fertilising Atlantic salmon (*Salmo salar*), sperm velocity has been shown to predict fertilisation success under competitive conditions (Gage et al., 2004). Similarly, in domestic fowl (*Gallus gallus domesticus*), a simple sperm mobility assay accurately predicts both competitive and non-competitive fertility outcomes, demonstrating that faster, more motile sperm have higher fertilisation potential (Froman et al., 2002). Comparable results have been reported in mice (*Mus musculus*), where sperm motility and morphology contribute significantly to the likelihood of reaching and fertilising the oocyte (Firman & Simmons, 2010). Whilst these results were originally shown between ejaculates from separate males, similar competition between sperm from the same ejaculate occurs (Alavioon et al., 2017). These findings illustrate that phenotypic variation among a male's own sperm can influence which haploid genotypes achieve fertilisation.

1.5.2 Interaction with the female reproductive environment

The female reproductive tract imposes powerful selective filters, meaning only a fraction of ejaculated sperm reach the fertilisation site. In mammals, sperm must traverse the cervix, uterotubal junction, and oviductal reservoir, each stage selecting for sperm with specific traits such as progressive motility, intact acrosomes, and appropriate membrane properties (Suarez & Pacey, 2006). For example, studies in mice have shown that thermotactic and chemotactic responses within the oviduct enrich for sperm with higher DNA integrity and improved embryo development rates (Bahat & Eisenbach, 2006; Pérez-Cerezales et al., 2018). Similarly, in cattle (*Bos taurus*), oviductal epithelial binding selects for sperm with more stable plasma membranes and higher fertilisation potential (Lefebvre & Suarez, 1996). These findings demonstrate that the female environment can act as a selective landscape for sperm phenotypes, indirectly filtering haploid genomes.

1.5.3 Selective binding and fusion with the oocyte

A third form of selection occurs at the level of sperm–oocyte interaction. The binding of sperm to zona pellucida (ZP) glycoproteins acts as a critical checkpoint that discriminates between competent and incompetent sperm. Studies in humans have shown that binding to solubilised ZP or recombinant ZP proteins enriches for sperm with lower DNA fragmentation and improved fertilisation rates (Huszar et al., 2007; F. Liu et al., 2011). At the plasma membrane, molecular recognition between IZUMO1 on sperm and JUNO on the oocyte represents an essential ligand–receptor pair required for fusion (Bianchi et al., 2014). Differences in the abundance or accessibility of IZUMO1 may therefore contribute to fertilisation bias within ejaculates (Sebkova et al., 2014). Collectively, these studies define the major points where selection can act on sperm prior to fertilisation, setting the stage for assessing whether these processes reflect true haploid selection.

While these processes define the points at which selective pressures can act on sperm, the extent to which they translate into differential reproductive success

remains a key question. Understanding whether variation in sperm phenotype leads to measurable differences in embryo viability or offspring performance is required to provide crucial evidence for haploid selection in action.

1.6 Evidence that Sperm Fitness Influences Offspring Quality

Sperm fitness is defined as the capacity of an individual sperm cell to reach, fertilise, and successfully support zygote and embryo development. It integrates multiple factors, including motility, longevity, morphology, metabolism, and the integrity of nuclear and mitochondrial genomes (Marcu et al., 2024). Experimental studies across species demonstrate that variation in sperm fitness within a single ejaculate can have measurable effects on embryo and offspring outcomes.

In zebrafish (*Danio rerio*), sperm longevity has been directly linked to offspring performance. When sperm were sorted into long-lived and short-lived fractions from the same ejaculate, offspring produced by long-lived sperm exhibited increased embryo viability and reduced developmental abnormalities (Alavioon et al., 2017). A subsequent study demonstrated that these offspring also showed increased gamete production, increased reproductive fitness during adulthood and delayed reproductive ageing, suggesting long-term transgenerational fitness effects of within-ejaculate sperm selection (Alavioon et al., 2019).

At the molecular level, emerging data indicate that sperm cells from the same male differ not only phenotypically but also in gene and protein expression. Early work in mice showed that the sperm membrane glycoprotein SPAM1 is not evenly shared across spermatids due to incomplete cytoplasmic bridge diffusion, leading to functional heterogeneity among mature sperm (Martin-DeLeon, 2006).

Epigenetic variation among sperm provides an additional mechanism for differential fitness. Human and mouse sperm exhibit non-random patterns of histone retention and DNA methylation at genes involved in embryonic development and metabolism (Jenkins & Carrell, 2012; Lismer & Kimmins, 2023).

In livestock such as boar (*Sus scrofa*) and bull (*Bos taurus*), specific methylation patterns and small RNA profiles have been associated with male fertility and early embryo development (Kropp et al., 2017). These findings suggest that both genetic and epigenetic heterogeneity among sperm can influence fertilisation outcomes and potentially contribute to offspring phenotype.

Taken together, these studies demonstrate that sperm are not functionally equivalent, and that selection acting among haploid gametes can produce measurable effects on embryo development and offspring fitness. These findings highlight sperm fitness as a powerful determinant of reproductive success and evolutionary potential.

1.7 The Male Fertility Crisis

One of the clearest applications for research into selection acting upon male gametes, and the influence of sperm fitness on offspring outcomes, is in the improvement of assisted reproductive technologies (ART). Procedures such as *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) are now among the most common clinical interventions for infertility, accounting for an estimated 3–6% of all births in high-income countries (European IVF Monitoring Consortium (EIM) et al., 2022).

Despite the widespread adoption of ART, success rates remain limited. Globally, only 20–30% of IVF cycles and around 25% of ICSI cycles result in a live birth per treatment attempt, with rates falling sharply to below 10% in women over 40 (Adamson et al., 2025; Alavioon et al., 2021; European IVF Monitoring Consortium (EIM) et al., 2022; Griesinger & Larsson, 2023; *HFEA*, 2022). Even when fertilisation occurs, 20–50% of embryos fail to implant, and miscarriage rates remain between 10-13% depending on patient age and clinical protocol (Gardner et al., 2015; Hu et al., 2018; Kalra & Molinaro, 2008).

A central limitation of these technologies is that they bypass many of the natural selective pressures, such as temperature gradients, pH variation, and biochemical

interactions within the female reproductive tract, that normally filter for the most functionally competent sperm under natural conception (Alavioon et al., 2021). In ICSI, for instance, sperm are selected based largely on subjective assessments of motility and morphology, allowing suboptimal sperm to be used in fertilisation (Palermo et al., 2017). The removal of these natural selection mechanisms has been associated with the use of lower-quality sperm and may contribute to poorer developmental outcomes in ART-derived embryos and offspring compared to natural conceptions (Alavioon et al., 2021; Nixon et al., 2023; van Oosterhout et al., 2022).

The importance of understanding sperm fitness and selection is amplified by the ongoing male fertility crisis, characterised by substantial global declines in sperm count, motility, and morphology (Levine et al., 2017; Sengupta et al., 2017, 2018). Meta-analyses indicate that sperm counts in men from Western countries have fallen by over 50% since the 1970s, with similar downward trends now reported across other continents (Levine et al., 2023). These widespread declines suggest that the reliance on ART will continue to increase, underscoring the need for more refined and biologically informed methods for assessing and selecting sperm in clinical contexts (De Jonge & Barratt, 2019; European IVF Monitoring Consortium (EIM) et al., 2022).

Environmental and lifestyle factors have also been strongly implicated in declining sperm quality. Exposure to endocrine-disrupting chemicals such as bisphenol A (BPA), phthalates, and pesticides has been shown to impair spermatogenesis and reduce motility (Skakkebæk et al., 2022). Similarly, obesity, heat exposure, chronic stress, and poor diet negatively affect both sperm development and function (Carvalho et al., 2021; Pini et al., 2020; Sharma et al., 2013). These factors alter the testicular environment and may contribute to the molecular and phenotypic heterogeneity observed among sperm within a single ejaculate. Understanding how such stressors influence sperm fitness is therefore critical for improving reproductive outcomes both in natural and assisted settings.

Much of the existing clinical fertility research treats ejaculate quality as a single metric per individual (Said & Land, 2011) averaging across all sperm cells and ignoring the within-ejaculate variation that might better predict fertilisation success (Alavioon et al., 2021). This population-level approach is useful for identifying general trends, helping in the categorisation and diagnosis of disease and other potential causes of infertility, but fails to identify which specific sperm are functionally superior and therefore fails to provide tailored solutions for individuals facing infertility.

1.8 Current Clinical Methods for Sperm Selection

With the increased application of IVF in humans, and the increasing information to suggest that selection for the highest fitness gametes is crucial to the success of these methods, many clinical practices have implemented methods to isolate "better" sperm (Said & Land, 2011). Clinics commonly use swim-up and density gradient centrifugation to isolate motile sperm under the assumption that movement reflects health and fertilising ability (Said & Land, 2011). However, while these methods effectively separate motile cells from immotile cells, they do not guarantee removal of sperm with DNA damage, epigenetic abnormalities, or mitochondrial dysfunction. Some studies have shown that sperm selected through these techniques still yield poor fertilisation outcomes or lead to early embryo arrest (Ribas-Maynou et al., 2021).

One of the critical issues in sperm selection is the assumption that motility equals functionality, however, whilst motility is necessary for sperm to reach and penetrate the oocyte, it is not sufficient on its own to ensure fertilisation success or embryo viability (Ribas-Maynou et al., 2021). This pattern is not restricted to humans. Similar findings have been reported in livestock species, including cattle (*Bos taurus*) (Samardzija et al., 2006) and boar (*Sus scrofa*) (Noguchi et al., 2015), where sperm selected for motility using density gradients showed mixed results for improved conception or embryo development rates. These results underscore that high motility alone does not guarantee functional competence and that other molecular or structural defects may persist undetected.

This discrepancy suggests that current selection methods are oversimplified and overlook key molecular aspects of sperm quality. Identifying molecular markers or phenotypic indicators of “functional” rather than just “live and moving” sperm remains a major unmet need in reproductive biology.

1.9 The Genomic and Transcriptomic Differences in Sperm Fitness

One key approach to linking sperm phenotype and haploid genotype is genomic and transcriptomic sequencing of sperm subpopulations. In mammals, single-cell RNA sequencing across spermatids in multiple species found that many genes exhibit allele-biased expression, i.e. they are not fully shared through cytoplasmic bridges, a pattern termed “genoinformative markers” (GIMs) (Bhutani et al., 2021). This result suggests that individual sperm cells may maintain haploid-specific gene expression potential.

In addition, in Mexican Tetra (*Astyanax mexicanus*) sperm subpopulations separated by phenotypic dye properties differed in their haploid genotype frequencies, indicating that phenotype classes correspond to distinct haplotypes (Borowsky et al., 2018). More recently, long-read sequencing of individual human sperm detected structural variation and genome-level heterogeneity at the haploid level (H. Xie et al., 2023). These findings reinforce the idea that sperm genomes are not identical within a male and may carry unique allelic compositions.

Together, these genomic observations imply that genetic heterogeneity within ejaculates is not random noise but a substrate on which selection might act. They support the hypothesis that haploid selection could modulate the transmission of genetic variants by favouring sperm with fitter haplotypes.

Alongside genomic approaches, transcriptomic analyses complement this view by examining how expression differences among sperm might contribute to fitness. In zebrafish (*Danio rerio*), single-cell genome and transcriptome sequencing of post-

meiotic spermatids revealed that a considerable fraction of genes (~6 %) show allele-specific expression, challenging the assumption of complete sharing via cytoplasmic bridges and reaffirming that haploid expression differences may drive phenotypic diversity (Marcu, 2024). These findings point to a potential mechanism by which sperm not only reflect the haploid genotype but also act as vectors of paternal environmental effects via regulatory RNAs. Collectively, these studies suggest that variation in the sperm transcriptome, including both coding and non-coding elements, may play a functional role in fertilisation success and early embryogenesis.

Together, genomic and transcriptomic studies provide converging evidence that haploid selection within ejaculates can shape sperm function and influence offspring phenotypes. These findings challenge the classical view of sperm as passive carriers of paternal DNA and instead support a model in which both genetic and regulatory variation among sperm contributes to reproductive success.

1.10 Sperm Proteomics

Whilst the most common methods to study the potential of selection, and the phenotype of gametes, have often been either genomic or transcriptomic in nature (Immler, 2019), another analytical method used to elucidate phenotypes has been proteomics (Hashemitabar et al., 2015). This method has often been underutilised due to its lack of material amplification methods and high cost; however, proteomics remains the clearest snapshot of the phenotype of a cell at the time of study, including post-translational modifications and total abundance of proteins and co-factors (Cannarella et al., 2020).

Proteomic studies of sperm have revealed significant variation in protein expression profiles between fertile and sub-fertile men (Amaral, Castillo, et al., 2014; Selvam et al., 2019), or between high- and low-motility sperm samples (57). In addition to humans, sperm proteomes have been investigated across species such as cattle (D'Amours et al., 2019a), boar (Zigo et al., 2023), and mice (Baker,

Hetherington, Reeves, & Aitken, 2008), all showing distinct proteomic signatures associated with sperm motility, longevity, or fertilisation success.

For example, in humans, sperm from asthenozoospermic individuals show altered expression in proteins linked to mitochondrial function, flagellar motility, and stress response (34). In both mice and humans, obesity and metabolic stress alter sperm protein profiles involved in mitochondrial respiration, chromatin condensation, and oxidative balance, reducing fertilisation success (Carvalho et al., 2021; Pini et al., 2020). These findings demonstrate that the sperm proteome is sensitive to both intrinsic genetic variation and external physiological conditions.

Understanding the sperm proteome could enable the development of non-invasive diagnostic tools for sperm function, especially in the context of ART. For instance, targeted identification of surface or secreted proteins may allow real-time selection of high-quality sperm using minimally invasive assays. Beyond fertility, sperm proteomics may also shed light on male reproductive aging, the effects of environmental exposures, or transgenerational inheritance mechanisms (Cannarella et al., 2020).

One major challenge in sperm proteomics is the small amount of protein present in each cell, combined with their high DNA content, compact chromatin and specialised morphology (Codina et al., 2015). Isolating enough protein for robust mass spectrometry without pooling across individuals, and therefore losing resolution of subpopulations, is technically difficult. Additionally, single-cell proteomics in sperm remains in its infancy due to these limitations, making non-destructive or high-throughput applications even more crucial for moving the field forward (Ahmad & Budnik, 2023). This is important because resolving protein expression at the level of individual sperm would allow functional heterogeneity to be directly linked to fertilisation potential, rather than being masked by population averages.

1.11 What is a Biomarker?

Whilst sperm proteomics remains technically difficult, one of its primary uses has been in the identification of biomarkers. A biomarker is a measurable indicator of a biological state or condition. In the context of sperm, biomarkers could be used to predict fertilisation potential, embryo viability, or even offspring health outcomes (Intasqui et al., 2018). They could also guide treatment decisions in ART, help identify the best sperm in a sample, or provide early indicators of male subfertility.

In humans, biomarkers such as the DNA degradation index (DDSi) have been correlated with fertility outcomes (Esteves et al., 2015). These markers are increasingly being tested as adjuncts to standard semen analysis in both clinical and research settings.

Current diagnostics in male fertility are limited in predictive power, especially in normozoospermic men who still experience unexplained infertility (Pandruvada et al., 2021). Molecular biomarkers could fill this gap by revealing functionally relevant differences not visible through morphology or motility analysis. Their development could also improve ART outcomes by enabling personalised selection strategies that go beyond population averages.

1.12 Beyond Sperm: The Testicular Origins of Sperm Quality

Whilst the identification of potential biomarkers for sperm fitness remains a large research target in reproductive biology, it must be remembered that the origin of sperm phenotypes and the understanding of the potential role of any biomarker identified relies not only on understanding sperm biology but also the biology of the testes. Sperm quality is not only shaped during ejaculation but also depends heavily on the testicular environment during spermatogenesis. Testis-specific gene expression profiles, protein turnover, and local stress responses (e.g., to heat or toxins) can all impact the molecular composition and function of mature sperm. Transient scrotal heat stress in mice for instance leads to widespread

transcriptomic and proteomic changes in developing germ cells, impairing motility and fertilisation capacity in mature sperm (Paul et al., 2008; Y.-F. Zhu et al., 2006). Similarly, in humans, testicular biopsies from infertile men frequently show dysregulation in stress-response and mitochondrial pathways that correspond with altered sperm proteomes (Davalieva et al., 2022; Liang et al., 2021). These examples illustrate that many features of sperm phenotype, and therefore potential biomarkers, originate upstream in the testes.

By combining testis and sperm datasets, researchers can better identify the origin and functional significance of candidate biomarkers. Integrative approaches linking testicular transcriptomes, sperm proteomes, and fertilisation outcomes are beginning to reveal which proteins persist into mature sperm because they serve continued roles, and which are simply developmental relics (Amaral, Castillo, et al., 2014; Castillo et al., 2023).

One of the central questions in sperm biomarker research is whether key proteins or transcripts are relics from the testis or have active functional roles in mature sperm. Given the compacted nature of sperm chromatin and their limited transcriptional capacity, many molecules may simply be remnants of development (Robles et al., 2017). However, others, particularly surface proteins or those involved in signalling, such as IZUMO1 (Bianchi et al., 2014), retain active roles during fertilisation and early embryo development, suggesting that their detection could carry strong predictive value.

1.13 The Importance of Animal Models

Given the ongoing decline in human fertility and the increasing demand for ART, understanding sperm biology in humans is a top research priority. However, direct studies on human gametes are often limited by ethical, legal, and technical constraints.

Unlike in model organisms, it is illegal and unethical to generate or manipulate human embryos beyond certain developmental stages, nor can we follow resulting

offspring. This restricts the ability to link sperm traits to developmental outcomes in humans and highlights the need for model systems (Stouffer & Woodruff, 2017a). Animal models allow for controlled manipulation of environmental conditions, genetic backgrounds, and experimental interventions that are not possible in humans. They provide the means to conduct hypothesis-driven experiments on fertility and reproduction.

Zebrafish are a powerful vertebrate model for reproductive biology due to their external fertilisation, high fecundity, transparent embryos, and generation times (Hoo et al., 2016). Their sperm can be collected non-lethally and fertilisation outcomes can be monitored in real time. Zebrafish are especially relevant in the context of environmental stress because they are sensitive to water temperature, pollutants, and endocrine-disrupting chemicals, all of which are known to affect fertility (Hajam, Rani, Sharma, Kumar, & Verma, 2021). Their use provides insight into how broader ecological changes may impact reproductive success in both wildlife and aquaculture. Combined with available genetic tools like CRISPR, transgenics, and lineage tracing, zebrafish offer a comprehensive platform for linking sperm phenotype to embryo outcome.

1.14 This Thesis: The Molecular Biomarkers of Sperm Fitness

This thesis investigates how molecular variation in sperm relates to functional sperm phenotypes and potential biomarkers of fertilisation success. Building on the theoretical framework of haploid selection, and previous genomic and transcriptomic empirical evidence of this, the following chapters use both zebrafish and human models to explore how environmental factors, developmental context, and phenotypic selection shape the sperm proteome, and how this can be leveraged for biomarker discovery.

Chapter 2 begins with a proteomic analysis of zebrafish testes under thermal stress, aiming to identify how environmental temperature affects protein expression during spermatogenesis. This chapter establishes the testicular context

in which sperm phenotypes arise and highlights stress-responsive proteins that may be carried into mature sperm.

Chapter 3 shifts focus to the mature sperm proteome in zebrafish. By profiling sperm from the same individuals used in Chapter 1, this chapter explores the molecular consequences of altered testicular conditions and identifies candidate proteins associated with sperm longevity, motility, and overall fitness. Together, Chapters 2 and 3 establish zebrafish as a model for understanding how environmental and developmental factors shape sperm molecular phenotypes.

Chapter 4 extends this approach to humans, comparing the proteomes of sperm populations that differ in phenotypes that can be separated through assays such as swim-up and methyl cellulose. This chapter identifies proteins with differential abundance between selected and un-selected sperm. The findings highlight biomarkers with translational potential for sperm proteomics in clinical fertility research.

Chapter 5 focuses on validating the candidate sperm proteins as biomarkers identified in chapter 4, using flow cytometry. This chapter tests whether protein expression levels can distinguish high- from low-fitness sperm within an ejaculate. It represents a step toward functional application, evaluating whether proteomic signals identified in earlier chapters can be used to develop improved sperm selection tools for assisted reproduction.

Overall, this thesis integrates proteomic profiling with functional assays to explore the molecular basis of sperm fitness and within-ejaculate variation. It aims to contribute both to fundamental understanding of haploid selection and to the development of practical tools for fertility diagnostics and treatment.

2 The Effects of Heat Stress on the Zebrafish Testes Proteome

2.1 Abstract

With global temperatures rising, a clear threat has emerged to reproductive fitness across species, with thermal stress emerging as a particularly important factor. However, the molecular consequences of these changes are still poorly understood. In ectotherm aquatic species, rising water temperatures can directly influence physiological processes as internal body conditions directly correlate with external environmental temperature. Male fertility appears to be particularly vulnerable as thermal fluctuations can impair spermatogenesis, alter sperm quality, and ultimately reduce reproductive fitness. While genomic and transcriptomic analyses have provided important insights into how organisms respond to heat stress, these approaches are limited in their ability to capture changes at the protein level, where much of the functional impact of temperature changes is realised.

Here, quantitative proteomic profiling via liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to assess the effect of a two-week period of elevated temperature on the testes proteome of adult male zebrafish *Danio rerio*. A global proteome of 5415 proteins was generated, and a total of 377 proteins were found to have significantly different abundance between the heat treated (34°C) and control groups (28°C). Notably, these molecular changes were accompanied by a consistent reduction in gonad size in heat-stressed individuals, independent of somatic growth. The abundance of peptides involved in processes of spermatogenesis was significantly reduced in the heat-treated testes whilst the abundance of molecular repair peptides was significantly increased. These findings suggest that heat stress alters the molecular composition of the testes in a way that is likely to impact sperm performance, alongside of morphological changes to the organ itself. Our results demonstrate that even a moderate increase in temperature over a short period of time has fundamental effects on the

molecular composition and function of testes, supporting the idea that male reproductive fitness is particularly vulnerable to increasing temperatures.

2.2 Introduction

Sexual reproduction is the result of on an intricate and delicate balance of molecular and physiological pathways and is known to be highly sensitive to environmental fluctuations, with a “reproductive stress axes” forming the interface of physiological stress response and reproductive output . This reproductive stress axis involves many different endocrine pathways depending on the taxa experiencing stress, however, these are primarily the hypothalamic-pituitary-adrenal (HPA) and the hypothalamic-pituitary-gonadal (HPG) axes, the effects of which have been noted in the reproductive and growth outcomes of various fish (Boni, 2019; Canosa & Bertucci, 2023; Chand & Lovejoy, 2011; Schreck, 2010), and mammals such as sheep *(Ovis aries*) (Naqvi et al., 2012), mice (*Mus musculus*), rhesus macaques (*Macaca mulatta*) and humans (Chand & Lovejoy, 2011; Toufexis et al., 2014).

One of the fundamental aspects of sexual reproduction in animals is gametogenesis, the generation and storage of gametes, which depends on the coordinated interaction between somatic and germ cells, typically occurring in the gonads (Oatley & Brinster, 2012), and is therefore directly influenced by the HPG stress axis. In males, spermatogenesis occurs in the testes (de Kretser et al., 1998), which serve as a physiological interface between the external environment, the organismal physiology and the germ cells (Godmann et al., 2009). Therefore, the testes are a critical focal point for understanding how environmental factors, including temperature, toxins, and nutrition, can affect both an individual organism and potentially a population as a whole (Boni, 2019; Rahman et al., 2018).

One environmental stressor that is of major current importance is thermal stress - particularly in aquatic species due to globally rising water temperatures (Alfonso et al., 2021; Barbarossa et al., 2021; Cheng et al., 2022). In aquatic animals, gonads are commonly internally located due to the nature of ectothermic physiology where

the body condition of the animal is directly influenced by environmental temperatures. This means that for most aquatic species, heat stress can have immediate and potentially dire effects on reproduction (Alix et al., 2020). In species such as the brown trout (*Salmo trutta)*, water temperature of 13°C – just 3°C above the optimal 0-10°C spawning temperature - can have both an indirect negative effect on fertility as the adult fish experiences a change in temperature causing physiological effects on the gonads, as well as direct effects on the gametes themselves as they are directly exposed to the higher temperature (Fenkes et al., 2017). Similar negative effects of increased water temperature have been noted across other aquatic species, with partial to complete regression in the testes of fish including yellowtail tetra (*Astyanax altiparanae*) (de Siqueira-Silva et al., 2015), European bullheads (*Cottus gobio*) (Dorts et al., 2012), and Pejerry (*Odontesthes bonariensis*) (Elisio et al., 2012).

In other ectothermic species such as insects including the red flour beetle (*Tribolium castaneum)* fertility output was found to reduce significantly before lethal thermal limits were achieved, with significant reductions of up to 40% in testes volume (Cole et al., 2025). Similar male sub-fertility was achieved in high temperatures before lethality in the parasitoid wasp (*Anisopteromalus calandrae*) with observation of up to 100 times less sperm produced by stressed males compared to the control (Nguyen et al., 2013). These findings illustrate that even moderate thermal stress can severely impair male fertility in ectothermic species, emphasising that reproductive function is often compromised well before survival thresholds are reached.

Notably, in zebrafish *Danio rerio*, prolonged increased heat exposure of 6° above the optimal 28°C over a period of two weeks caused reductions in overall male fertility rates, although the offspring were found to have no significant differences in survival or abnormality rates compared to the offspring of control males (Irish et al., 2024). Similarly, assays including heat stress in combination with other stressors such as predatory cues, overcrowding and starvation for a period of 21 days revealed negative consequences for sperm production, number and quality in

zebrafish (Valcarce et al., 2023). Zebrafish are not only a prime example of the effects of stress on reproduction in aquatic and ectothermic species, they are also a commonly used model organism for mammalian and human reproduction, despite the differences in modality of reproduction as over 70% of genes are homologous (Hoo et al., 2016).

Inbreeding is an important consideration when interpreting reproductive phenotypes in fish, as laboratory-maintained strains typically experience far higher levels of inbreeding than wild populations. Captive lines bred over many generations often accumulate elevated homozygosity and reduced genetic diversity, which can lead to inbreeding depression affecting reproductive traits such as sperm count, morphology, and motility (Kincaid, 1983; Wright et al., 2008). As a result, laboratory fish may be more susceptible to environmental or physiological stressors, including those that affect spermatogenesis or sperm function.

Conversely, the reduced genetic variability within inbred laboratory strains may lead to lower inter-individual variation, making phenotypic trends easier to detect and less obscured by background noise. Wild populations typically show broader individual variability because of their heterogeneous genetic backgrounds (Gjedrem & Baranski, 2009). Taken together, these differences underline the importance of considering both genetic background and domestication history when interpreting results from laboratory zebrafish and comparing them to studies using wild strains.

However, despite their utility as a model organism, another key point of note is that zebrafish and other ectotherms lack the protective adaptations seen in internally fertilising endotherms, where external testes help buffer gametogenesis against heat stress. In many terrestrial mammals for example, testes are located externally for better temperature regulation (Kastelic & Rizzoto, 2021). Nevertheless, high temperatures can still have negative consequences for the fertility of these species. For example, in humans, heat stress has been found to result in a decrease in both sperm motility and quality in men exposed to high temperatures

as a result of their professional occupation, leading to potential fertility issues (Hoang-Thi et al., 2022). Similarly, in house mice (*Mus musculus)*, exposure to higher temperatures was found to cause a reduction in sperm quality as well as morphological changes to both testes structure and size (Rizzoto et al., 2020).

Besides direct assays on reproductive output, molecular analyses of heat stress to date have largely focused on genetic effects, or effects at the transcriptomic level (Guan et al., 2025; Jayakodi et al., 2019). Genetic analysis of dairy cattle *Bos taurus* for example revealed six specific genomic regions which were found to correlate with the likelihood of conception under heat stress conditions, including candidate genes *BRWD1, EXD2, ADAM20, EPAS1, TAOK3*, and *NOS1* implicated in both fertilisation and heat stress response, providing a useful guide for future breeding (Sigdel et al., 2020). In the Mexican four-eyed octopus, *Octopus maya,* transcriptome analysis revealed several genes including *ZMYND15* involved in histone interactions and haploid gene regulation, and *KLHL10* involved in sperm maturation through protein ubiquitination, that were downregulated when exposed to thermal stress leading to infertility (López-Galindo et al., 2019).

While gene expression data provides information of the possible effects of temperature on molecular processes, the translation to effects at the protein level are often not as direct and require more in depth analyses at the proteome level. Proteomics, the identification, classification and quantification of proteins in a tissue, can help elucidate these details. However, the general high cost and high input material requirements have typically precluded the possibility of proteomic experiments on low abundance tissue, especially as proteins cannot currently be amplified in volume like RNA and DNA (Chalmel & Rolland, 2015). Despite these limitations, the number of proteomic studies of reproductive responses to stress is growing (Panner Selvam et al., 2019). For example, in the Taiwanese country chicken *Gallus gallus domesticus*, mass spectrometry revealed 92 distinct proteins to be differentially expressed in the testes as a result of acute heat stress, potentially linked to a decrease in fertility (Wang et al., 2014). Proteomics has also

revealed specific post-translational modifications such as acetylation of lysine can change in response to heat stress in mouse testes (C. Xie et al., 2018).

Here, we exposed male zebrafish to two-week periods of increased temperature of 34°C and compared these to males maintained at 28°C. We performed two experimental blocks, one to collect testes for weight measurements, and one to collect testes for quantitative proteomic profiling using liquid chromatography tandem mass tag spectrometry (TMT LC-MS/MS) to measure the changes in abundance across the testes proteome.

Tandem Mass Tag (TMT) labelling was selected because it enables multiplexed quantification of peptides across multiple samples within a single LC–MS/MS run, substantially reducing technical variation and improving the accuracy of relative protein abundance measurements (Zhang & Elias, 2017). This workflow was paired with an Orbitrap mass spectrometer, as Orbitrap instruments provide high mass accuracy, high resolving power, and excellent dynamic range, all of which enhance peptide identification confidence and quantification precision (Eliuk & Makarov, 2015). Together, these features make TMT-labelled Orbitrap-based proteomics well suited for detecting subtle shifts in protein expression across biological samples.

Further analyses including STRING and gene ontology (GO term) were performed to provide evidence at both a proteomic and physiological level that changes to molecular mechanistic regulation are key to the changes observed in sperm function and quality for zebrafish instead of physiological changes in terms of gonad size.

2.3 Methods

2.3.1 Zebrafish husbandry

We used sexually mature AB wild-type male zebrafish obtained from the European Zebrafish Resource Centre (EZRC) and aged between 18 to 24 months for this study. Prior to experiments, the fish were maintained in 3.5L tanks at a 10:6 male to female sex ratios kept at 28°C for two weeks with a 14:10 light schedule. All fish were kept in a recirculating aquatic system with filtered, UV-treated water and maintained under standard zebrafish husbandry conditions. Fish were fed *ad libitum* with a mix of dry feed (ZEBRAFEED 400–600 by SPAROS, Área Empresarial de Marim, Lote C, 8700-221 Olhão, Portugal) and live artemia (Sep-Art Artemia Cysts, Ocean Nutrition). All fish used for experiments were kept under Home Office license P0C37E901.

2.3.2 Experimental set up

Male zebrafish were randomly allocated to experimental tanks in the standard 10:6 male:female ratio (16 fish per 3.5 L tank). For each experimental run, six tanks were established (three replicate blocks), and within each block, the two tanks were randomly assigned to either the control temperature (28 °C) or the heat-stress treatment (34 °C). To minimise environmental confounds, each treatment tank was physically paired with its corresponding control tank from the same block; paired tanks were positioned adjacent to one another on the same rack and exposed to identical light conditions, room temperature, and flow rate. Submersible heaters were added to both tanks in each pair, activated at 34 °C for treatment tanks or 28°C in control tanks to control for potential heater effects. Fish remained in their assigned conditions for two weeks.

For the proteomics analyses, we collected the testes from a total of 60 males and pooled the testes from 10 males kept in the same tank as one replicate sample resulting in a total of three replicates.

For analysis of the change in testes weight, we used 42 sexually mature ABWT male zebrafish obtained from the Zebrafish International Resource Center (ZIRC) aged one and a half years. Fish were kept in a sex ratio of 6 males to 6 females with 12 fish per 3.5L tank. Prior to the experiment, fish were anaesthetised using metomidate hydrochloride (Aquacalm by Syndel, #9, 4131 Mostar Rd, Nanaimo, BC V9T 6A6 Canada), weighed to the nearest mg, and photographed for identification. Fish were arranged into three replicate blocks, each consisting of one control tank and one heat-treatment tank, positioned adjacent to one another on the same rack and exposed to identical light conditions, room temperature, and flow rate. Following the two-week treatment period, fish IDs were anonymised and fish were euthanised, weighed, photographed for identification and gonads were dissected on ice and weighed. Following data analysis, identification photographs were used to reveal treatment identities and match weights pre- and post-treatment.

2.3.3 Protein extraction and LC-MS/MS

Dissected testes were flash frozen in liquid nitrogen upon collection with no added buffer and stored at -80°C prior to extraction. Protein was extracted from pooled testes samples , using an SDC-based in-solution digestion approach (Masuda et al., 2008), by the addition of 500 μL 10% SDS with three cycles of vortexing and boiling at 99°C as well as pestle disruption. The samples were then vortexed and centrifuged to remove debris and Proteins were precipitated using methanol/chloroform and acetone. Protein pellets were resuspended in 2.5% sodium deoxycholate (SDC; Merck) in 0.2 M EPPS-buffer (Merck), pH 8.5, and vortexed under heating for a total of three cycles.

Protein concentration was estimated using a Direct Detect fluorometric assay (MERCK MILLIPORE, Dorset, UK). Equal amounts of protein for each replicate were reduced, alkylated, and digested with trypsin in the SDC buffer according to standard procedures. After digestion, the SDC was precipitated by adjusting to 0.2% TFA, and the clear supernatant subjected to C18 SPE (OMIX tips; Agilent). Peptide concentration was further estimated by running an aliquot of the digests

on LCMS. Tandem Mass Tag (TMT) labelling was performed using a TMT™ 6plex kit (Lot VB290974, ThermoFisher Scientific, Hemel Hempstead, UK) according to the manufacturer's instructions with slight modifications; for each sample approx. 100 µg of the testes peptides were dissolved in 90 µl of 0.2 M EPPS buffer (MERCK)/10% acetonitrile, and 200 µg TMT reagent dissolved in 22 µl of acetonitrile was added. Samples were assigned to the TMT channels in two separate experiments, as follows: replicates from Control males were run in channels 126, 127 and 128 and replicates from heat stress males were run in channels 129, 130 and 131.

After 2 h incubation, aliquots of 1.5 µl from each replicate from each individual TMT experiment were combined in 500 µl 0.2% TFA, desalted, and analysed on the mass spectrometer to check the labelling efficiency and estimate total sample abundances. The sample aliquots were quenched by adding 8 µl of 5% hydroxylamine and combined to roughly level abundances. The peptides were desalted using a C18 Sep-Pak cartridge (200 mg, Waters, Wilmslow, UK). The eluted peptides were dissolved in 500 µl of 25 mM NH_4HCO_3 and fractionated by high pH reversed phase HPLC.

The replicates were loaded to an XBridge® 5 µm BEH C18 130 Å column (250 x 4.6 mm, Waters). Fractionation was performed on an ACQUITY Arc Bio System (Waters) with the following gradient of solvents A (water), B (acetonitrile), and C (25 mM NH_4HCO_3 in water) at a flow rate of 1 ml min^{-1}: solvent C was kept at 10% throughout the gradient; solvent B: 0-5 min: 5%, 5-10 min: 5-10%, 10-80 min: 10-45%, 80-90 min: 45-80%, followed by 5 min at 80% B and re-equilibration to 5% for 24 min. Fractions of 1mL were collected every 1 min and concatenated by combining fractions of similar peptide concentration to produce 29 final fractions testis samples for MS analysis.

Aliquots were analysed by nanoLC-MS/MS on an Orbitrap Eclipse™ Tribrid™ mass spectrometer coupled to an UltiMate® 3000 RSLCnano LC system (Thermo Fisher Scientific, Hemel Hempstead, UK). The samples were loaded onto a trap

cartridge (Pepmap 100, C18, 5um, 0.3x5mm, Thermo) with 0.1% TFA at 15 µl min^{-1} for 3 min. The trap column was then switched in-line with the analytical column (nanoEase M/Z column, HSS C18 T3, 1.8 µm, 100 Å, 250 mm x 0.75 µm, Waters) for separation using the following gradient of solvents A (water, 0.1% formic acid) and B (80% acetonitrile, 0.1% formic acid) at a flow rate of 0.2 µl min^{-1} : 0-3 min 3% B (parallel to trapping); 3-10 min linear increase B to 8 %; 10-75 min increase B to 37%; 75-90 min linear increase B to 50 %; followed by a ramp to 99% B and re-equilibration to 3% B.

Data were acquired with the following parameters in positive ion mode: MS1/OT: resolution 120K, profile mode, mass range *m/z* 400-1800, AGC target 100%, max inject time 50 ms; MS2/IT: data dependent analysis with the following parameters: top10 in IT Turbo mode, centroid mode, quadrupole isolation window 0.7 Da, charge states 2-5, threshold 1.9e4, CID CE = 35, AGC target 1e4, max. inject time 70 ms, dynamic exclusion 1 count for 15 sec mass tolerance of 7 ppm; MS3 synchronous precursor selection (SPS): 10 SPS precursors, isolation window 0.7 Da, HCD fragmentation with CE=65, Orbitrap Turbo TMT and TMTpro resolution 30k, AGC target 1e5, max inject time 105 ms, Real Time Search (RTS): protein database *Danio rerio* (Uniprot reference proteome UP000000437, August 2022, 46,842 entries), enzyme trypsin, 1 missed cleavage, oxidation (M) as variable, carbamidomethyl (C) and TMT as fixed modifications, Xcorr = 1, dCn = 0.05.

2.3.4 Weight data analysis

Weight data were analysed and visualised using R version 4.4.3 (The R Foundation for Statistical Computing, Vienna, Austria, URL https://www.R-project.org/) for generalised linear model analysis. Model assumptions were checked using DHARMa (Florian, 2016). We ran a series of statistical models to assess the effects of heat treatment on the size of the testes. Assumption violations in residuals led us to refit all models using Gamma GLMs with a log link, which better accommodate the positive, right-skewed distribution of testes weight. DHARMa diagnostics confirmed substantially improved model fit and residual

uniformity for these models. We used stepwise reduction using the AIC as an indicator of fit to determine the final models.

2.3.5 Proteome data analysis

The acquired raw proteome data were processed and quantified in Proteome Discoverer 3.0 (Thermo) using the incorporated search engines Comet and CHIMERYS (MSAID, Munich, Germany). The processing workflow for both engines included recalibration of MS1 spectra (RC), reporter ion quantification by most confident centroid (20 ppm) and a search on the *Danio rerio* fasta database (as above) and a common contaminants database. For CHIMERY, the Top N Peak Filter was used with 20 peaks per 100 Da and parameters were precursor tolerance 6 ppm, enzyme trypsin with 1 missed cleavage, variable modification oxidation (M), fixed modifications carbamidomethyl (C) and TMT 6plex on N-terminus and K. For Comet the version 2019.01 rev. 0 parameter file was used with default settings except precursor tolerance set to 6 ppm and trypsin missed cleavages set to 1. Modifications were the same as for CHIMERYS.

The consensus workflow included the following parameters: assigning the three replicates/channels as described above per condition (cold and heat), only unique peptides (protein groups) for quantification, intensity-based abundance, TMT channel correction values applied (Lot VB290974),), co-isolation/SPS matches thresholds 50%/70%, normalised CHIMERYS Coefficient Threshold 0.8, normalisation on total peptide abundances, protein abundance-based ratio calculation, missing values imputation by low abundance resampling, hypothesis testing by t-test (background based), adjusted p-value calculation by BH-method. All data filtering was performed in accordance with current proteomics best practice guidelines (Burger, 2018).

Following data acquisition from the consensus workflow, all identified Uniprot IDs were re-mapped using the Uniprot ID-mapping software (The UniProt Consortium, 2021) to ensure removal of depreciated and obsolete protein IDs. Processed data

were further analysed and visualised using R version 4.4.3 (The R Foundation for Statistical Computing, Vienna, Austria, URL https://www.R-project.org/), using a modified Dseq2 differential expression pipeline (Love et al., 2014) to normalize abundances and determine data significance. Gene Ontology (GO) term analysis was performed using BioMart (Haw et al., 2011; Smedley et al., 2015) and Bioconductor (Gentleman et al., 2004) packages through R.

2.4 Results

2.4.1 Relative testes weight

Visual inspection of the allometric relationship between somatic body weight and testis mass (Figure 2-1) showed an approximately linear association with variance increasing at higher testis mass. This supports the suitability of the Gamma GLMs used to test treatment effects on corrected testes weights.

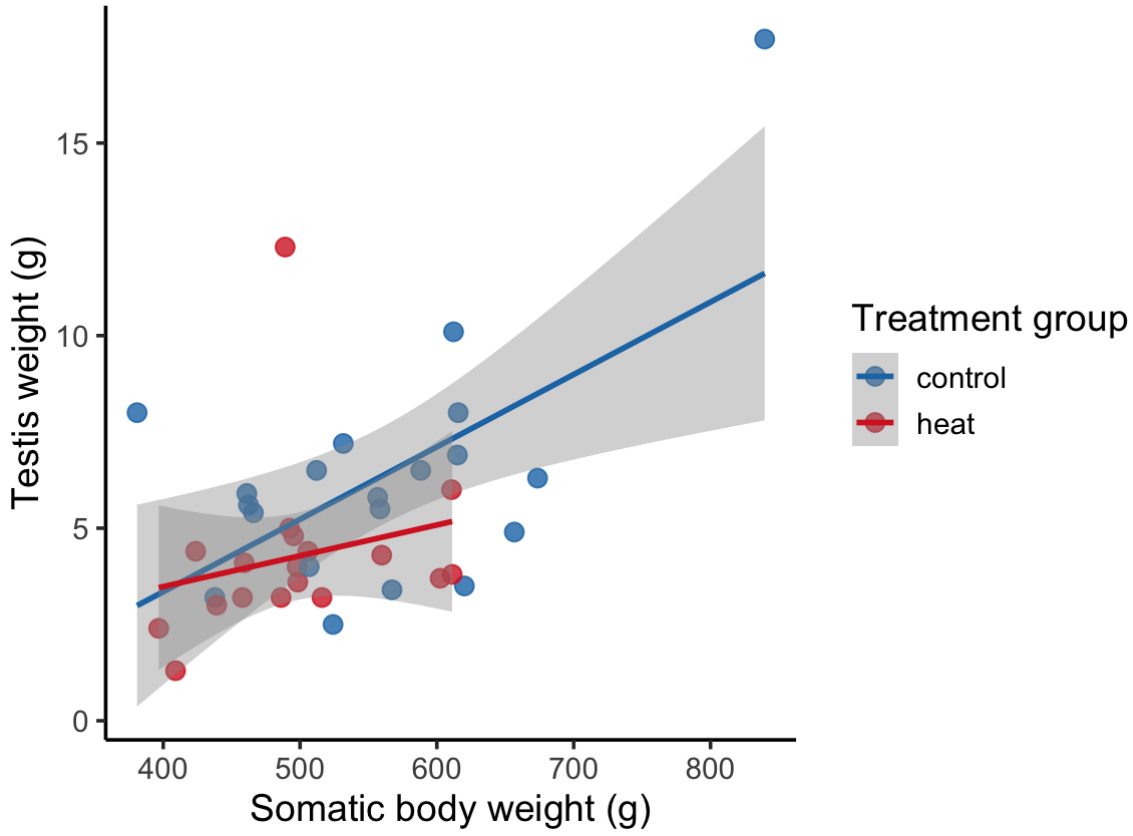


Figure 2-1: Scatterplot showing the relationship between somatic body weight (total body mass minus testes; x-axis) and corrected testis mass (y-axis) for individual males in the control (28°C, blue) and heat-treated (34°C, red) groups. Points represent individual fish, and coloured lines show group-specific linear regression fits with 95% confidence intervals.

Across all final Gamma GLMs (Tables 2-1-2-4), the effect of heat treatment on testes weight was consistently negative, with varying significance depending on the covariates included. The models included Treatment (Heat vs Control), Batch (B1–B3), and one of three body-weight covariates (final weight, somatic weight, or weight change), depending on the model. When controlling for final body mass, the effect was not significant (Δ Deviance = 3.579, df = 33, p = 0.094, Table 2-1), but body mass itself was a strong positive predictor of testes weight (p = 0.010). A similar trend was seen when using post-testes body mass (Δ Deviance = 3.433, df = 33, p = 0.088, Table 2-2). In contrast, when modelling testes weight as a proportion of body mass, the treatment effect remained non-significant (Δ Deviance = 1.258, df = 33, p = 0.089, Table 2-3), suggesting a consistent but modest reduction in reproductive investment under heat stress.

Table 2-1: Results for Gamma GLM model 1- comparing corrected testes weights against the final body weight.

Dependent variable:	**testes_corrected**
Treatment (Heat)	-0.256
t = -1.729	*p < 0.1*
Weight_T2W	0.002
t = 2.759	***p < 0.01***
Batch (B2)	-0.025
t = -0.147	
Batch (B3)	0.235
t = 1.380	
Constant	0.496
t = 1.044	
Null deviance	8.848 (df = 37)
Residual deviance	5.269 (df = 33)
Δ Deviance	3.579
Observations	38
Akaike Inf. Crit.	164.359
Note:	**p < 0.1; **p < 0.05*; ***p < 0.01*

Table 2-2: Results for Gamma GLM model 2- comparing corrected testes weights against the adjusted final body weight (with the weight of the testes deducted from the measured final body weight).

Dependent variable:	**testes_corrected**
Treatment (Heat)	-0.265
t = -1.759	*p < 0.1*
Weights_after	0.002
t = 2.593	**p < 0.05**
Batch (B2)	-0.029
t = -0.172	
Batch (B3)	0.235
t = 1.354	
Constant	0.549
t = 1.131	
Null deviance	8.848 (df = 37)
Residual deviance	5.415 (df = 33)
Δ Deviance	3.433
Observations	38
Akaike Inf. Crit.	165.424
Note:	**p < 0.1; **p < 0.05*; ***p < 0.01*

Table 2-3: Results for Gamma GLM model 3- comparing proportion of the final weight that was the testes against adjusted final body weight.

Dependent variable:	**Proportion**
Treatment (Heat)	-0.269
t = -1.757	*p < 0.1*
Weights_after	0.0004
t = 0.470	
Batch (B2)	-0.038
t = -0.220	
Batch (B3)	0.228
t = 1.293	
Constant	-4.767
t = -9.639	***p < 0.01***
Null deviance	8.848 (df = 37)
Residual deviance	5.269 (df = 33)
Δ Deviance	1.258
Observations	38
Akaike Inf. Crit.	–309.107
Note:	**p < 0.1; **p < 0.05; **p < 0.01*

We also modelled testes weight as a function of weight change during the experiment. While weight gain was not itself a significant predictor (Δ Deviance = 2.382, df = 33, $p = 0.187$, Table 2-4,), the treatment effect remained significant (Estimate = –0.396, $p = 0.016$), supporting the interpretation of a direct effect of heat on testes development, rather than an indirect effect via somatic growth.

Table 2-4: Results for Gamma GLM model 4- comparing weight of testes corrected against change in body weight.

Dependent variable:	**testes_corrected**
Treatment (Heat)	-0.396
t = -2.540	**p < 0.05**
Weight_dif	0.001
t = 1.348	
Batch (B2)	0.023
t = 0.115	
Batch (B3)	0.191
t = 0.999	
Constant	1.796
t = 12.407	***p < 0.01***
Null deviance	8.848 (df = 37)
Residual deviance	6.466 (df = 33)
Δ Deviance	2.382
Observations	38
Akaike Inf. Crit.	172.338
Note:	*$p < 0.1$; **p < *0.05*; ***p < 0.01*

Overall, our models consistently show that heat treatment leads to a reduction in relative testes weight across multiple body size metrics and model structures, corresponding to an estimated 20–33% decrease in testes mass relative to controls (Figure 2-2). The magnitude of the effect varies slightly depending on whether final or adjusted (final weight of the fish with the weight of the testes deducted) body mass is used, but the direction of the treatment effect is consistent. Importantly, the observed reduction in testes size persists even after controlling for variation in somatic growth, supporting the conclusion that elevated temperature imposes a specific reproductive cost independent of body condition or size.

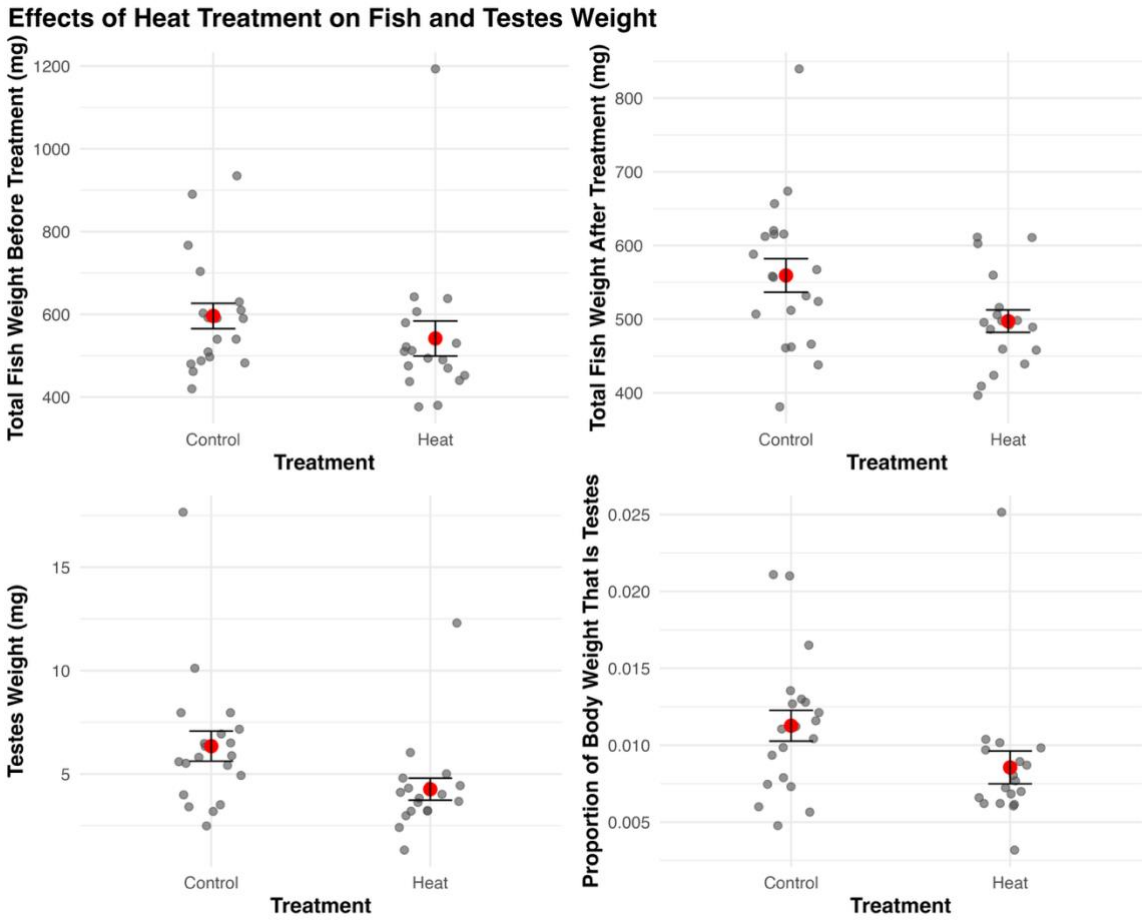
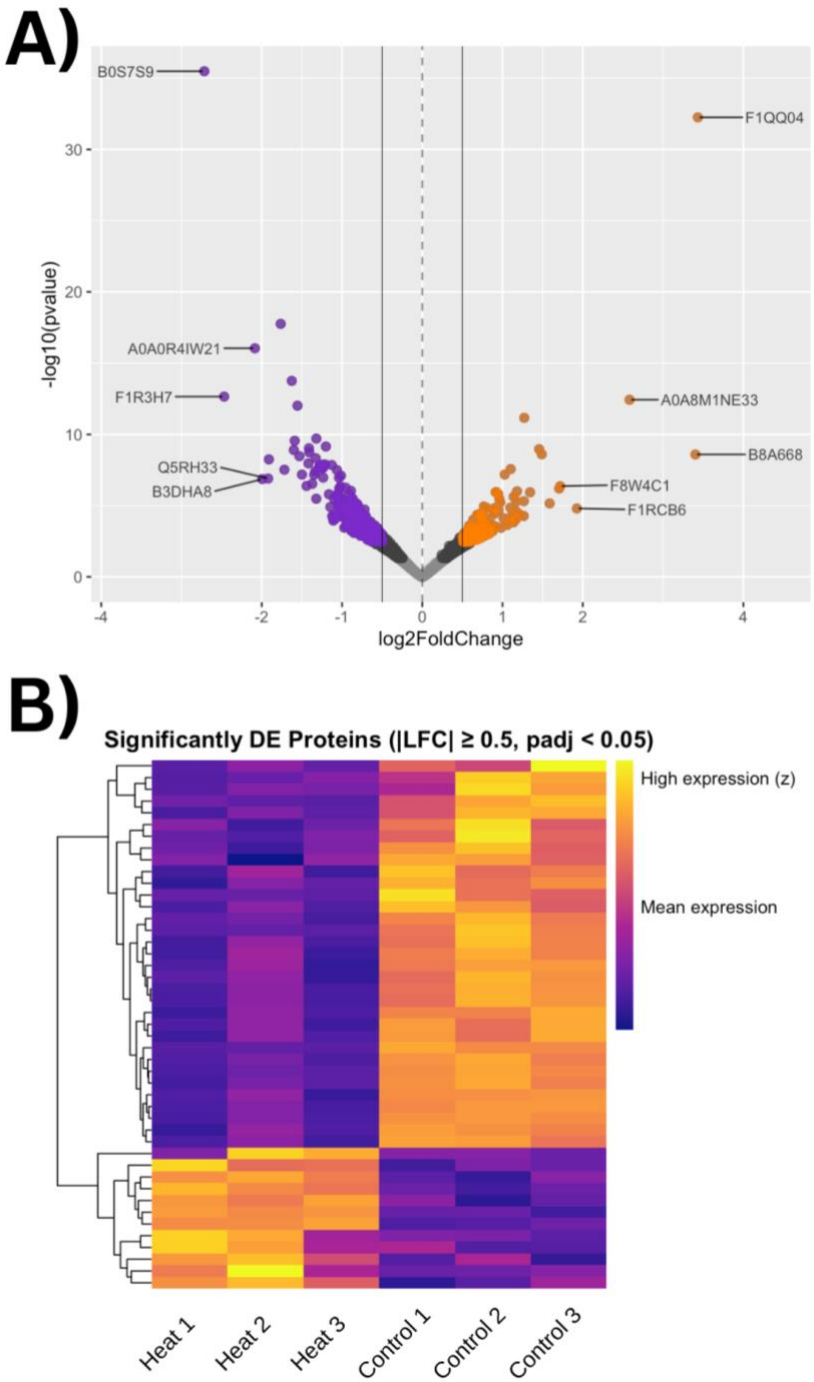


Figure 2-2: Effect of heat treatment on zebrafish body weight and testes weight- Scatter plots show individual values for control (n = 21) and heat-treated fish (n =21), with red dots indicating the mean and error bars representing standard error of the mean. A) **total fish** weight before treatment (T0), B) total somatic weight after treatment (T2W), calculated as total body weight minus testes, C) testes weight after treatment and D) proportion of total body weight accounted for by testes mass.

2.4.2 Testes Proteome

The initial TMT quantitative proteome produced for the six pooled testes samples resulted in 11,434 peptide matches using Uniprot database version 2022_05. Following re-mapping of the proteome to the more recent Uniprot database version 2024_06, and filtering to remove medium and low FDR confidences as well as entries with unique peptide numbers less than two, a total of 5415 proteins were confidently identified (Supplementary Table 1). We compared protein

abundance between the two experimental groups using the software *DESeq2* (Love et al., 2014) of the 5,415 identified proteins with non-zero total read counts, a total of 414 proteins (7.6%) were identified as significantly differentially abundant ($p_{adj} < 0.05$) between testes of males kept at 28°C and 34°C (Supplementary Table 2). Of these, 146 proteins (2.7%) were significantly more abundant in heat treated testes ($\log_2$ fold change > 0), and 268 proteins (4.9%) were significantly less abundant in heat treated testes ($\log_2$ fold change < 0). No outliers were detected based on Cook's distance, and no features were filtered out due to low counts (mean count < 410), as determined by independent filtering in *DESeq2*. We added an additional filtering step to retain only proteins with an absolute $\log_2$ fold change (|LFC|) ≥ 0.5 to ensure only proteins that substantially changed as a result of treatment are included. This produced a final list of 377 proteins that showed significantly different abundances between the heat treated and control testes, of which 244 were found to be significantly more abundant in Control testes, and 133 were significantly more abundant in heat-treated testes (Figure 2-3).



1189

1190 Figure 2-3- Heatmap and Volcano plot of differences in testes proteome as a
1191 result of heat stress. (A) Volcano plot showing the 5415 identified proteins, with
1192 significantly more abundant proteins in heat treated testes (p_{adj} < 0.05, LFC > 0.5)
1193 shown in orange, whilst significantly less abundant proteins in heat treated testes
1194 (p_{adj} < 0.05, LFC <-0.5) shown in violet. (B) A heatmap with all 5415 identified
1195 proteins LFC >0.5 and p_{adj} < 0.05.

The five proteins with most significantly lower abundance in testes of heat-treated males were F1R3H7, Q5RH33, A0A0R4IW21, B0S7S9, and B3DHA8 (Figure 2-3A). Of these, F1R3H7 and Q5RH33 correspond to annotated proteins (RIB43A-like with coiled-coils protein 1, and FYVE, RhoGEF and PH domain-containing 6, respectively), while the remaining three were listed as uncharacterised or identified via Sanger codes.

The five proteins with most significantly higher abundance in testes of heat-treated males were F1QQ04, A0A8M1NE33, B8A668, F8W4C1, and F1RCB6 (Figure 2-3A). Based on UniProt annotation, these correspond to Serpin H1 (collagen-binding protein), Heat shock cognate 71 kDa protein, Calponin, Myelin basic protein, and Actin alpha cardiac muscle 1a, respectively.

2.4.3 The GO terms and STRING analysis

Protein IDs were mapped using *BioMart* (Smedley et al., 2015) to retrieve corresponding Ensembl and Entrez gene identifiers for both significantly upregulated and downregulated proteins in the heat-treated testes proteome, compared to Controls. The resulting gene IDs were analysed for functional enrichment using the full zebrafish genome as the background. Enriched Gene Ontology (GO) terms were identified across the categories of Biological Process, Cellular Component, and Molecular Function, along with KEGG pathways (Supplementary Tables 3 & 4).

Gene ontology (GO) enrichment analysis of proteins with lower abundance in heat-treated testes identified significant enrichment for biological processes including cilium organization, cilium movement, oxidative phosphorylation, electron transport chain, and mitochondrial complex I assembly (Figure 2-4). Corresponding cellular component terms included cilium, motile cilium, axoneme, respiratory chain complex, and NADH dehydrogenase complex. Molecular function terms were enriched for nucleosomal DNA binding and nucleosome binding. KEGG pathway analysis highlighted downregulation of oxidative phosphorylation,

cardiac muscle contraction, cytoskeletal elements, motor proteins, and adrenergic signalling in cardiomyocytes (Figure 2-4).

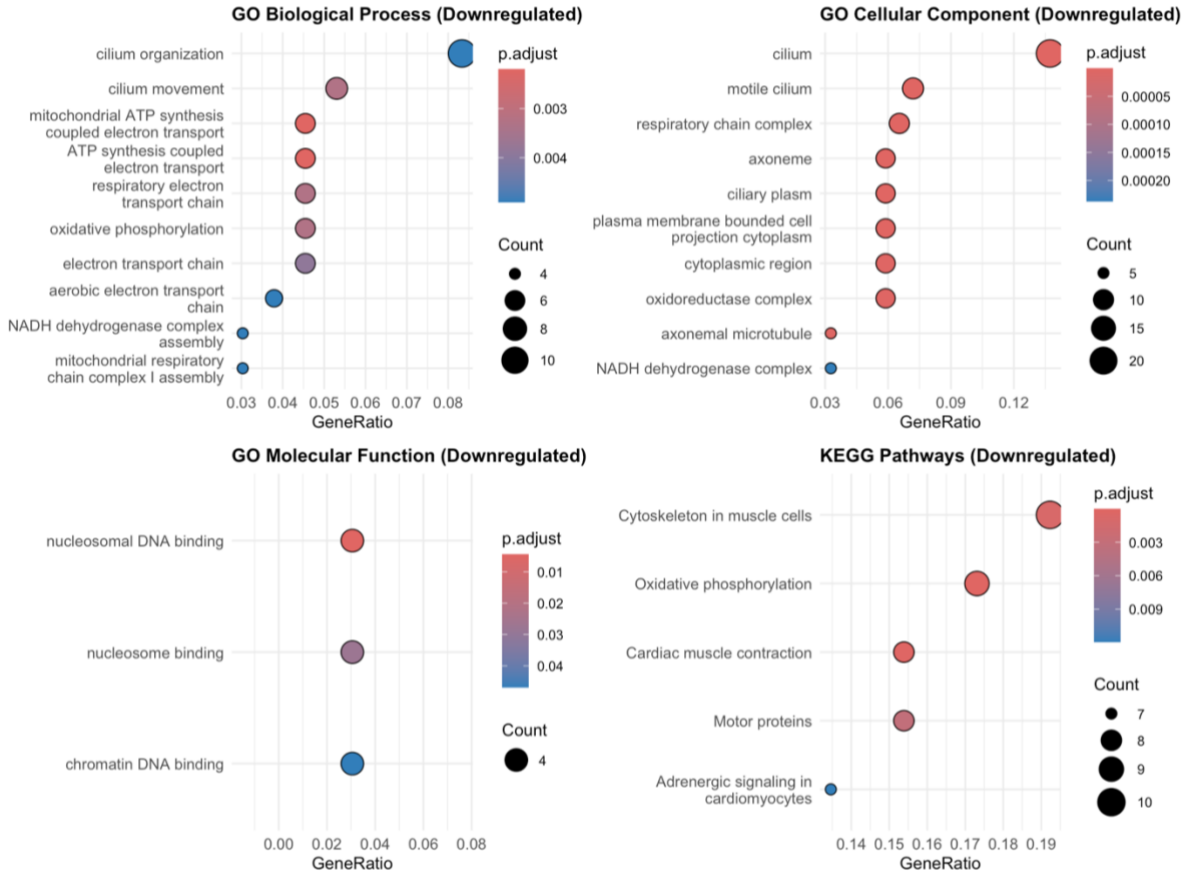


Figure 2-4: GO terms of genes linked to proteins that are downregulated in heat-stressed zebrafish testes. Dot plots showing the top 10 terms for each GO analysis (biological processes, cellular components and molecular function) and KEGG pathways enriched for proteins that were less abundant in heat treated testes compared to control testes. The GO terms were calculated against a background of the entire zebrafish genome.

For proteins with higher abundance in heat-treated testes, enriched GO biological processes included microtubule cytoskeleton organization, protein folding, spindle assembly and organization, chromosome segregation, and organelle assembly (Figure 2-5). Cellular component terms included centrosome, spindle, microtubule, and sarcomere, while molecular function terms were enriched for ATP-dependent protein folding chaperones, ATP hydrolysis activity, and microtubule binding.

KEGG pathway analysis identified enrichment for protein processing in the endoplasmic reticulum and tyrosine metabolism (Figure 2-5).

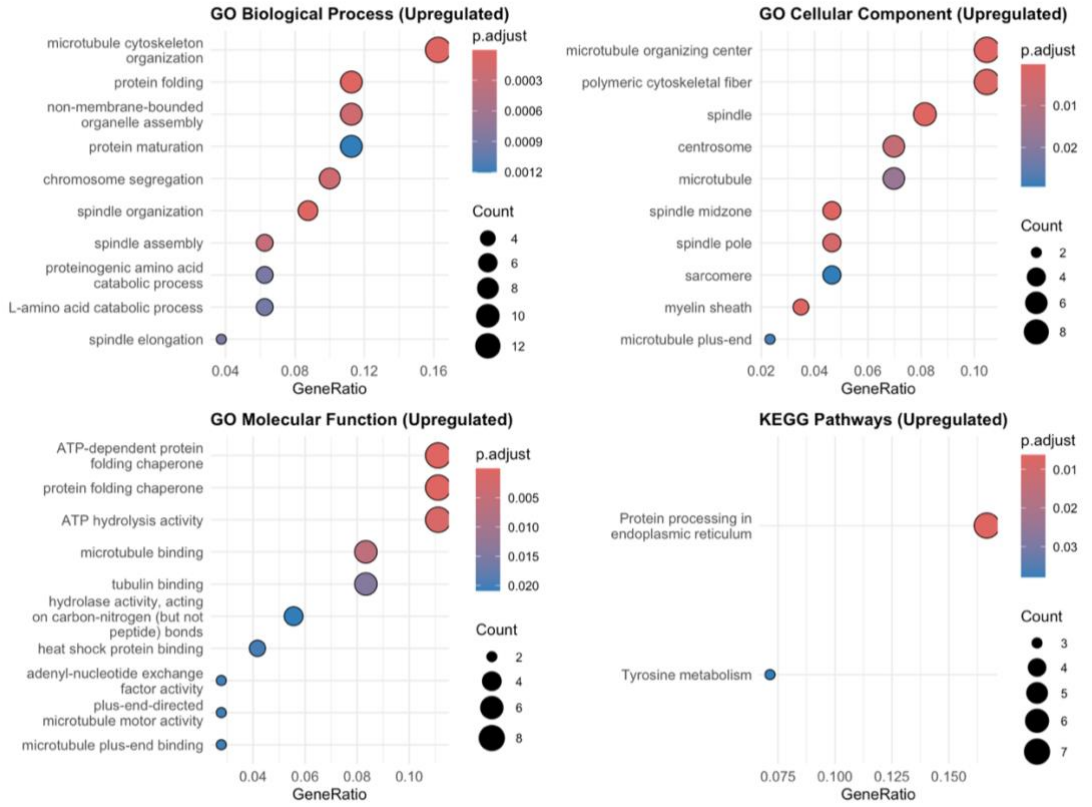


Figure 2-5: GO terms of genes linked to proteins that are upregulated in heat-stressed zebrafish testes. Dot plots showing the top 10 terms for each GO term (biological processes, cellular components and molecular function) and KEGG pathways enriched for proteins that were more abundant in heat treated testes compared to control testes. These GO terms were calculated against a background of the entire zebrafish genome.

STRING network analysis of downregulated proteins revealed a cluster of interactions among oxidative phosphorylation-related proteins, including cytochrome c oxidase subunit (Cox6b2) and multiple components of the NADH dehydrogenase (Nduf) complex (Figure 2-6). In the network of upregulated proteins, two main clusters were detected: one comprising heat shock proteins (including Hsp90) and another comprising kinesin-like proteins (kif11, kif17) (Figure 2-7).

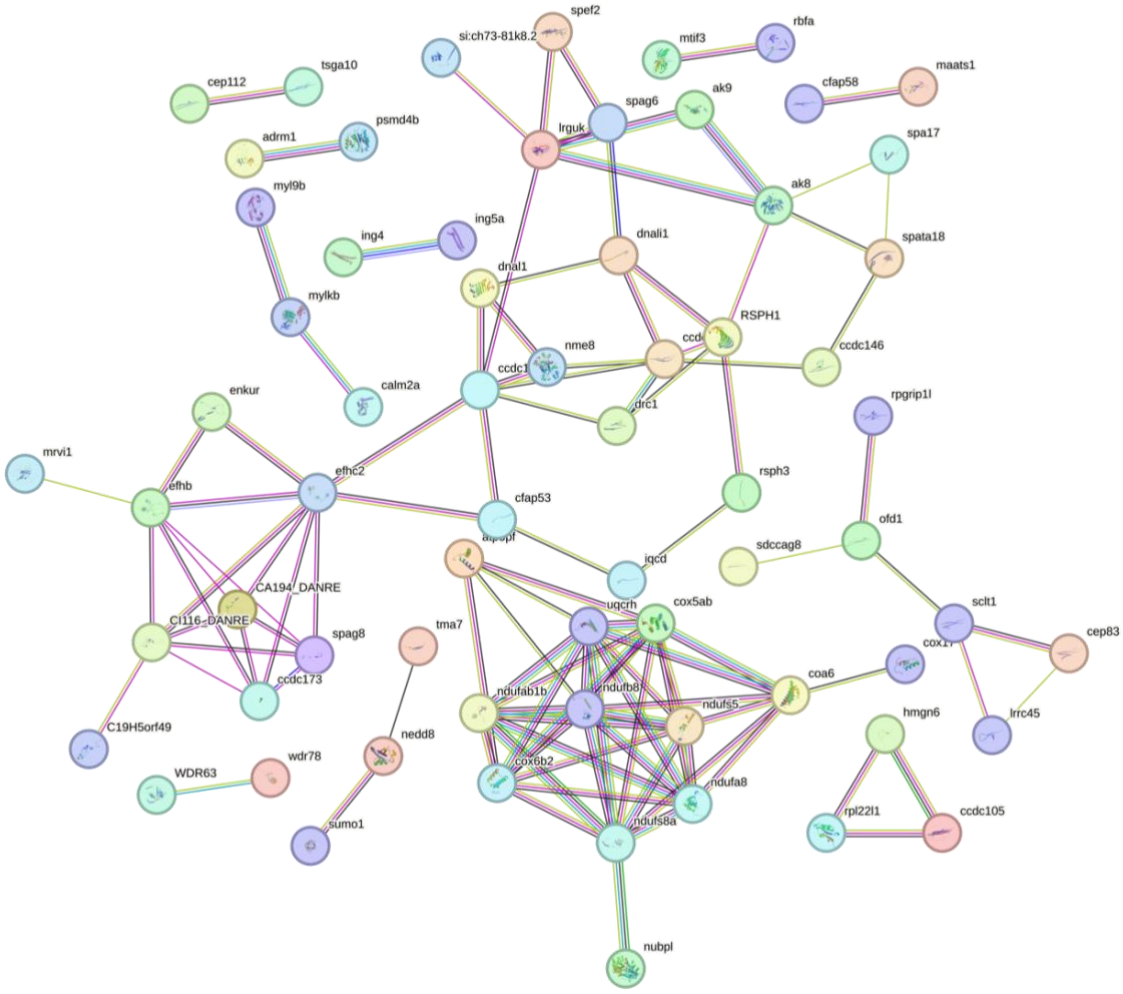


Figure 2-6- STRING network of protein–protein interactions for proteins significantly less abundant in testes of heat-treated males (p_{adj} f< 0.05, LFC ≤ –0.5). Nodes represent proteins and are colour-coded according to STRING functional enrichment clusters. Each node contains the predicted tertiary structure generated by the STRING/PDB model. Edges represent protein–protein associations, with edge colours indicating the evidence source; known interactions: curated databases (light blue), experimentally determined (pink), predicted interactions: gene neighbourhood (green), gene fusions (red), gene co-occurrence (blue), other evidence: text mining (yellow), co-expression (black), protein homology (purple). A minimum interaction confidence score of 0.7 (high confidence) was applied, and all disconnected proteins were hidden.

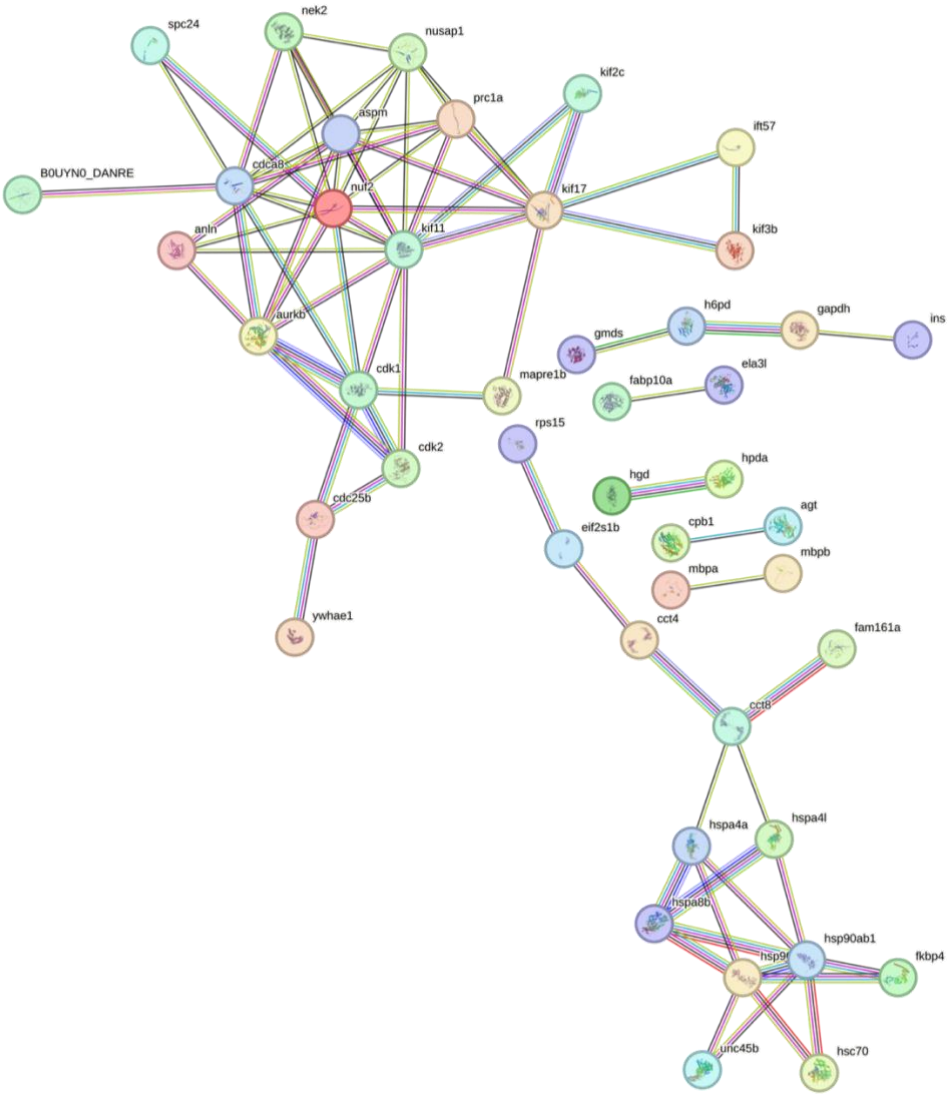


Figure 2-7- STRING network of protein–protein interactions for proteins significantly more abundant in testes of heat-treated males (p_{adj} < 0.05, LFC ≥ 0.5). Nodes represent proteins and are colour-coded according to STRING functional enrichment clusters. Each node contains the predicted tertiary structure of the protein, as generated by the STRING/PDB modelling pipeline. Edges represent predicted or known protein–protein associations, and edge colours correspond to the specific underlying evidence source: known interactions: curated databases (light blue), experimentally determined (pink), predicted interactions: gene neighbourhood (green), gene fusions (red), gene co-occurrence (blue), other evidence sources: text mining (yellow), co-expression (black), protein homology (purple). A minimum interaction confidence score of 0.7 (high confidence) was applied. Disconnected proteins were hidden to improve clarity of the interaction network.

2.5 Discussion

2.5.1 The global zebrafish testes proteome

Previous global proteomes of zebrafish testes identified 2214 proteins (Groh et al., 2011a) compared to 5,415 proteins identified in our study. However, these previous results were published 14 years before the analysis of our data and whilst similar methods of LC-MS/MS were used, the technology for identification of lower abundance proteins has greatly improved, leading to the increase in proteins identified in our study. Similarly in more recent targeted proteomic analyses of the zebrafish testes response to microplastic exposure, 1347 proteins were identified (Li et al., 2024), however, the number of fish per pool used in the recently published paper was lower than our study, potentially suggesting that our data provide a broader snapshot of the testes proteome. Although our data do not classify or characterise the proteins identified within this proteome in depth, the addition of these proteins to the confirmed zebrafish testes proteome can act as a resource for continued research.

Comparable upregulation of molecular chaperones and cytoskeletal regulators has been reported in the testes of other ectothermic species exposed to thermal stress, including tilapia (*Oreochromis niloticus*), common carp (*Cyprinus carpio*), and rainbow trout (*Oncorhynchus mykiss*)**,** indicating a conserved protective strategy to maintain spermatogenic function (Deane & Woo, 2011). These parallels suggest that activation of protein-folding and cytoskeletal stabilization pathways represents a general vertebrate response to elevated temperature, rather than one unique to zebrafish.

2.5.2 The effects of heat stress on the testes proteome

Among the top five proteins with significantly lower abundance in heat stressed testes, F1R3H7 is a RIB43A-like protein with coiled-coils protein 1, which has been linked to the formation and function of cilia or flagella-like structures in both humans and zebrafish (Frohnhöfer et al., 2016). The second identifiable protein was Q5RH33, which is a FYVE, RhoGEF and PH domain-containing 6 protein.

This protein has been previously identified as important in embryogenesis although the exact role has not been investigated (Lo et al., 2003). These proteins would suggest that the formation of flagella and therefore the processes of spermatogenesis may be decreased in zebrafish testes in response to prolonged heat exposure.

The highest-ranked protein by LFC was Serpin H1 (F1QQ04)**,** also known as Heat shock protein 47 (HSP47)**,** which functions as a molecular chaperone involved in the biosynthesis and proper folding of collagen, a role which is conserved across many aquatic species including rainbow trout *Oncorhynchus mykiss***,** and zebrafish . In addition to its role in collagen maturation, HSP47 contributes to the preservation of cellular architecture and function under stress conditions including thermal stress and hypoxia (Lim & Bernier, 2022). Another highly-ranked protein by LFC A0A8M1NE33 is commonly known as heat shock 70 kDa protein-like and has a role as a molecular chaperone in maintaining cellular functions during stress response, in a highly conserved process across aquatic species such as the Nile tilapia *Oreochromis niloticus*, with both proteins regulated through the heat-shock factor 1 pathway (Delaney & Klesius, 2004). The presence of these two heat-shock proteins among the most strongly upregulated in heat-treated testes indicates a substantial cellular investment in molecular repair and stress mitigation mechanisms compared to the control group.

The third most upregulated protein, Calponin (B8A668), is an actin-binding protein that regulates actomyosin interactions and smooth muscle contraction. Calponin has been implicated in cytoskeletal reorganization and cell motility, processes that may be particularly important under stress conditions where structural stability of the testes is compromised (Q. Zhu et al., 2004). The fourth, Myelin basic protein (F8W4C1), is a structural protein previously noted in the nervous system of zebrafish (Brösamle & Halpern, 2002), but it may participate in membrane stabilization and signalling during spermatogenesis, as it has previously been identified in the testes of *Xenopus laevis* (Nanba et al., 2010). Finally, Actin alpha cardiac muscle 1a (F1RCB6) is a cytoskeletal protein central to contractile and

structural integrity, with expression in multiple tissue types (Arora et al., 2023). Upregulation of this isoform of actin may suggest effects on the ability for the testes to contract and expand during ejaculation.

Together, these proteins highlight a combined response to heat stress in zebrafish testes that involves both canonical molecular chaperones (HSP47, HSP70-like) and structural or cytoskeletal elements (Calponin, Myelin basic protein, and Actin), pointing to a coordinated effort to preserve testicular architecture and maintain spermatogenic potential under elevated temperatures.

2.5.3 The effects of heat stress on testes size

To determine if changes in the testes proteome reflected a general reduction in testes size or a more specific disruption of processes such as spermatogenesis, body weights were measured pre- and post-treatment and testes weights measured post-treatment. Our analysis showed a reduction in relative testes size in heat-treated males. Therefore, the reduction in sperm yield is likely to reflect both impaired spermatogenesis and a measurable decrease in gonad size under heat stress. These findings echo results found in other species such as red flour beetles, where up to a 40% reduction in testes volume were found after sublethal heat treatment (Cole et al., 2025). In Nile tilapia (*Oreochromis niloticus*), exposure to elevated temperatures (36–37 °C) led to pronounced testicular atrophy, vacuolization and the loss of spermatogenic germ cells, although partial recovery was observed after return to control temperatures (Jin et al., 2019). Similar reductions in testes size under elevated temperatures have been documented in the European bullhead (*Cottus gobio*), where thermal stress impaired reproductive physiology and reduced gonadal investment (Dorts et al., 2012).

Our findings align with a growing body of research showing that heat stress can directly impair spermatogenesis in ectothermic taxa. In freshwater ectotherms such as guppies (*Poecilia reticulata*), experimental heatwaves have been shown to reduce testis size, sperm number, and sperm velocity, resulting in a cumulative deterioration of reproductive performance (Breedveld et al., 2023). Similarly,

comparative reviews of teleost fishes highlight that elevated temperatures disrupt gametogenesis at multiple levels, from germ cell proliferation to spawning output, with consequences for population dynamics under climate change scenarios (Alix et al., 2020).

2.5.4 The GO terms and STRING analyses of heat-stressed testes

The idea that the heat-treated testes show decreased spermatogenesis and increased stress response compared to the control testes is further supported by GO term analysis (Figure 2-3, 2-4). The terms noted for GO terms related to less abundant proteins are mainly directly related to both the function of mature sperm and the processes of spermatogenesis which suggests that not only was spermatogenesis decreased in heat-treated testes, but the overall number of mature sperm was lower as well. Decreases in spermatogenic output as a response to heat stress have been widely observed across teleost species. For example, in medaka *Oryzias latipes* with a loss of developing sperm cells being noted alongside the importance of the Notch pathway in maintaining spermatogonial stem cells (and therefore spermatogenesis) during the application of thermal stress (Moreno Acosta et al., 2023).

The GO terms related to higher abundance proteins in the heat-treated testes are suggestive of cellular repair and stress response. Notably the upregulation of cellular repair, as well as the higher abundance of HSP47 and HSP70 is contrary to patterns that have previously been reported. It has previously been reported that other teleost including the minnow *Puntius sophore* demonstrate a downregulation of HSP proteins in the gonads in response to thermal stress, possibly due to an increase in testes apoptosis (Mahanty et al., 2019). In contrast, the combination of upregulated repair-associated GO terms and elevated HSP expression in our dataset suggests that the zebrafish in this study may possess a greater reproductive tolerance to elevated temperatures, mounting a repair response rather than entering a degenerative state. This interpretation is further supported by the fact that testes weight was reduced in heat-treated males, with

some of this change potentially attributable to overall somatic effects and spermatogenic reduction instead of direct gonadal destruction.

These GO term sets are supported by STRING analysis of the significantly more and less abundant proteins (Figures 2-6 and 2-7). The STRING analysis of significantly less abundant proteins in heat-treated testes includes cytochrome c oxidase subunit (Cox6b2) and several components of the NADH dehydrogenase (Nduf) complex**,** both key elements of the oxidative phosphorylation pathway. This finding suggests a reduction in oxidative phosphorylation activity in the heat-treated testes, which may reflect a state of oxidative stress in the testes. The direct link between heat stress and oxidative stress has previously been shown in mice, with developing round spermatids being directly vulnerable to these stressors (Houston et al., 2018). The vulnerability of developing spermatids to both heat and oxidative stress further implicates that the processes of spermatogenesis and sperm maturation are likely to be highly affected by thermal stress, reducing the spermatogenic output of testes.

The STRING analysis of the more abundant proteins also supports the mechanisms presented by the GO terms, with nodes containing two Hsp90 proteins, suggestive of a heat-stress response and another node containing both kif11 and kif17 (kinesin-like proteins) which is suggestive of cellular proliferation and repair, similarly to the GO terms. Kinesins and kinesin-like proteins have previously been reported to be heavily involved in spermatogenesis, with kif17 implicated in chromatoid body movement and RNA metabolism (Ma et al., 2017). This seems counter-intuitive to the reduction in spermatogenesis noted earlier, however, these proteins are also capable of forming complexes with HSP proteins and are therefore also integral to the thermal stress response (Hirokawa & Tanaka, 2015).

Another line of evidence supporting the hypothesis that heat-stressed testes undergo reduced spermatogenesis comes from the overlap between the zebrafish sperm proteome (Chapter 3) and the testicular proteome presented here. Notably,

the sperm proteome was generated from sperm stripped directly from the same testes used in this study. However, due to difficulties in collecting sperm from heat-treated males, primarily due to low sperm counts and small ejaculate volume, no treatment-based comparison could be made within the sperm proteome dataset. Despite this limitation, the comparison of protein overlap is revealing: 150 proteins of lower abundance in the heat-treated testes overlapped with the sperm proteome, compared to only 47 proteins of higher abundance (Figure 2-8). This suggests that many of the proteins reduced in abundance in heat-stressed testes are likely sperm-associated, further supporting the idea of reduced spermatogenic output under heat stress.

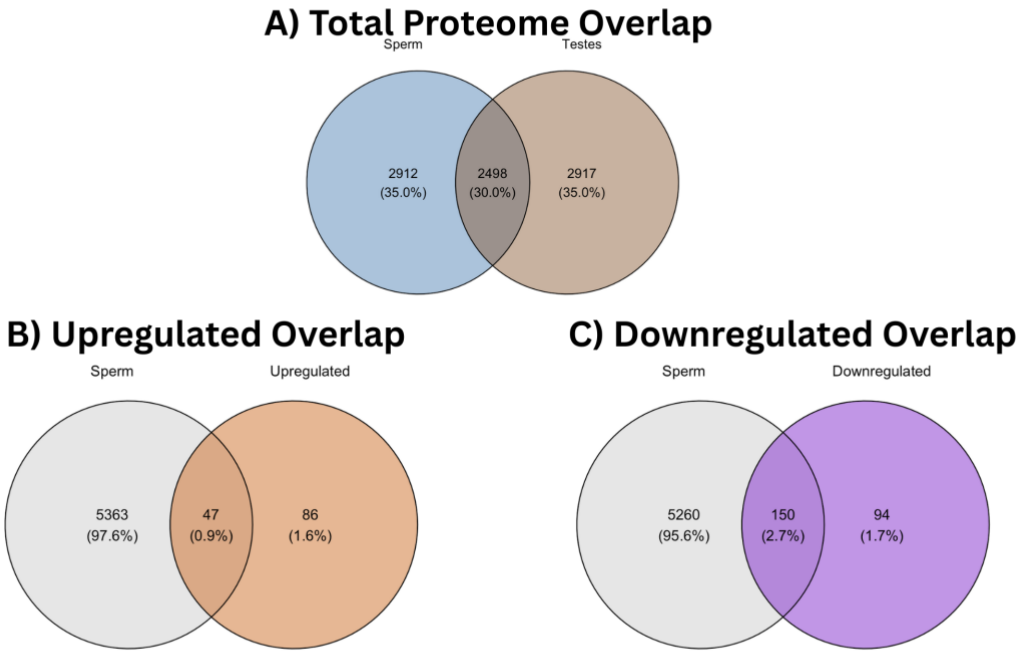


Figure 2-8: Venn diagrams comparing the zebrafish testes proteome and zebrafish sperm proteome (Chapter 3). (A) Venn diagram representing the overall overlap between the zebrafish sperm proteome (Chapter 3) with the combined zebrafish testes proteome. (B) Venn diagram showing the overlap of the sperm proteome (Chapter 3) with the significant proteins of higher abundance in the heat-treated testes. (C) Venn diagram showing the overlap of the sperm proteome (Chapter 3) with the significant proteins of lower abundance in the heat-treated testes.

The continued evidence of a reduction in spermatogenesis in the testes in response to thermal stress directly links to previous data showing decreased fertility in male zebrafish in response to the same heat stress parameters (Irish et al., 2024). However, the upregulation of repair-associated proteins in the testes shown here may help explain the lack of observed effects on offspring quality in those same experiments. This suggests that while spermatogenesis was impaired, active cellular repair mechanisms may have mitigated the production or release of damaged sperm, thereby buffering against downstream reproductive consequences.

2.5.5 Conclusion

Overall, our study provides both a comprehensive global proteomic profile of the zebrafish testes and protein-level evidence for the physiological effects of heat stress on the male gonads. These effects include clear signatures of impaired spermatogenesis and heightened stress-induced cellular repair. Notably, the most differentially abundant proteins included classical heat shock proteins such as Hsp70 and Serpin H1 (HSP47), consistent with the activation of protective chaperone systems in response to thermal insult. In contrast, GO terms associated with proteins of significantly lower abundance in heat-treated testes were enriched for processes related to cilia formation and motility, functions strongly linked to mature sperm cells.

Importantly, the hypothesis that molecular differences were solely due to reduced testis size in heat-treated males was not supported. Although gonad size decreased by 20–33% in heat-treated fish, this morphological change alone is unlikely to explain all the molecular alterations detected. This strengthens the conclusion that heat stress directly impairs the cellular and molecular processes of spermatogenesis, rather than causing changes through generalised shrinkage of the gonad.

Taken together, our findings offer a detailed insight into the molecular and physiological impacts of heat stress on male zebrafish reproduction, with broader

implications for understanding thermal sensitivity in teleost fish. This dataset lays important groundwork for future studies into reproductive resilience under climate change and may help predict the reproductive consequences of rising water temperatures for externally fertilising species.

3 The Zebrafish Sperm Proteome

This chapter is based on the following published manuscript:

Cohen-Krais, J., Martins, C., Bartram, J., Crighton, Z., de Coriolis, J.-C., Godden, A., Marcu, D., Robak, W., Saalbach, G. and Immler, S. (2025), The Zebrafish Sperm Proteome. Proteomics, 25: e202400310. https://doi.org/10.1002/pmic.202400310 (Appendix 1)

3.1 Abstract

One of the key processes that forms the basis of fertilisation is the tight interaction between sperm and egg. Both sperm and egg proteomes are known to evolve and diverge rapidly even between closely related species. Understanding the sperm proteome therefore provides key insights into the proteins that underpin the mechanisms involved during fertilisation and the fusion between sperm and egg, and how they can differ across individuals of the same species. Despite being a commonly used model organism for reproductive research, little is currently understood about the sperm proteome of the zebrafish *Danio rerio*. We performed proteomics analysis using nanoLC-MS/MS after off-line sample fractionation with six pooled samples containing sperm from ten males each. A total of 5410 proteins were confidently identified, from which a total of 3900 GeneIDs were generated leading to 1720 Gene Ontology terms. A high abundance of histone proteins was noted, with 8 identified within the top 25 most abundant proteins. This proteome also provides evidence of the retention of proteins that have previously been identified as important for spermatogenesis.

3.2 Introduction

Sperm are highly complex cells with key functions essential for successful reproduction and fertility. The proteome of sperm provides insight into functional aspects such as motility, capacitation, and fertilisation (Amaral, Castillo, et al., 2014). Sperm morphology, and therefore the sperm proteome, shows considerable variation not only across species and individuals but also between ejaculates from the same individual, influenced by factors such as age (Kleshchev et al., 2023), environmental conditions (Kleshchev et al., 2021), and overall health status (Morrow & Gage, 2001). Identifying and quantifying the proteins present in sperm can therefore help pinpoint potential biomarkers associated with male reproductive performance and fertility.

According to Web of Science (Clarivate (Web of Science). Clarivate 2024. All rights reserved.), “sperm proteomes” have been described in 891 papers, including 34 human papers and 857 papers on other species. The current total number of proteins identified in the human sperm proteome is 6871 combined across all accessible human sperm proteomics datasets, with a maximum of 5685 identified from a single experiment (Luo et al., 2022). Similarly, in the domestic bull *Bos taurus*, proteomic analyses of high and low motility sperm populations revealed that 498 proteins differed between high and low fertility bulls (D’Amours et al., 2019a). Furthermore, the number of proteins identified in sperm proteomes differ by up to 21.6% across three mouse species (Aitken et al., 2007; Baker, Hetherington, Reeves, Müller, et al., 2008; Chauvin et al., 2012; Vicens et al., 2017), exhibiting divergent mating systems thought to drive the evolution in the sperm proteome. In the oyster *Crassostrea hongkongensis*, the sperm proteome was used to establish the molecular mechanisms underlying species-specific sperm and oocyte binding in free-spawning organisms (Mu et al., 2020), and similarly, high levels of variation in the sperm proteome have been identified across marine mussel species *Mytilus edulis* and *Mytilus galloprovincialis* (Romero et al., 2019).

Previous studies highlight the diversity and rapid divergence of sperm proteomes even between closely related species as an indicator of their key role in reproduction (Vicens et al., 2017). In teleost fish species such as the rainbow trout *Oncorhynchus mykiss*, 206 proteins have been identified in sperm (Nynca et al., 2014), whereas in carp *Cyprinus carpio*, 348 proteins were identified (Dietrich et al., 2014). This variation in protein numbers is likely not only due to biological differences between species but also the analytical methods used to determine the proteomes. More accurate data are therefore needed to allow further research into the role of the sperm proteome in sperm function and sperm-egg interactions.

The zebrafish *Danio rerio* is a popular model organism for biomedical research ranging from ecotoxicology, evolutionary and developmental biology, cancer and neurodegenerative diseases, and ageing (Hajam et al., 2021). As an externally fertilising vertebrate, collection of gametes and *in vitro* fertilisation is particularly easy and can be performed mimicking natural conditions, rendering the zebrafish a prime model organism to study fertility and reproduction (Meyers, 2018). Proteomic profiles have been developed for a wide range of zebrafish tissues including the testes (2214 described proteins) and the ovaries (1379 described proteins) (Groh et al., 2011b). In addition, the zebrafish oocyte proteome has been described with 1568 proteins differing between mature and immature oocytes (C. Ge et al., 2017) and the proteome profiling of zebrafish embryos has been used to study the effects of environmental stressors such as toxins (Vieira et al., 2020). The available proteomic profile of the zebrafish currently includes 53,918 proteins on the NCBI Refseq protein database (O'Leary et al., 2016). However, despite the proliferation of proteomic profiling of zebrafish tissues and cell types, the global proteomic profiling of zebrafish sperm is currently lacking.

Here, the global proteomic profile of zebrafish sperm was obtained through tandem liquid chromatography-mass spectrometry (LC-MS/MS) and analysed through the comparison of relative peptide abundance as well as Gene Ontology.

3.3 Methods

3.3.1 Zebrafish husbandry and experimental set up

Sperm were collected from sexually mature ABWT (AB Wild Type strain) male zebrafish, originally obtained from the Zebrafish International Resource Center (ZIRC, Oregon) and maintained at the Controlled Environment Facility at the University of East Anglia, UK. The males were between 1 and 1.5 years old and at the peak of reproductive age. All fish were kept in a recirculating aquatic system with filtered, UV-treated water and maintained under standard zebrafish husbandry conditions. Prior to gamete collection, the fish were maintained in 3.5 L tanks at a 10:6 male to female sex ratio kept at 28°C (standard temperature) or 34°C (high temperature) to test for a potential role of environmental factors shaping the sperm proteome with a 14:10 light schedule and fed ad libitum with a mix of dry feed (ZEBRAFEED 400–600 by SPAROS, Área Empresarial de Marim, Lote C, 8700-221 Olhão, Portugal) and live *Artemia* (Sep-Art Artemia Cysts, Ocean Nutrition). All fish used for experiments were kept under Home Office license P0C37E901.

Males were housed as part of a three-block design, with each block consisting of one control tank (28 °C) and one heat-treatment tank (34 °C). Fish were randomly distributed into tanks in the standard 10:6 male:female ratio (16 fish per 3.5 L tank). Within each block, control and heat-treatment tanks were positioned adjacent to one another on the same rack to ensure identical light, flow, and environmental conditions. Submersible heaters were added to all tanks activated at 34 °C for treatment tanks or 28°C in control tanks to control for potential heater effects. Fish were maintained under their assigned temperature for two weeks prior to sperm collection.

Following anaesthesia using metomidate hydrochloride (Aquacalm by Syndel, #9, 4131 Mostar Rd, Nanaimo, BC V9T 6A6 Canada), males were stripped for sperm through gentle squeezing in a cranio-caudal direction and the ejaculates were collected immediately into a microcapillary tube. Due to the low number of cells produced in a single zebrafish ejaculate, sperm samples from ten males were pooled into one replicate sample to ensure sufficient material for mass

spectrometry, and we collected a total of six biological replicates (60 males). These sperm pools were washed in Hanks' Balanced Salt Solution to remove seminal fluid and pelleted through centrifugation for 8 min at 8000 rpm, before freezing in N_2, followed by storage at -80°C.

3.3.2 Proteomics

After thawing, sperm pool pellets were precipitated in acetone (50 µL + 900 µL, 10 mM NaCl) under vortexing on ice for 2 h. The pellets were then resuspended in 50 µL of SDC buffer (2.5% sodium deoxycholate (SDC; Merck) in 0.2 M EPPS-buffer (Merck), pH 8.5) and vortexed under heating, using an SDC-based in-solution digestion approach (Masuda et al., 2008). Aiming at 50 µg protein per sample, estimated by a Direct Detect fluorometric assay (MERCK MILLIPORE, Dorset, UK), equal amounts of protein per sample were reduced, alkylated, and digested with trypsin in the SDC buffer according to standard procedures adapted from Shevchenko et al. (Shevchenko et al., 2006). After the digest, the SDC was precipitated by adding an equal volume of 0.4% TFA, and the clear supernatant subjected to C18 SPE (OMIX tips; Agilent). Peptide concentration was further estimated by analysing a small aliquot of the digests by LCMS, as described below, to estimate the total peptide abundance per sample. Sample aliquots corresponding to estimated equal peptide abundances were used for TMT labelling using a TMT 6plex kit (Lot VB290974, ThermoFisher Scientific, Hemel Hempstead, UK) according to the manufacturer's instructions with slight modifications. However, this labelling was not used for quantification and is not relevant for the analysis presented in this paper. Six differently labelled samples were pooled and desalted using a C18 Sep-Pak cartridge (200 mg, Waters, Wilmslow, UK). The eluted peptides were dissolved in 500 µL of 25 mM NH_4HCO_3 and fractionated by high pH reversed phase HPLC. For this, the samples were loaded to an XBridge 3.5 µm C18 column (150 × 3.0 mm, Waters).

Fractionation was performed on an ACQUITY Arc Bio System (Waters) with the following gradient of solvents A (water), B (acetonitrile), and C (25 mM NH_4HCO_3 in water) at a flow rate of 0.5 mL/min: solvent C was kept at 10%

throughout the gradient; solvent B: 0–5 min: 5%, 5–10 min: 5%–10%, 10–60 min: 10%–40%, 60–75 min: 40%–80%, followed by 5 min at 80% B and re-equilibration to 5% for 24 min. Fractions of 0.5 mL were collected every 1 min and concatenated by combining fractions of similar peptide concentration to produce 19 final fractions of the sperm samples for MS analysis. Aliquots were analysed by nanoLC-MS/MS on an Orbitrap Eclipse Tribrid mass spectrometer coupled to an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific, Hemel Hempstead, UK). The samples were loaded onto a trap cartridge (Pepmap 100, C18, 5um, 0.3 × 5 mm, Thermo) with 0.1% TFA at 15 µL/min for 3 min. The trap column was then switched in-line with the analytical column (nanoEase M/Z column, HSS C18 T3, 1.8 µm, 100 Å, 250 mm × 0.75 µm, Waters) for separation using the following gradient of solvents A (water, 0.1% formic acid) and B (80% acetonitrile, 0.1% formic acid) at a flow rate of 0.2 µL/min: 0–3 min 3% B (parallel to trapping); 3–10 min linear increase B to 8%; 10–75 min increase B to 37%; 75–90 min linear increase B to 50%; followed by a ramp to 99% B and re-equilibration to 3% B. Data were acquired with the following parameters in positive ion mode: MS1/OT: resolution 120K, profile mode, mass range *m*/*z* 400–1800, AGC target 100%, max inject time 50 ms; MS2/IT: data dependent analysis with the following parameters: top10 in IT Turbo mode, centroid mode, quadrupole isolation window 0.7 Da, charge states 2–5, threshold 1.9e4, CID CE = 35, AGC target 1e4, max. inject time 70 ms, dynamic exclusion 1 count for 15 s mass tolerance of 7 ppm; MS3 synchronous precursor selection (SPS): 10 SPS precursors, isolation window 0.7 Da, HCD fragmentation with CE = 65, Orbitrap Turbo TMT and TMTpro resolution 30k, AGC target 1e5, max inject time 105 ms, Real Time Search (RTS): protein database *D. rerio* (Uniprot reference proteome UP000000437, August 2022, 46,842 entries), enzyme trypsin, 1 missed cleavage, oxidation (M) as variable, carbamidomethyl (C) and TMT as fixed modifications, Xcorr = 1, dCn = 0.05. The LCMS analysis was done in 2022 using the database available at that time. The final processing reported here was done in 2024 using the updated database from 2024.

3.3.3 Data analysis

The acquired raw data from the 19 HPLC fractions were processed and quantified using peptide peak intensities in Proteome Discoverer 3.1 (Thermo); all mentioned tools of the following workflows are nodes of the proprietary Proteome Discoverer (PD) software. The *D. rerio* fasta database (Uniprot UP000000437_Danio_7955.fasta, April 2024, 25,991 entries) was imported into PD adding a reversed sequence database for decoy searches. The processing workflow included Minora Feature Detection with min. trace length 7, S/N 5, PSM confidence high, and Top N Peak Filter with 20 peaks per 100 Da. For the database search the CHIMERYS search engine (MSAID, Munich, Germany) was used with the inferys_3.0.0_fragmentation prediction model with FDR targets 0.01 (strict) and 0.05 (relaxed), a fragment tolerance of 0.3 Da, enzyme trypsin with one missed cleavage, variable modification oxidation (M), fixed modifications carbamidomethyl (C), and TMT 6plex on N-terminus and K.

The consensus workflow was set up to quantify peptides and proteins based on their ion count intensity combined from all HPLC fractions of the six pooled samples. For identification, an FDR of 0.01 was used as strict threshold. Protein abundance was calculated using the top three most abundant peptides unique to protein groups. The results were exported into a Microsoft Excel table including data for protein abundances, number of peptides, protein coverage, the search identification score and other important values. All data filtering was performed in accordance with current proteomics best practice guidelines (Burger, 2018).

Processed data were then further analysed and visualised using R version 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria, URL **https://www.R-project.org/**). ID mapping was performed using Uniprot (Bateman et al., 2023) as well as the STRING database (Szklarczyk et al., 2023). Gene Ontology (GO) term analysis was performed using ShinyGO 0.76.2 (S. Ge et al., 2020).

3.4 Results

Initial mass-spectrometry runs had been designed to compare the sperm proteomes of males from 28 °C and 34 °C treatments. However, preliminary quality-control assessments showed that individual tank-level samples displayed high preparation-related variability, including elevated CV values and unstable adjusted p-values. This variability made differential expression analysis unreliable and indicated that the dataset was better suited to generating a high-confidence global sperm proteome rather than testing for temperature effects.

To maximise protein coverage and increase depth of identification, we pooled all six tank replicates to produce a combined dataset. The observed variation in the 34 °C samples most likely reflected a combination of biological heterogeneity and technical noise during sample preparation, further supporting the decision to combine the dataset into one global proteome rather than pursue treatment-level comparisons.

Following filtering to remove any single peptide matches and potential contaminants, a total of 5410 proteins could be confidently identified from the combined six pooled sperm samples (Supplementary Table 5). Of these, 4168 could be quantified, while 1242 could not be quantified, (Figure 3-1).

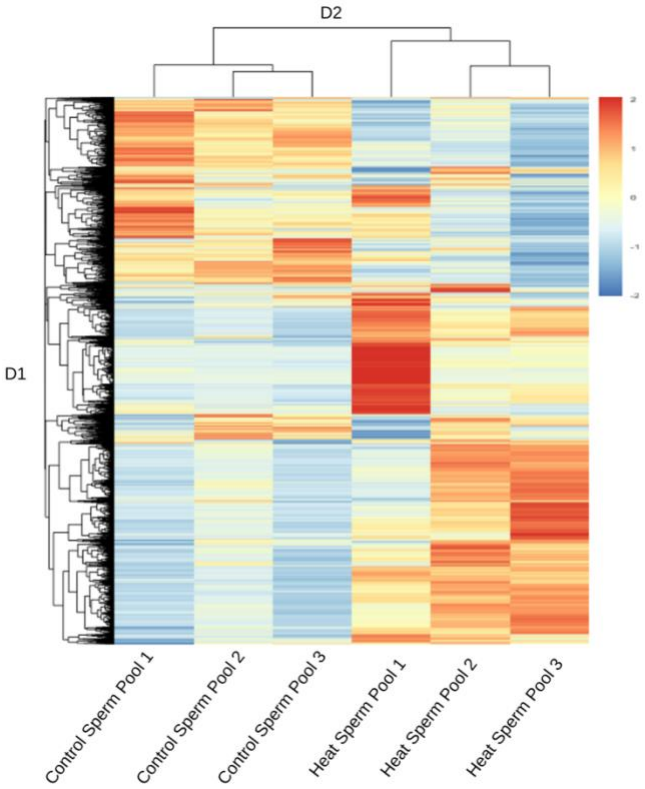


Figure 3-1: Heatmap comparing heat-treated and control sperm proteomes, showing the 4168 quantifiable proteins identified. Log scale.

The Uniprot UP000000437_Danio_7955.fasta reference proteome of the zebrafish used in this study contains one protein per gene (25,991 entries) [19]. The GRCz11 reference genome [22] for zebrafish includes a total of 25,545 coding genes. In this study, 5410 proteins were detected, which suggests that roughly 20% of the potential zebrafish gene products were detected.

According to the Uniprot ID database (Bateman et al., 2023), of the 5410 proteins that were confidently identified in this dataset, 3449 (63.8%) were listed as having previously been identified with evidence at the protein level, 1042 (19.3%) were found at the transcript level, 473 (8.7%) were inferred from homology, and 446 (8.2%) were predicted from the zebrafish genome but have no previously recorded protein evidence.

The top 25 most abundant proteins from the sample pools include eight different histone related proteins including Histone H3-like (*A0A8M6Z1M4)*, H1.0 linker histone (*Q6NYV3*), Histone H2B (*X1WHF1*), Histone H1-like (*A0A8M6Z7Z5*), Histone H1-like (*Q568D0*), Histone H2A (*Q0Z946*), Histone H2AX (*Q7ZUY3*), and Core histone macro-H2A (*Q4V914*).

These top 25 proteins were also mapped with the STRING database (Szklarczyk et al., 2023) (Figure 3-2), and while 11 of these could not be identified, the largest continuous network determined by this database included five of the histone related proteins mentioned above as well as the alpha (Q08BA1) and beta (A8WGC6) subunits of ATP-synthase and heat shock protein HSP 90- beta (O57521).

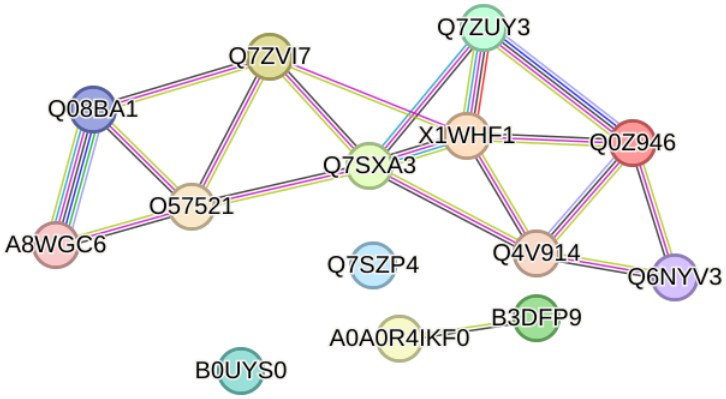


Figure 3-2: The STRING database map generated through the input of the protein accession ID of the 25 most abundant proteins detected in this data.

The 5410 proteins were matched to 4844 NCBI GeneIDs using the Uniprot ID mapping software. A total of 566 proteins could not be assigned NCBI GeneIDs. Using the assigned GeneIDs, a total of 1919 GO terms were identified among the three categories of GO terms, including 395 cellular components (Figure 3-3A), 1001 biological processes (Figure 3-3B), and 523 molecular functions (Figure 3-3C). Two of the most fold-enriched cellular component GO terms were the endopeptidase and peptidase complexes. Of the top 20 cellular component GO terms, six were mitochondria related. The biological processes included in the top

20 GO terms by fold-enrichment include a number of biosynthetic processes for molecules such as peptides and amides as well as the establishment of protein localisation. The highest fold-enrichment molecular function GO term was ligase activity, and nine of the top 20 molecular functions were molecular binding terms such as anion binding and small molecule binding.

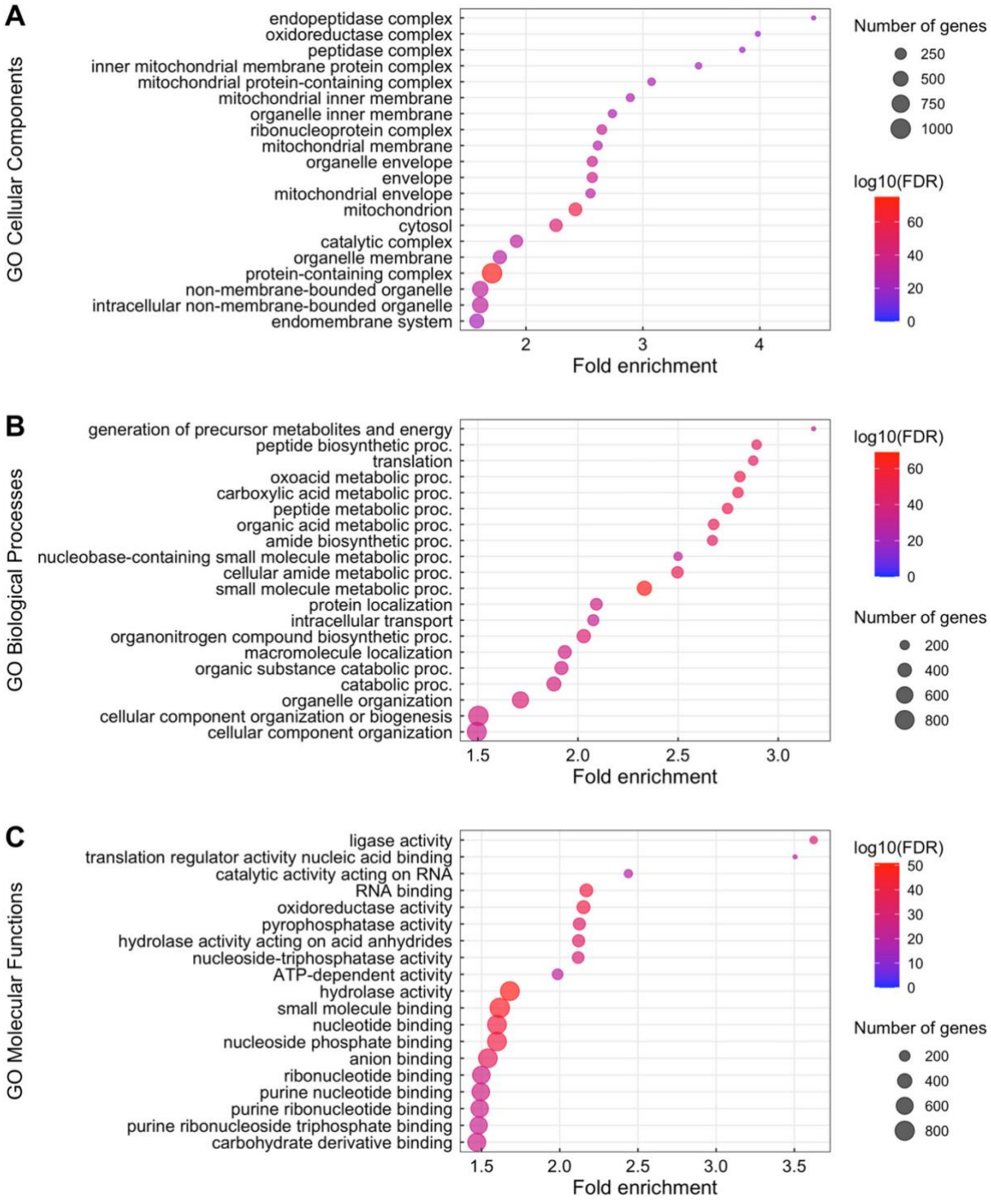


Figure 3-3: Top 20 GO terms ranked by fold-enrichment for the zebrafish sperm proteome, based on the 4844 assigned GeneIDs for (A) cellular component GO

terms, (B) biological process GO terms and (C) molecular function GO terms for the sperm proteome.

3.5 Discussion

Initial TMT-based proteomic analysis revealed no significantly differentially expressed proteins between sperm from males maintained at 28°C or 34°C (p_{adj} < 0.05), likely due to substantial biological variability among the high-temperature replicates. As a result, all six pooled sperm samples were combined for downstream interpretation. After filtering to exclude single-peptide identifications and contaminants, 5410 proteins were confidently identified, representing approximately 20% of the predicted protein-coding genes in the zebrafish genome. Of these, 4168 proteins were quantifiable. According to UniProt classification, most identified proteins had existing protein- or transcript-level evidence, while ~8% were predicted with no prior protein-level confirmation. The most abundant proteins included multiple histone variants, with STRING analysis revealing a central interaction network comprising histones, ATP-synthase subunits, and heat shock proteins. GO annotation of the mapped proteins (n = 4844) identified enrichment in mitochondrial components, biosynthetic processes, and molecular binding functions, highlighting key functional themes in the zebrafish sperm proteome.

Despite the inability to compare temperature treatments directly, the combined dataset provides valuable insight into the zebrafish sperm proteome under standard laboratory conditions. Compared to previous studies in other teleost fish, the depth of coverage achieved here is substantially higher. For example, proteomic profiling in rainbow trout (*Oncorhynchus mykiss*) sperm identified 206 proteins using a combined SDS-PAGE and LC-MS/MS method (Nynca et al., 2014), while a similar study in carp (*Cyprinus carpio L*) spermatozoa identified 348 proteins (Dietrich et al., 2014). In contrast, our study confidently identified 5410 proteins, of which 4168 were quantified. These differences are likely attributable to methodological advancements, including the use of tandem mass tag (TMT) labelling, deeper mass spectrometry acquisition, and a more complete zebrafish

reference genome and proteome. This expanded proteome offers a valuable resource for understanding zebrafish sperm biology and represents one of the most comprehensive teleost sperm proteomes to date.

Even when compared to proteomic profiles of other tissue types from zebrafish, our study presents a more complete proteomic profile. For example, proteomic profiles of other zebrafish organs identified ~1300 proteins in muscle tissue (5% of gene products), ~1500 proteins in skin tissue (6% of gene products) and ~2400 proteins in gill tissue (9% of gene products) resulting in a total of over 5000 proteins across all tissues (Nolte et al., 2014). In contrast, the present study identified 5410 proteins in zebrafish sperm alone.

Supporting the idea of a functionally distinct and complex sperm proteome, eight histone variants were among the 25 most abundant proteins identified. The high abundance of histones in zebrafish sperm is to be expected due to the lack of protamine-replacement and maintenance of histone packaging during sperm chromatin condensation during spermatogenesis (González-Rojo et al., 2018a). This contrasts with the protamine-based DNA compaction observed in many other species, including mammals such as humans and mice, where histones are replaced to achieve tighter genome condensation (Elango et al., 2022).

One key process shared by the sperm of many species is capacitation. Capacitation refers to the physiological changes sperm undergo such as changes to the membrane, motility patterns, and protein phosphorylation states, in order to become able to fertilise an oocyte (Bailey, 2010). While capacitation is a common process amongst sperm, the exact mechanisms and activation triggers can vary greatly across species, especially dependent on the internal or external mode of fertilisation used. In mammals, capacitation typically occurs post-ejaculation within the female reproductive tract (Stival et al., 2016). In contrast, in many fish species, sperm capacitation is activated in response to environmental cues such as osmotic shock (Alavi & Cosson, 2006). Therefore, while zebrafish (*Danio rerio*) are often regarded as a useful model for human reproductive biology due to their ~70% genetic homology with humans (Howe et al., 2013), differences in key

reproductive processes such as capacitation, differences in reproduction due to internal vs external environments, and reproductive strategy must be carefully considered. This may provide some explanation as to the differences noted between mammalian sperm DNA compaction and the histone-rich compaction observed in zebrafish.

The STRING network generated from the top 25 most abundant proteins (Figure 3-2) further supports the central role of histones in zebrafish sperm biology. The largest interaction cluster consisted predominantly of core histones and histone variants, reflecting their dominance among the most abundant proteins in the dataset and reinforcing the histone-based chromatin organisation characteristic of this species. Notably, most mammalian sperm contains DNA that has been repackaged from histones to protamines during spermatogenesis to increase DNA compaction (Moritz & Hammoud, 2022), although this repackaging does not occur in zebrafish. The presence of ATP synthase subunits and the heat shock protein HSP90-beta within the same network suggests that proteins involved in mitochondrial energy production and protein stabilisation are also maintained at high abundance in mature sperm. These findings highlight the dual importance of chromatin structure and protein stabilisation or stress response in the functional proteome of zebrafish sperm.

GO term analysis of the identified proteins further reinforced the importance of chromatin structure, mitochondrial function, and protein processing in mature zebrafish sperm. Among the top fold-enriched cellular component GO terms (Figure 3-3 A) were the endopeptidase and peptidase complexes, both of which are known to be important in sperm motility due to their roles in mediating protein–protein interactions in response to activation stimuli (Ren et al., 2023). Six of the top ten highly enriched cellular component GO terms were associated with mitochondrial components including; mitochondrion, mitochondrial membrane, mitochondrial envelope, mitochondrial inner membrane, inner mitochondrial membrane protein complex, and mitochondrial protein-containing complex , consistent with the role of mitochondria in ATP production required for flagellar movement (Kumar, 2022). In zebrafish sperm specifically, mitochondria are known

to undergo ultrastructural changes during activation including bursting of the plasma membrane, further underscoring their importance in motility (Sáez-Espinosa et al., 2022). These ultrastructural effects, specifically changes in the plasma membrane, may be reflected as four of the top 10 highly enriched cellular component GO terms were directly linked to mitochondrial membranes.

Since this proteome was generated from mature sperm, many of the biological process terms may reflect proteins retained from earlier stages of spermatogenesis (Figure 3-3B), including organelle organisation, cellular component localisation or biosynthesis, and protein localization. This raises the possibility that proteins initially involved in spermatogenic processes may have alternative or residual roles in the mature sperm cell. For example, enriched molecular function terms (Figure 3-3C) included ligase activity and various binding functions, such as anion and small molecule binding, which may relate to the known role of ion flux in sperm activation. Zebrafish sperm are activated by a hypo-osmotic shock triggered by a drop in extracellular potassium upon ejaculation into water, initiating rapid changes in membrane potential and motility (Sáez-Espinosa et al., 2022; Wilson-Leedy et al., 2009). The presence of proteins associated with these functions supports the idea that the mature zebrafish sperm proteome is primed for environmental responsiveness and rapid activation.

The initial experimental design for this study aimed to compare the sperm proteomes of samples extracted from the same testes used to generate the proteomic dataset presented in Chapter 2, with males grouped in pools of ten by tank and treatment. However, this temperature-based comparison was ultimately not possible due to both high variability and low sperm availability in samples from males kept at 34°C. As shown by the GO term analysis in Chapter 2, spermatogenesis appeared to be downregulated in the testes following heat treatment. This likely resulted in reduced sperm output, and it is possible that the limited sperm produced were also more susceptible to damage under thermal stress, contributing to the high variation observed across samples. Other studies have previously reported measurable differences in sperm proteomes as a result of heat stress, for example in *Drosophila melanogaster* where 96 proteins were

found to differ in abundance between multigenerational heat stress groups, although this approach was not quantitative (Khan & Mishra, 2024). This suggests that higher sample sizes may help identify more subtle differences in sperm proteomes between temperature conditions, and future studies will be needed to address this question in greater detail.

The quality and reliability of the dataset were further supported by the levels of existing evidence associated with the identified proteins. Of the 5410 proteins confidently identified, 63.8% had previously been validated at the protein level, while an additional 19.3% had transcript-level evidence. A smaller proportion were inferred from homology (8.7%) or predicted from genome annotations without prior empirical support (8.2%). These proportions reflect a high level of agreement with existing zebrafish proteomic and transcriptomic resources and indicate that the dataset is both comprehensive and robust, with the potential to highlight novel or under-characterised sperm-specific proteins.

In conclusion, quantitative mass spectrometry-based profiling of zebrafish sperm yielded a comprehensive dataset of 5410 proteins. The high abundance of histone variants reflects the distinctive chromatin architecture of zebrafish sperm, which lacks protamine-based DNA compaction. In addition, many proteins associated with earlier stages of spermatogenesis appear to be retained in mature sperm, as suggested by the enrichment of relevant biological process GO terms. Enrichment of cellular component and molecular function terms related to mitochondrial activity, protein complexes, and binding functions further highlights the importance of energy production and environmental responsiveness in sperm activation. Together, these findings provide a valuable resource for future protein-level investigations of zebrafish sperm biology and reinforce the utility of this species as a model for reproductive proteomics.

4 The Proteomic Biomarkers of Human Sperm Quality

4.1 Abstract

Sperm quality plays a critical role in male fertility, yet much remains unknown about the molecular mechanisms that determine which sperm are most functionally competent. Previous work has shown that haploid selection within ejaculates, where selection acting upon phenotypic differences among genetically distinct sperm to influence fertilisation outcomes, can impact offspring fitness. While genomics and transcriptomics have provided insight into sperm development and gene expression, proteomics offers a direct window into functional cellular mechanisms. In particular, identifying proteins associated with sperm motility and longevity can enable the discovery of biomarkers for sperm quality, with potential applications in diagnostics and assisted reproductive technologies.

This study investigates the proteomic signatures underlying sperm quality in humans by identifying protein biomarkers associated with motility and longevity. Using Tandem Mass Tag (TMT)-based quantitative proteomics, sperm subpopulations were isolated from the same ejaculate via methyl cellulose and swim-up assays, methods that respectively select for high motility and prolonged viability. Comparative proteomic analysis identified 4,616 high-confidence proteins, with 1,178 showing differential abundance between selected and unselected sperm. Proteins with lower abundance in selected sperm were strongly enriched for markers of cellular immaturity and stress, such as those involved in RNA processing and immune signalling. Whereas proteins with higher abundance in selected sperm were associated with structural and functional components critical for motility and membrane stability. A shortlist of 68 candidate biomarkers was generated, with OLFM4 emerging as the most consistently downregulated protein in high-quality sperm. These findings provide a molecular foundation for

developing non-destructive diagnostic tools for sperm selection, with implications for improving male fertility assessments and assisted reproduction outcomes.

4.2 Introduction

The effects of competition between individuals during sexual reproduction as a driving force for evolution forms the basis of the theory of sexual selection (Darwin, 1871). This theory was later expanded to include competition between the sperm of different males, termed sperm competition (Parker, 1970). More recently, this framework has been extended to recognise that competition can also occur within a single ejaculate, where genetically distinct sperm from the same male vary in their capacity to fertilise an egg (Wigby & Chapman, 2004). The concept of haploid selection extends the framework of sexual selection to the gametic stage, proposing that genetically distinct gametes within an ejaculate can differ in fertilisation success, allowing selection to act at the haploid stage even in primarily diploid organisms (Immler & Otto, 2018; Joseph & Kirkpatrick, 2004).

However, the concept of haploid selection has previously been overlooked in evolutionary theories, as haploid sperm were assumed to be transcriptionally and translationally inactive (Hecht, 1998). At the time, the regulation of sperm phenotype was thought to be entirely determined by the diploid male during spermatogenesis, and differences observed among mature spermatozoa were generally attributed to random developmental variation rather than a reflection of genetic variation and a possible target of selection (Immler, 2019).

Contrary to earlier assumptions, mature sperm are not merely passive carriers of paternal DNA. Post-meiotic sperm can be highly varied, and haploid selection acts as an important evolutionary tool in removing deleterious alleles from a population (Alavioon et al., 2017; Immler, 2019; Immler & Otto, 2018). Molecular studies have shown that sperm retain a diverse repertoire of RNAs and proteins, many of which are functionally active and can influence fertilisation and early embryogenesis (Miller & Ostermeier, 2006; Ostermeier et al., 2004). This variation provides a

direct link between haploid genotype and phenotype, making sperm legitimate targets of natural selection. Rather than representing developmental noise, heterogeneity among spermatozoa can therefore have adaptive consequences, both for immediate fertilisation success and for the genetic quality of subsequent offspring (Sendler et al., 2013).

Growing empirical evidence supports the idea that haploid selection is not just a theoretical possibility, but an observable process across a wide range of taxa (Immler, 2019; Immler & Otto, 2018). Previous work in zebrafish (*Danio rerio*) provided evidence that haploid selection operates within a single ejaculate, with sperm longevity linked to consistent effects on offspring phenotype (Alavioon et al., 2017, 2019). Comparable evidence has been reported in Atlantic salmon (*Salmo salar*), where genetic variation among sperm influences fertilisation success, indicating that haploid selection can act during external fertilisation in natural populations (Immler et al., 2014). Evidence from mammals further supports that the haploid sperm genotype plays a role in shaping sperm function and fertilisation ability (Ramón et al., 2014). In cattle (*Bos taurus)*, comparisons between X- and Y-chromosome–bearing sperm have revealed consistent differences in several mitochondrial proteins, including enzymes central to oxidative phosphorylation and energy metabolism (Scott et al., 2018). These observations suggest that variation at the haploid genome level can influence metabolic performance, a functional trait directly tied to motility and therefore fertilisation potential. Other studies have shown that sperm genotype can have a direct effect on the ability of a sperm to overcome selective barriers to reach the oocyte during natural conception (Nixon et al., 2023). In mice (*Mus musculus*) for example, sperm that reached the oviduct exhibited lower levels of DNA fragmentation than those that did not (Hourcade et al., 2010), while in boar (*Sus scrofa*), sperm with compromised chromatin integrity were under-represented among those capable of penetrating oocytes (Ardon et al., 2008). Collectively, these findings indicate that mammalian sperm with higher genetic quality are more likely to reach later stages of fertilisation, consistent with haploid selection acting within the ejaculate.

Following previous research, genomic studies in humans identified parallel signatures of haploid selection, with longevity-based sperm selection also being found to be associated with genomic divergence and improvements in key sperm traits such as motility, morphology, and DNA integrity (Marcu, 2024). The implications of sperm selection extend beyond evolutionary theory and are highly relevant to fertility in both humans and animals. Assisted reproductive technologies (ART) currently rely on criteria such as morphology and motility to select sperm, but these measures are poor predictors of fertilisation and pregnancy success (Ribas-Maynou et al., 2021). As a result, embryos generated through ART may carry a higher genetic load, reducing implantation rates, increasing miscarriage risk, and potentially compromising offspring health (van Oosterhout et al., 2022).

Incorporating principles of within-ejaculate sperm selection could help to address the limitations of current ART methods by favouring sperm that demonstrate better function, and therefore possibly a lower genetic load, thereby lowering the transmission of deleterious alleles. Recent conceptual work has argued that developing non-destructive molecular markers for sperm quality would be transformative, improving both diagnostic assessment of male infertility and the efficiency of clinical procedures such as IVF and ICSI (Alavioon et al., 2021).

High throughput approaches such as genomics, transcriptomics, and proteomics have provided important insights into sperm biology (Amaral, Castillo, et al., 2014; Cannarella et al., 2020). Proteomics complements both genomic and transcriptomic approaches by directly measuring all proteins present within a cell at the time of study, providing a more accurate depiction of cellular function and phenotype (Aebersold & Mann, 2003). Methods such as Tandem Mass Tag (TMT) quantitative proteomics allow for the measurement of specific protein abundances (Pagel et al., 2021), which offers critical insights into the actual biological processes occurring within the cell, making it a useful tool for understanding the molecular mechanisms underlying sperm function and haploid selection.

Proteomic studies have provided important insights into the molecular basis of human male infertility, firstly by comparing ejaculates from men with different clinical diagnoses (Cannarella et al., 2020). Contrasts between normozoospermic and asthenozoospermic human samples exhibit consistent differences in proteins associated with metabolism, motility, oxidative stress, and structural integrity (Martínez-Heredia et al., 2008; Saraswat et al., 2017). While these studies have advanced understanding of infertility, they treat ejaculates as homogeneous units and overlook the substantial heterogeneity that exists among sperm from the same individual. More recent work started to focus on this heterogeneity by examining subpopulations within single ejaculates where distinct proteomic signatures have been identified between functional states of sperm from the same ejaculate (Parkes & Garcia, 2024). Sequential analyses before and after capacitation and the acrosome reaction revealed dynamic changes in protein abundance in human sperm (Castillo et al., 2023; Secciani et al., 2009), while comparisons of mature versus immature sperm within a single sample showed distinct molecular profiles (Cui et al., 2016).

In this chapter, two distinct selection methods were used to select for human sperm subpopulations from the same ejaculate. The first was a methyl cellulose assay, which enriched for progressively motile sperm through migration within a viscous medium over four hours. The second approach involved a swim-up assay, allowing comparison of sperm that were motile both before and after a four-hour incubation, thus differentiating between shorter- and longer-lived sperm. These subpopulations were analysed using TMT-based quantitative proteomics to characterise the sperm proteome and to detect differences in protein abundance between subpopulations from the same ejaculate. The aim was to determine whether proteomic differences could be detected between sperm subpopulations isolated from the same ejaculate, and to identify candidate molecular markers associated with sperm motility and longevity.

4.3 Methods

4.3.1 Sperm parameter analysis

Samples were collected from two self-reported healthy donors at the University of East Anglia, UK, under ethics permit no. ETH2324-0997, and two self-reported healthy donors from Cape Town, South Africa (ZA). Following collection, samples were warmed to 37°C in a Midi 40 CO_2 incubator (Thermo Fisher, UK). Measurements of volume and pH were taken. Sperm concentration, motility and velocity parameters were assessed with the motility module of Integrated Semen Analysis System (ISAS) v1 (Poiser, Spain), and the data was captured with an ISAS 782M camera connected to a UB203i microscope with a 10x phase contrast objective and a pre-heated stage. Preheated (37°C) disposable 75 mm x 25 mm glass chamber slides with 20-micron depth chambers (Cytonix, USA) were loaded with 2µl of sample and at least 200 sperm were analysed from at least two fields for one second at 50 frames per second (f/s). Kinematics parameters recorded for each sample included: curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN) = (VSL/ VCL)*100, straightness (STR) = (VSL/ VAP)*100, wobble (WOB) = (VAP/ VCL)*100, amplitude of lateral head displacement (ALH) and beat cross frequency (BCF).

4.3.2 Sperm selection assays

For the two samples collected at UEA, methyl cellulose (MC) assays were conducted by using a 10 cm-diameter watch glass filled with 10 mL of 1% (w/v) methylcellulose (Sigma, Denmark), distributed evenly up to a 4 cm radius. Raw ejaculate (300 µL) was carefully pipetted into the centre, forming a central deposit of approximately 0.5 cm in diameter. For each donor, five watch glasses were prepared in parallel to allow recovery of sufficient cell numbers. Samples were incubated at 37°C under 5% CO_2 for two hours in a Midi 40 CO_2 incubator (Thermo Fisher, UK). Motile sperm were collected from the outer edge of the methylcellulose dome at radii ranging from 2 to 3.5 cm from the centre depending on relative sperm density. Sperm density at different radii was determined by taking 2 µL samples from each measured point on the radius of the dish and examining using CASA (ISAS v1 Poiser, Spain). The furthest point with at least 10

visible sperm per field of view was selected, and 4mL of sperm were collected using extra-long bevelled 200 µL pipette tips and pooled. A 2 mL sample of sperm that did not pass the selection criteria was taken from the central point of the dish, also using extra-long bevelled 200 µL pipette tips, avoiding debris at the bottom and without touching the dish. To extract sperm from the viscous methyl cellulose, 1 µL of cellulase was added per 1 mL of collected sample, mixed, and incubated at 50°C for two hours with shaking at 50 rpm using a Thermomixer C (Eppendorf, Germany). The samples were centrifuged at 14,000 rpm for 5 minutes. Supernatant was discarded and sperm pellets were stored at -80°C until protein extraction.

For the two sperm samples collected in South Africa and subjected to swim up (SW) assays, aliquots of 1 mL raw ejaculate were mixed 1:1 with human tubal fluid (HTF) medium and incubated at 37°C in 5% CO_2 for 4 hours. At both T0 (prior to incubation) and T4 (following the 4 hour incubation) a 0.5 mL aliquot was removed from the sample, was gently placed under 1 mL of fresh HTF in a new tube and incubated for 60 minutes to allow motile sperm to swim up. The top 0.5 mL were collected using a 1 mL Pasteur pipette, centrifuged at 700 x g for 10 minutes at room temperature, and resuspended in HTF. The samples were then centrifuged at 14000 rpm for 5 minutes. Supernatant was discarded and sperm pellets were stored at -80°C until protein extraction.

4.3.3 Sample preparation for proteomics

Sperm sub-population pellets collected from the methyl cellulose and swim-up assays were washed in HTF and resuspended in 200 µL HTF. Two samples per donor were used for proteomic comparison: one representing the less motile or unselected population (Centre (MC) or T0 (SW) and one representing the motile, selected population (Edge (MC) or T4 (SW)). All samples were stored at -80°C.

4.3.4 Protein extraction and peptide preparation

Frozen sperm pellets were thawed and precipitated with 450 µL acetone, vortexed for 15 minutes, and centrifuged at 10,000 rpm for 10 minutes at room temperature. Samples were then prepared using an SDC-based in-solution digestion approach (Masuda et al., 2008). Wherein, after removal of the supernatant, 500 µL acetone was added, and samples were gently mixed, centrifuged at 10,000 rpm for 10 minutes, and air-dried at room temperature. Pellets were resuspended in 100 µL 2.5% sodium deoxycholate (SDC) in 0.2 M EPPS buffer (pH 8.5). Samples were reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin. SDC was removed by acid precipitation, and peptides were desalted by C18 solid-phase extraction.

4.3.5 TMT labelling and fractionation

Peptides were quantified and labelled using an 8-plex TMT10 kit (ThermoFisher Scientific). Each sample was dissolved in EPPS/acetonitrile, labelled with 200 µg TMT reagent, and combined after a 2-hour incubation. Aliquots were checked for labelling efficiency via LC-MS. Samples were quenched with hydroxylamine, desalted using C18 Sep-Pak cartridges, and fractionated by high pH reverse-phase HPLC. Fractions were concatenated into 20 final pools. Each sample replicate was fractionated using a Pierce High pH Fractionation kit to yield 7 fractions.

4.3.6 Mass spectrometry Acquisition

Samples were analysed on an Orbitrap Eclipse Tribrid mass spectrometer with FAIMS Pro Duo and UltiMate 3000 RSLCnano LC. Samples were loaded on a C18 trap cartridge and separated on an Aurora Frontier UHPLC column. A detailed gradient of increasing acetonitrile (0-105 min) was used for peptide elution. MS acquisition included MS1 at 120K resolution, FAIMS CVs of -35V, -50V, and -65V, MS2 CID fragmentation with rapid mode acquisition, and SPS-MS3 detection for quantification. The sample for donor 3 (SW) was repeated due to issues with initial data acquisition.

4.3.7 Data processing and protein identification

Raw data files were analysed in Proteome Discoverer 3.1 using CHIMERYS and Comet search engines against the UP000005640_human_2024_9606.fasta database. Recalibration, quantification, and database searching were performed with fixed (carbamidomethylation, TMT) and variable (oxidation) modifications. Peptide-spectrum matches were filtered at 1% FDR. Quantification was performed using intensity-based abundance with correction for co-isolation, missing value imputation, and t-tests with BH correction.

4.3.8 Filtering and structural analysis

Proteins with fewer than two unique peptides (excluding NAs) or detected in fewer than three of five samples (four donors with donor 3 repeated) were excluded. Structural models for shortlisted proteins were visualised using PyMOL (version 3.1.5.1) (Schrödinger, LLC, 2015). All data filtering was performed in accordance with current proteomics best practice guidelines (Burger, 2018). Gene Ontology (GO) term analysis was performed using BioMart (Haw et al., 2011; Smedley et al., 2015) and Bioconductor (Gentleman et al., 2004) packages through R.

4.4 Results

A total of 8,161 peptide matches were initially identified across all sperm samples using TMT-labelled LC-MS/MS. To ensure confidence in protein identification, the dataset was filtered to retain only proteins supported by at least two unique peptides per experiment and detected in a minimum of three donors. This resulted in a high-confidence dataset of 4,616 proteins (Figure 4-1, Supplementary Table 6), which was carried forward for downstream comparative analyses.

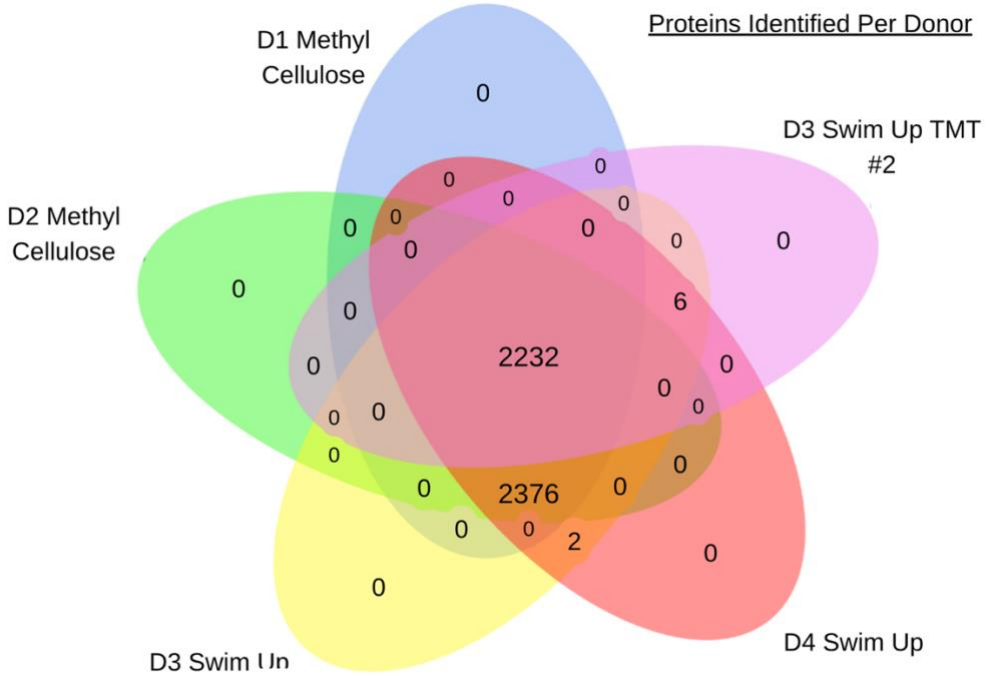


Figure 4-1: Overlap of confidently identified proteins across all donors. Venn diagram showing the intersection of proteins identified in each of the five human donors following TMT-labelled LC-MS/MS. Proteins were retained in the final dataset if they were supported by at least two unique peptides and detected in a minimum of three donors, resulting in a high-confidence proteome of 4,616 proteins used for downstream comparative and enrichment analyses.

The high-confidence protein dataset was then analysed for differences in abundance between selected sperm samples (T4, Edge) and unselected samples (T0, Centre) to investigate the effects of selection on the sperm proteome. Filtering criteria required a consistent twofold change in the same direction across at least three donors, and a $p_{adj} < 0.05$ in at least one donor. This analysis identified 634 proteins with significantly lower abundance and 544 proteins with significantly higher abundance in selected sperm.

The combined set of 1,178 proteins showing differential abundance was further refined to generate shortlists of candidate biomarkers. For proteins with lower abundance in selected sperm, more stringent filtering was applied due to the strength of observed directional change. This resulted in a shortlist of 62 proteins.

In contrast, although 544 proteins were initially identified with significantly higher abundance, these changes were less consistent across donors. Therefore, a looser criterion was applied, including only proteins with a fold-change ratio above 2 in at least two donors, and significance in at least one donor. This added a further six proteins to the shortlist (Supplementary Table 7).

From the combined shortlist of 68 proteins, top candidates were selected using additional criteria. The top five proteins with lower abundance in selected sperm were present in all five samples (four donors with one repeated), statistically significant in at least four donors, and had a median fold-change ratio below 0.2. Similarly, the top four proteins with higher abundance were present in all five donors, significant in at least four donors, and had a median fold-change ratio above 2 (Figure 4-2).

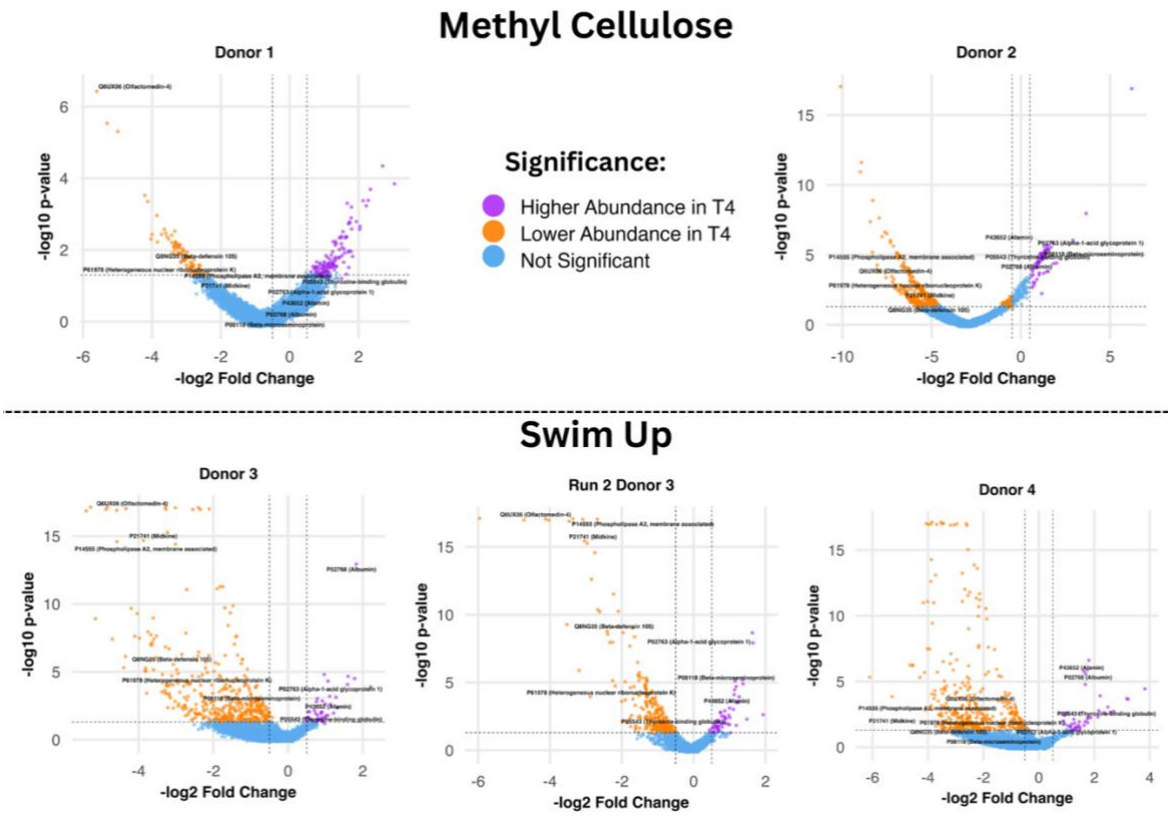


Figure 4-2: Volcano plots showing differential protein abundance between selected and unselected sperm pools. The plots display the -$\log_2$ fold-change in protein abundance (x-axis) against the $-\log_{10}$ p_{adj} (y-axis) for each donor, comparing selected (T4/Edge) and unselected (T0/Centre) sperm subpopulations. Proteins

with significantly higher (right, purple) or lower (left, orange) abundance in selected sperm are highlighted. The top candidate proteins, shortlisted based on consistency across donors and magnitude of change, are labelled on each plot. Cut-off thresholds for differential abundance included a minimum twofold change across at least three donors and a $p_{adj} < 0.05$ in at least one donor.

Following the identification of differentially abundant proteins across the whole dataset, changes in abundance were also analysed within each experimental replicate. For each experiment, proteins were considered to be higher in abundance if the fold-change ratio was greater than one in both donors, and lower in abundance if the ratio was below one. Proteins that were not statistically significant in both donors were excluded.

This filtering identified 52 proteins with higher abundance and 425 with lower abundance in the methyl cellulose assay (donors 1–2), 33 higher and 594 lower in the first swim-up assay (donors 3 & 4), and 59 higher and 276 lower in the second swim-up TMT replicate (donor 3). When considered separately, the number of significant proteins per donor ranged from 43-113 higher and 86-431 lower abundance proteins (Table 4-1). These experiment- and donor-specific protein lists were then annotated with Entrez Gene IDs and used for Gene Ontology (GO) enrichment analysis against the full human genome background.

Table 4-1: Number of Proteins of significantly higher and lower abundance in selected sperm by donor.

Donor and Experiment	Proteins Significantly Higher in Selected Sperm	Proteins Significantly Lower in Selected Sperm
Donor 1 Methyl Cellulose	113	86
Donor 2 Methyl Cellulose	101	430
Donor 3 Swim Up	43	427
Donor 4 Swim Up	45	431
Donor 5 Swim Up TMT 2	59	276

We performed enrichment analyses for proteins with significantly different abundances between sperm pools for biological processes, cellular components, molecular functions, and KEGG pathways (Figures 4-3–4-6). Gene Ontology enrichment of biological processes for proteins less abundant in selected sperm were strongly associated with RNA processing and protein translation (Figure 4-3A). In contrast, proteins more abundant in selected sperm were enriched for biological processes related to membrane remodelling and regulation of enzymatic activity following selection via methyl cellulose. The first swim up TMT enriched for lipid transport and cholesterol efflux pathways characteristic of capacitation, whilst the second TMT for swim up enriched for motility and membrane organisation. (Figure 4-3B).

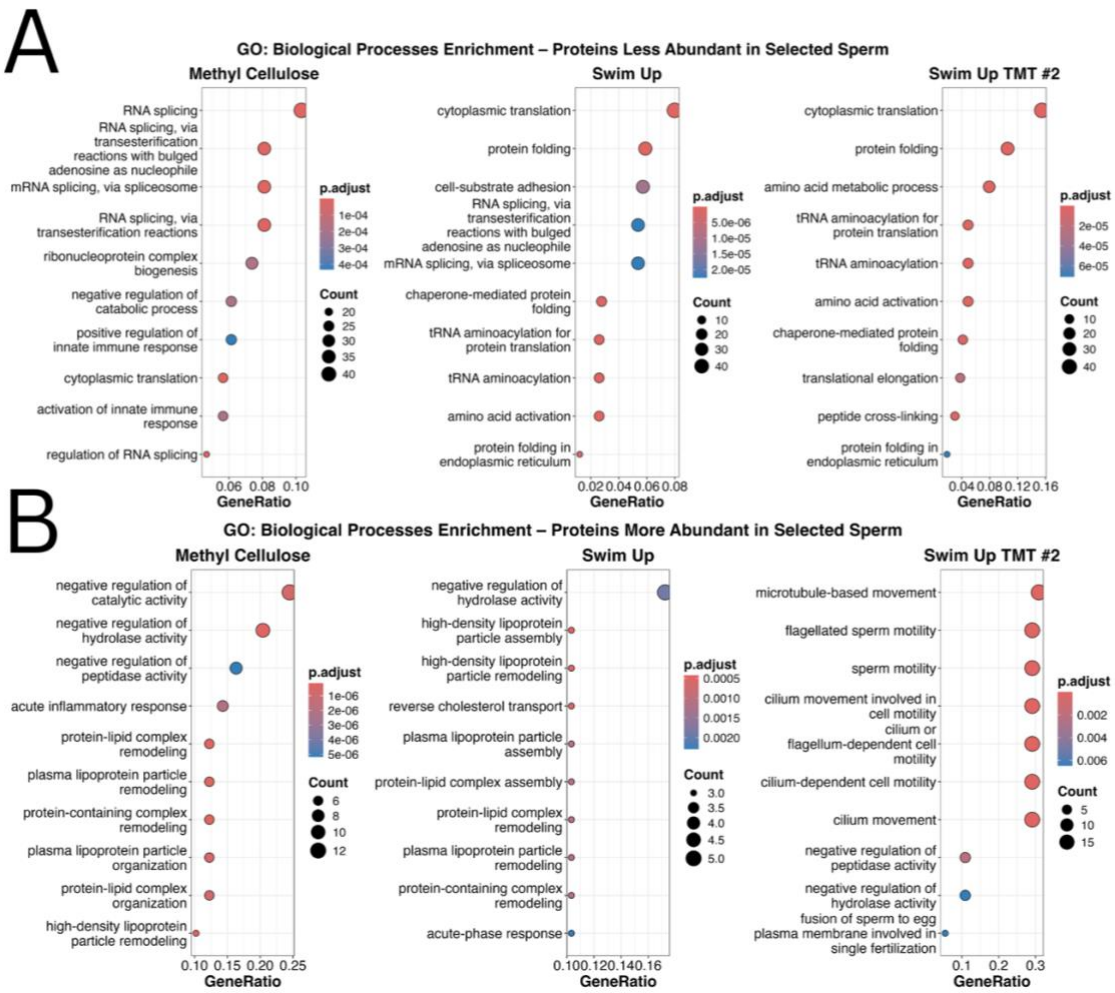


Figure 4-3: A) Bubble plot showing the top 10 significantly enriched GO Biological Process terms among proteins with lower abundance in selected sperm compared to unselected sperm. B) Bubble plot showing the top 10 significantly enriched GO Biological Process terms among proteins with higher abundance in selected sperm compared to unselected sperm. Gene Ontology (GO) term analysis was performed using BioMart (Haw et al., 2011; Smedley et al., 2015) and Bioconductor (Gentleman et al., 2004) packages through R.

For cellular component terms, proteins depleted in selected sperm were associated with ribosomal and spliceosomal structures, including “cytosolic ribosome,” “ribosomal subunit,” and “spliceosomal complex” (Figure 4-4A). Proteins enriched in selected sperm mapped to structures such as “sperm

flagellum," "motile cilium," and "axoneme," with strong signals in swim-up replicate 2 (Figure 4-4B).

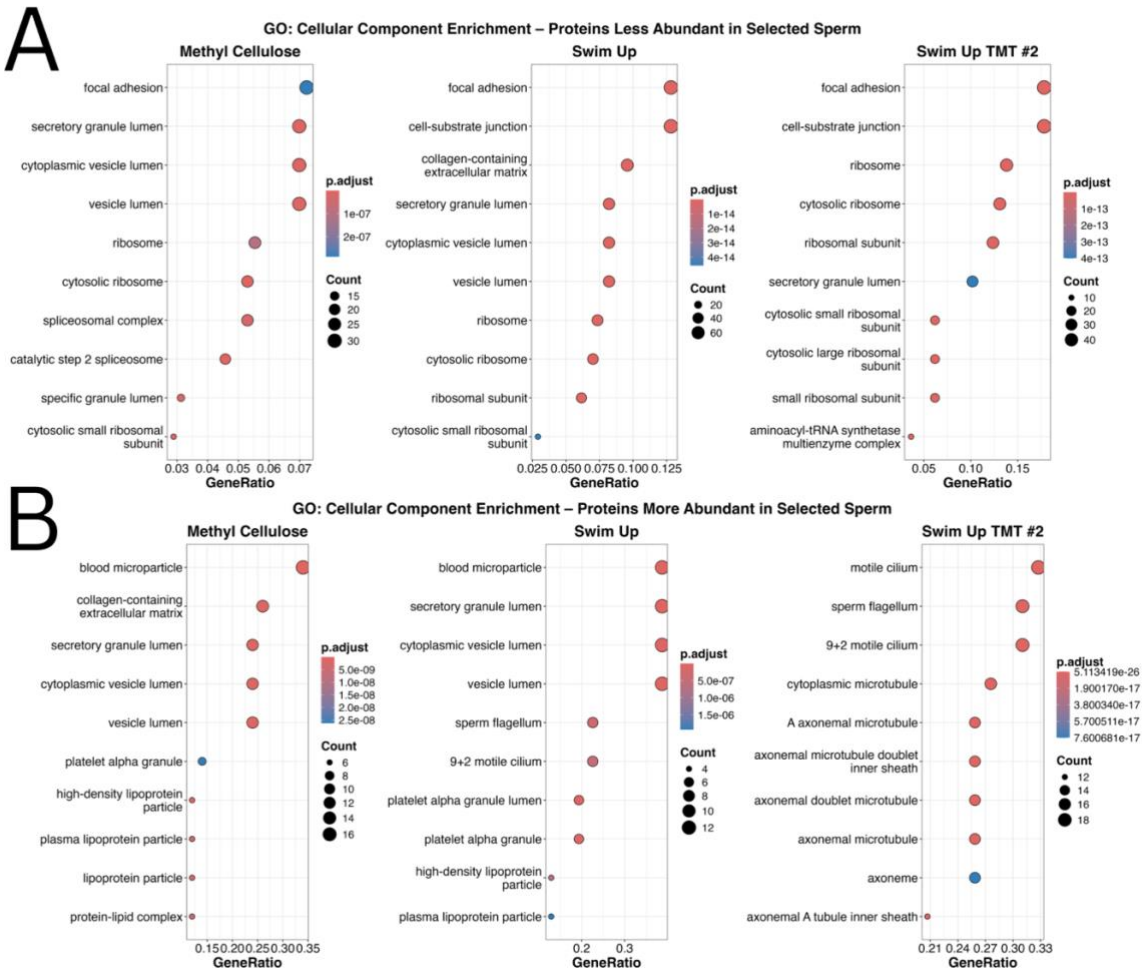


Figure 4-4: A) Bubble plot displaying the top 10 GO Cellular Component enrichment for proteins with lower abundance in selected sperm. B) Bubble plot displaying the top 10 GO Cellular Component enrichment for proteins with higher abundance in selected sperm. Gene Ontology (GO) term analysis was performed using BioMart (Haw et al., 2011; Smedley et al., 2015) and Bioconductor (Gentleman et al., 2004) packages through R.

Molecular function analysis showed that proteins less abundant in selected sperm were enriched for RNA- and ribosome-binding activities, including "ribonucleoprotein complex binding" and "structural constituent of ribosome" (Figure 4-5A). Adhesion-related terms such as "collagen binding" and "cell-

substrate junction" were also observed. Proteins more abundant in selected sperm were enriched for functions related to enzyme inhibition and structural support, including "endopeptidase inhibitor activity," "peptidase regulator activity," and "antioxidant activity" (Figure 4-5B).

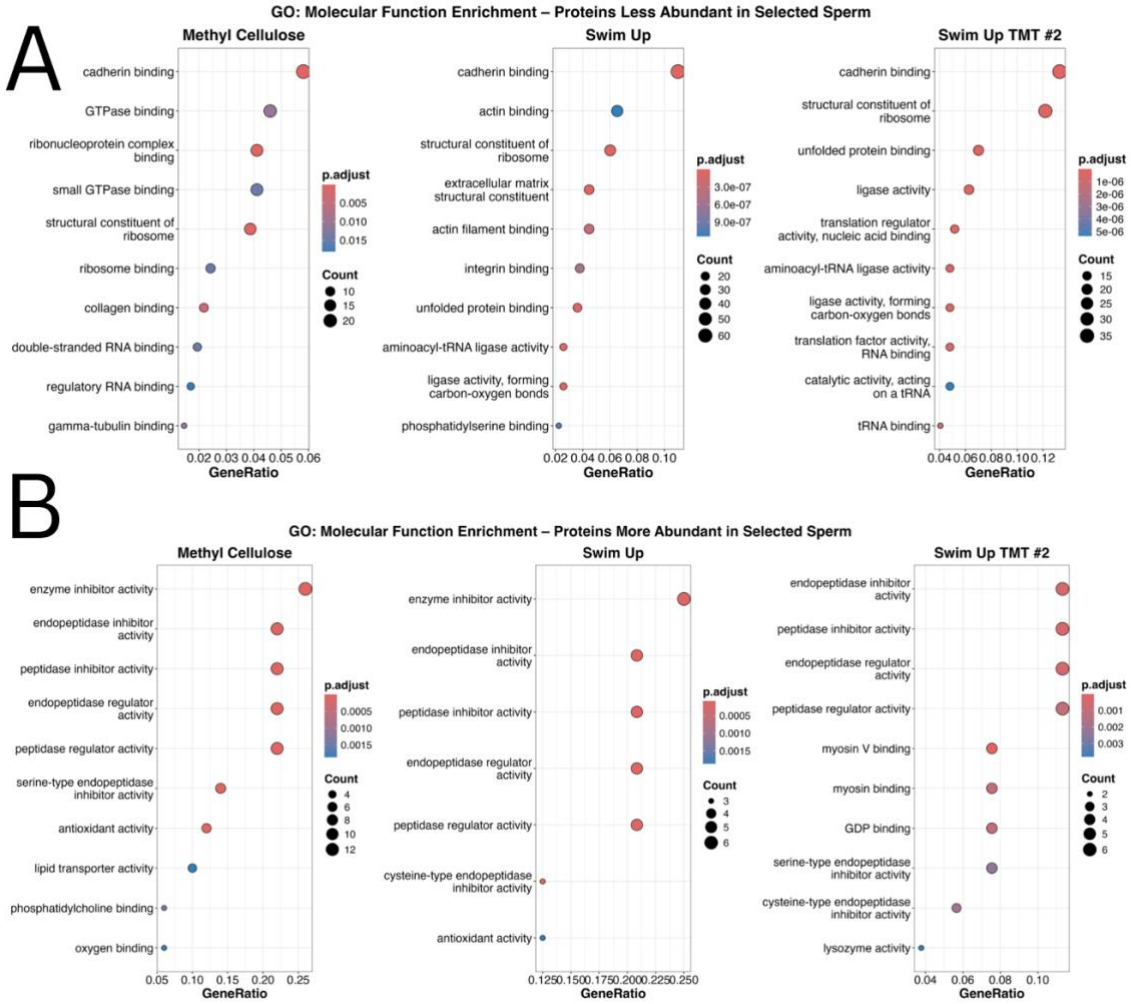


Figure 4-5: A) Bubble plot displaying the top 10 GO Molecular Function enrichment for proteins with lower abundance in selected sperm. B) Bubble plot displaying the top 10 GO Molecular Function enrichment for proteins with higher abundance in selected sperm. Gene Ontology (GO) term analysis was performed using BioMart (Haw et al., 2011; Smedley et al., 2015) and Bioconductor (Gentleman et al., 2004) packages through R.

KEGG pathway analysis of proteins depleted in selected sperm revealed enrichment for "spliceosome," "ribosome," "protein processing in endoplasmic

reticulum," and "aminoacyl-tRNA biosynthesis" (Figure 4-6A). In contrast, proteins enriched in selected sperm were associated with pathways including "cholesterol metabolism," "complement and coagulation cascades," and "thyroid hormone synthesis" (Figure 4-6B).

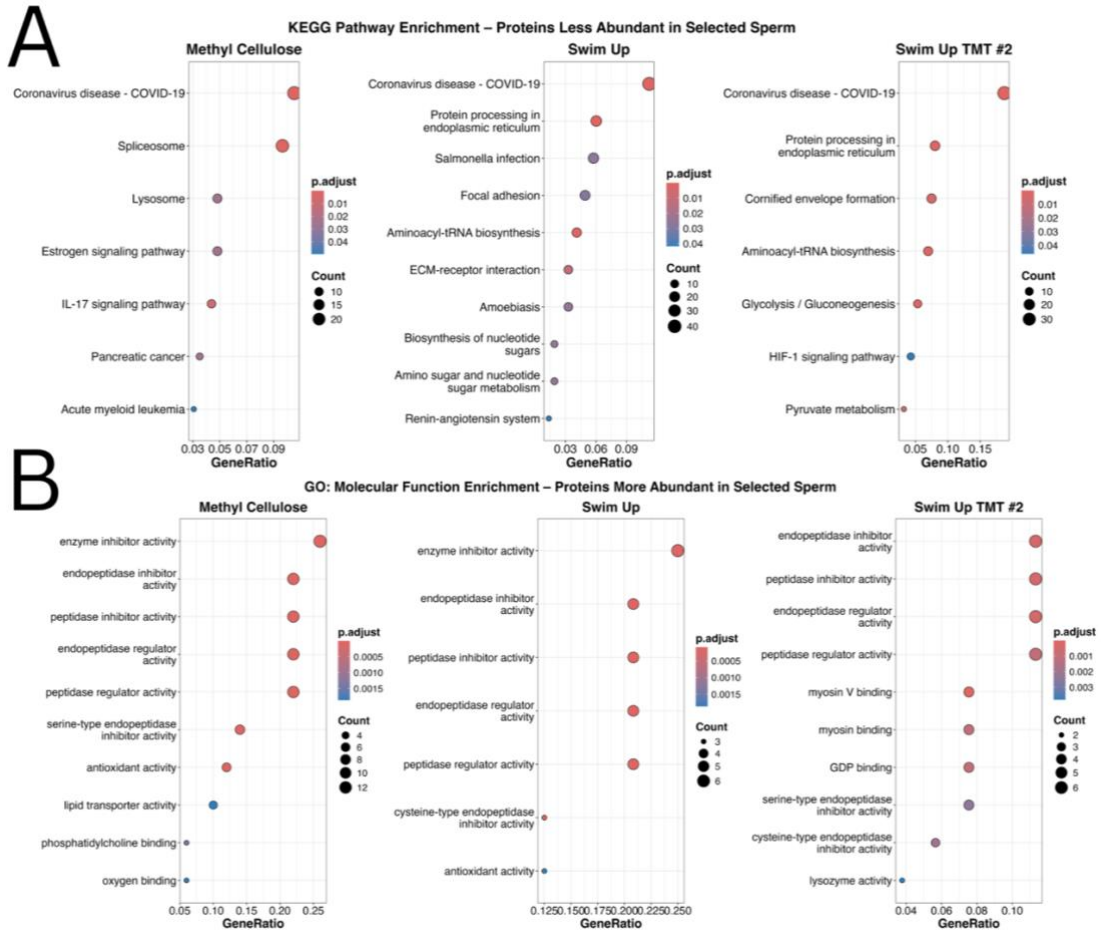


Figure 4-6: A) Bubble plot displaying the top 10 KEGG pathway enrichment for proteins with lower abundance in selected sperm. B) Bubble plot displaying the top 10 KEGG pathway enrichment for proteins with higher abundance in selected sperm. Gene Ontology (GO) term analysis was performed using BioMart (Haw et al., 2011; Smedley et al., 2015) and Bioconductor (Gentleman et al., 2004) packages through R.

Analysis of shortlisted proteins showed no significant enrichment for biological processes among the six proteins more abundant in selected sperm, although

Cellular Component terms such as “secretory granule lumen” and “platelet alpha granule” were identified, together with Molecular Function terms including “vitamin binding” and “toxic substance binding” (Figure 4-7). By contrast, the 62 proteins with lower abundance in selected sperm showed consistent enrichment across all GO categories, including “RNA splicing,” “mRNA processing,” “regulatory RNA binding,” and “helicase activity,” alongside significant enrichment for the KEGG pathway “Spliceosome” (Figure 4-8).

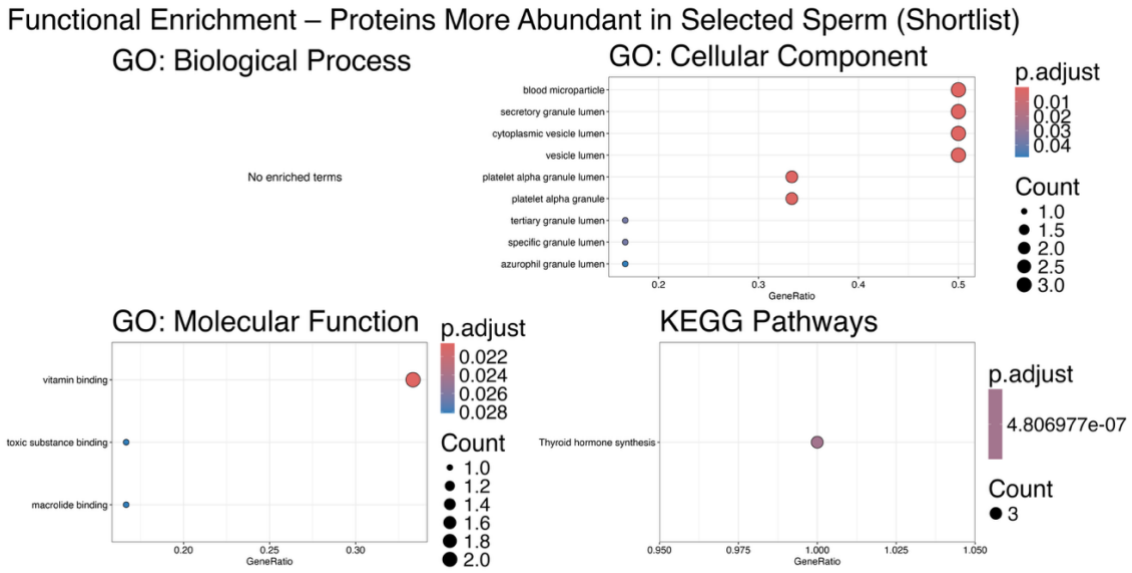


Figure 4-7: Bubble plots displaying the top 10 GO terms enriched for Biological Processes, Cellular Components, Molecular Functions and KEGG Pathways for shortlisted proteins higher in abundance in selected sperm. Gene Ontology (GO) term analysis was performed using BioMart (Haw et al., 2011; Smedley et al., 2015) and Bioconductor (Gentleman et al., 2004) packages through R.

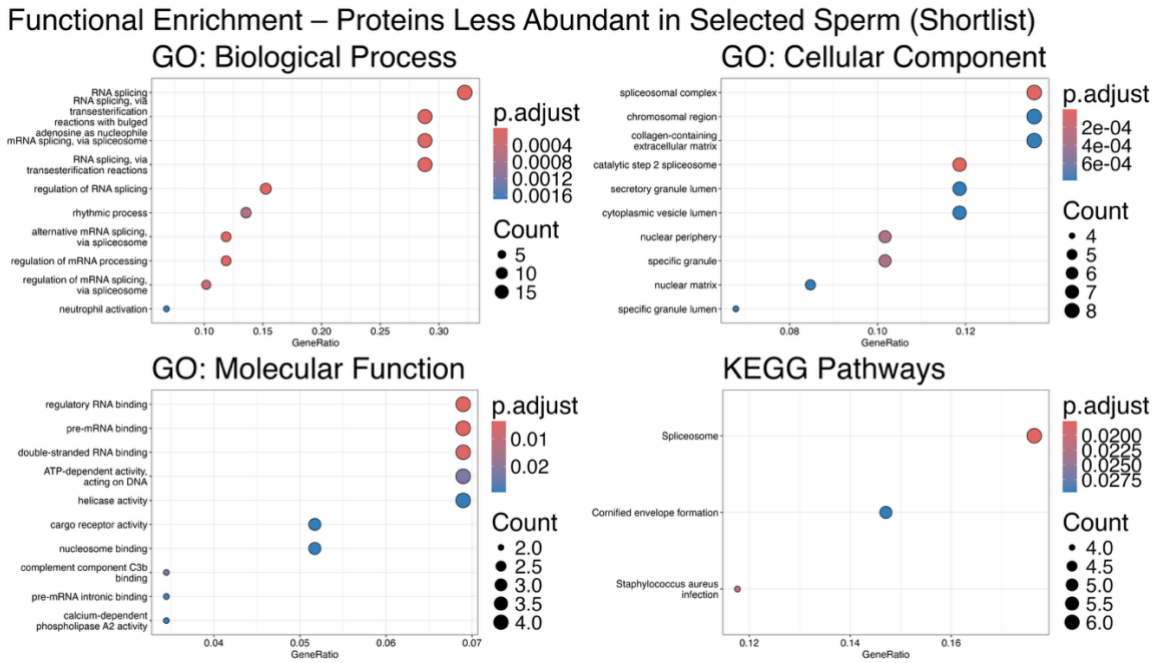


Figure 4-8*:* Bubble plots displaying the top 10 GO terms enriched for Biological Processes, Cellular Components, Molecular Functions and KEGG Pathways for shortlisted proteins lower in abundance in selected sperm. Gene Ontology (GO) term analysis was performed using BioMart (Haw et al., 2011; Smedley et al., 2015) and Bioconductor (Gentleman et al., 2004) packages through R.

Comparison of proteomic data (4,616 confidently identified proteins) to previously generated genomic datasets from methyl cellulose and swim-up assays revealed no overlap between the two (Figure 4-9).

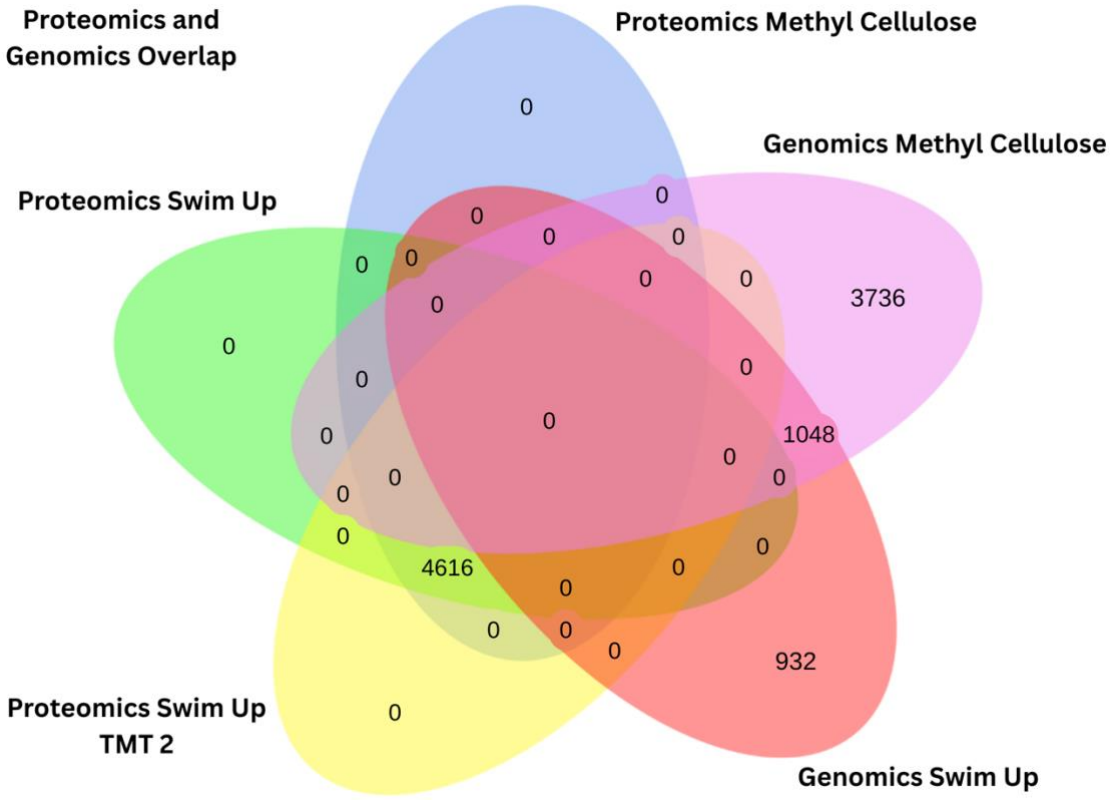


Figure 4-9: Overlap of confidently identified proteins across the three TMT experiments and two genomics datasets *(Marcu, 2024)*. Venn diagram showing the intersection of proteins identified in each of the three TMT-labelled LC-MS/MS. Protein IDs were converted to Entrez IDs which were compared with the Entrez IDs determined by Marcu et al. 2024.

4.5 Discussion

4.5.1 Overview of findings and proteomic depth

We show that selection for sperm subpopulations based on sperm quality from the same ejaculate is associated with consistent proteomic differences. Across four donors, quantitative proteomic analysis identified 1,178 proteins with altered abundance between selected and unselected sperm. The most consistent signal was a depletion of proteins involved in RNA processing and translation in selected sperm, while proteins linked to motility and membrane organisation were enriched. Refinement of the dataset yielded a shortlist of 68 candidate biomarkers, including nine top candidates present across all donors. These findings provide direct molecular evidence that functionally distinct sperm subpopulations within a single

ejaculate differ in their proteomic profiles. Of the 1,178 proteins with consistent differential abundance across all donors, 634 proteins were less abundant and 544 more abundant in selected sperm. Notably, the magnitude of the fold-change ratio and consistency of lower abundance were stronger, with many proteins showing fold-change ratios < 0.2 across multiple donors.

A total of 4,616 proteins were confidently identified from human sperm samples using TMT-labelled quantitative proteomics. While this number is lower than the ~6,000 proteins reported across the full known human mature sperm proteome, it is important to note that the larger figure was compiled through meta-analyses of multiple studies rather than a single dataset (Castillo et al., 2023). In contrast, the current dataset was derived from only four biological replicates yet still captures over 75% of the known human sperm proteome, demonstrating substantial depth and coverage.

4.5.2 Comparison of selection methods

Differences between the methyl cellulose and swim up selection methods influenced both the number and identity of proteins with significantly different abundances between sperm pools. The more mechanically stringent methyl cellulose assay likely selects for sperm with enhanced motility traits, as migration through viscous media requires high progressive motility and is correlated with fertilisation competence in vivo (Quill et al., 2003; Suarez & Pacey, 2006). In contrast, the swim-up assay selects sperm that are both progressively motile in a less viscous medium and able to maintain that motility over extended incubation. Several studies have shown that swim up assays isolate sperm with improved morphology, DNA integrity, and fertilising potential compared to the raw ejaculate (Henkel & Schill, 2003). The requirement for sustained swimming over time means that swim up fractions are enriched for sperm with increased longevity and resilience, traits directly linked to higher fertilisation rates (McDowell et al., 2014).

This difference in selective pressures between the two methods is demonstrated by the differences observed in the GO terms generated for proteins more

abundant in selected sperm, with the swim-up experiments enriched for “cilium motility” and related terms, consistent with previous evidence that sperm flagellar beating and ciliary function are central determinants of fertilising ability (Amaral et al., 2013; Lindemann & Lesich, 2016). By contrast, In the methyl cellulose experiments, enrichment for terms such as “oxygen binding” is consistent with the importance of redox regulation for sperm quality, as oxygen-binding proteins can support mitochondrial respiration while protecting against reactive oxygen species (ROS), which are key modulators of capacitation and fertilisation but detrimental in excess (O’Flaherty & Matsushita-Fournier, 2017). Lipid–protein interactions are also critical for capacitation, acrosome exocytosis, and membrane fusion with the oocyte, with cholesterol efflux and lipid remodelling representing established hallmarks of sperm functional maturation (Gadella & Luna, 2014). Together, these functional enrichments highlight that the two assays isolate sperm populations with distinct molecular signatures: swim-up favouring motility and longevity, and methyl cellulose selecting for resilience in viscous environments and optimal metabolic and membrane properties.

4.5.3 Candidate biomarkers

The most consistently and significantly less abundant protein in selected sperm across all donors and experiments was Olfactomedin-4 (*OLFM4*, Q6UX06). OLFM4 is a glycoprotein that has been found to be implicated in cell adhesion, immune regulation, and apoptotic resistance in the context of the gastrointestinal epithelium and cancer biology (Wei et al., 2024). The presence of OLFM4 in mammalian spermatozoa and epididymal vesicles has been previously reported, suggesting a role in sperm maturation or intercellular signalling within the reproductive tract (Zmudzinska et al., 2022). Its lower abundance in sperm that successfully passed through our selection assays may indicate that elevated levels of OLFM4 are deleterious to sperm function or structural integrity, potentially interfering with motility due to inappropriate cell adhesion. Given its consistent significance across donors and the lowest observed median fold-change ratio, OLFM4 may represent a key marker of poor-quality sperm.

Two of the other most significantly less abundant proteins were phospholipase A2 (*PLA2G2A*, P14555) and Midkine (*MDK*, P21741), which also showed consistent and notable depletion in selected sperm. Phospholipase A2 is a secretory enzyme, known to be present in human seminal plasma, and also known to be closely related to various male reproductive malignancies including penile, prostate and testicular cancer (Dahiya et al., 2023). Midkine is a protein growth factor classically involved in embryonic development, tissue regeneration, and inflammatory signalling. Many studies have focussed on the importance of midkine during fertilisation (Torabi et al., 2017). This makes the lower abundance of midkine in selected sperm surprising although it has also been implicated as a potential prognostic biomarker for embryonal carcinoma (Jono & Ando, 2010). The lower abundance of both phospholipase A2 and midkine in the selected sperm population further supports the previous evidence that sperm that survive selective pressures may relate to increased health benefits in the offspring (Alavioon et al., 2019).

The final two proteins in the top five are Beta-defensin 105 (*DEFB105A*, Q8NG35) and Heterogeneous nuclear ribonucleoprotein K (*HNRNPK*, P61978). Beta-defensin 105 is an antimicrobial peptide that has previously been identified in the mouse epididymis (Yamaguchi et al., 2002), with some members of the beta-defensin protein family believed to be positive markers of human fertility (Solanki et al., 2023), although the exact impact of beta-defensin 105 has not been categorised. Heterogeneous nuclear ribonucleoprotein K has previously been identified in the human sperm nucleus (de Mateo et al., 2011), and mutations in the gene responsible for this protein have previously been identified in men with impaired spermatogenesis (Westerveld et al., 2004). Both heterogeneous nuclear ribonucleoprotein K and beta-defensin 105 have been implicated as having positive effects on male fertility (Solanki et al., 2023; Westerveld et al., 2004) so their conserved lower abundance between selected sperm samples of different donors is puzzling. This trend and the exact effects of these proteins abundances on mature sperm require more investigation to determine what molecular effects are conferred and if they may act as suitable biomarkers of sperm quality.

Among the six proteins (Thyroxine-binding globulin, Afamin, Beta-microseminoprotein, Alpha-1-acid glycoprotein 1, Albumin, and Transthyretin) shortlisted as higher in abundance in selected sperm, four met the additional selection criteria of being detected across all donors, significant in at least four, and with a median fold-change ratio above 2. However, due to variability across individuals, none of these proteins were consistently identified in more than two donors within the initial discovery set. As a result, their utility as biomarker candidates remains uncertain, but they could warrant further investigation in larger cohorts where inter-individual variation can be more robustly accounted for.

Proteins selected for downstream validation by flow cytometry (Chapter 5) were chosen based on their strong and consistent differential expression, predicted or known membrane localisation, and potential relevance to sperm function or fertility. The combination of differential abundance, biological plausibility, and accessibility in intact sperm made these candidates suitable for further study.

4.5.4 Functional enrichment analysis

Functional enrichment analyses revealed two broad and consistent patterns. Firstly, proteins linked to RNA processing were consistently depleted. This aligns with previous work showing that translational and splicing activity are features of immature sperm and are typically absent in fully mature, high-quality spermatozoa (Nixon et al., 2023). The depletion of these proteins in selected fractions therefore supports the interpretation that sperm surviving the assays represent a more functionally mature population.

Secondly, proteins more abundant in selected sperm were enriched for terms related to motility. These included components of the flagellum and cilium, consistent with evidence that flagellar structure and activity are central to sperm motility and fertilisation competence (Lindemann & Lesich, 2016). Other enriched functions included lipid metabolism and membrane-associated protein complexes, processes closely linked to capacitation and the regulation of membrane fluidity (Gadella & Luna, 2014). Enrichment for antioxidant and enzyme inhibitor activities

is also consistent with the requirement to withstand oxidative and proteolytic stress during transit through the female tract, factors well known to compromise fertility when unregulated (Bisht et al., 2017; Cesari et al., 2010).

KEGG pathways reinforced these patterns, linking less abundant proteins to translational machinery and more abundant proteins to lipid metabolism and hormonal regulation. Specific KEGG pathways mentioned such as cholesterol efflux and membrane fluidity have been recognised hallmarks of capacitation (Cross, 1998), while thyroid hormones are known to modulate testicular and epididymal function, with downstream effects on sperm quality (Jasim, 2021).

Taken together, these enrichment results suggest that sperm selection favours cells with molecular profiles optimised for motility, membrane remodelling, and stress resistance, while excluding sperm that retain features of immaturity or dysregulation. This provides further support for the idea that within-ejaculate variation in sperm function is reflected at the proteomic level and can be detected by differential selection assays.

4.5.5 Integration with genomic data

Comparison of the proteomic dataset (4,616 confidently identified proteins) with previously generated genomic datasets from methyl cellulose and swim-up assays revealed no direct overlap between the two (Figure 4-9). This is expected, as genomic and proteomic analyses capture different but complementary layers of haploid selection: variation at the DNA sequence level versus the resulting functional phenotypes at the protein level. Importantly, however, both approaches converge on the same biological signal. In the genomic study, selected sperm showed a reduced representation of alleles associated with human disease, consistent with haploid selection filtering out genetically compromised cells (Marcu et al., 2024). In the present proteomic dataset, several of the top five proteins depleted in selected sperm are annotated as disease biomarkers, suggesting that sperm excluded during selection also carry molecular signatures linked to pathological processes. Together, these independent lines of evidence support the

conclusion that haploid selection reduces the transmission of deleterious or disease-associated variants, with implications both for reproductive success and for the genetic health of offspring.

4.5.6 Experimental design limitations

It should also be noted that due to logistical constraints, the swim-up assays were performed on donor samples obtained in Cape Town, South Africa, while the methyl cellulose assays were carried out on samples collected in Norwich, United Kingdom. Rather than limiting the study, this design was chosen to avoid restricting the dataset to a single age group or ethnic background, thereby capturing a broader range of variation in sperm proteomes. In line with this, each donor was treated as an independent experiment and overlaps in protein changes across donors and assays provide evidence for robust signals and promising biomarker candidates. The observed variation between donors and between assays likely reflects both biological differences among individuals and assay-specific selective pressures. Such variation between donors is well documented, with previous proteomic studies showing extensive inter-individual differences in protein composition and abundance (Amaral, et al., 2014 ; Saraswat et al., 2017). Importantly, the presence of this variation emphasises the need to investigate sperm function at the level of the individual ejaculate, since pooling or averaging across donors could obscure meaningful haploid-level differences.

Another limitation is the current inability to define the sperm cell surface proteome. Although biomarkers should ideally be surface expressed to enable non-destructive selection, distinguishing true surface proteins from intracellular components remains technically challenging. single-cell proteomics of sperm is not yet feasible, and many of the proteins identified to date lack functional annotation or localisation data. Furthermore, functional predictions are often based on somatic data, which may not accurately reflect sperm biology. This uncertainty limits the ability to distinguish surface-expressed candidates from intracellular proteins, highlighting the need for further studies that combine proteomics with imaging or biochemical validation of protein localisation.

4.5.7 Conclusion

Our findings provide proteomic evidence that within-ejaculate sperm selection is associated with systematic molecular differences, identifying candidate biomarkers that distinguish higher-quality sperm from those of lower quality. By showing that sperm excluded during selection are enriched for proteins linked to immaturity, dysregulation, and disease processes, this study demonstrates that haploid selection acts at the protein level to shape sperm quality. These insights move beyond descriptive catalogues of the sperm proteome and establish a functional link between molecular composition and selective fertilisation potential. Future work to validate the localisation and function of these candidates, particularly those expressed at the sperm surface, will be essential for translating these findings into clinical tools. Incorporating proteomic biomarkers into assisted reproduction technologies would provide a non-destructive means of identifying sperm with superior functional capacity and lower genetic risk, improving both fertility treatment outcomes and the long-term health of offspring. More broadly, the integration of genomic, transcriptomic, and proteomic layers will be key to building a comprehensive understanding of haploid selection in humans and its evolutionary and biomedical significance.

5 Validation of Cell Surface Biomarkers for Sperm Longevity and Fitness

5.1 Abstract

Within-ejaculate variation in sperm quality presents both a biological challenge for understanding male fertility, and a clinical opportunity for improved selection in assisted reproductive technologies (ART). In Chapter 4, 68 candidate sperm surface biomarkers were identified via Tandem Mass Tag (TMT)-based quantitative proteomics in sperm selected in two assays selecting for sustained motility and longevity, thereby serving as a functional proxy for sperm quality. To validate these candidates and assess their potential functional relevance, three biomarkers were selected for further investigation: Olfactomedin-4 (OLFM4), junction plakoglobin (JUP/CTNG), and Amine oxidase copper containing 3 (AOC3). These markers were prioritised based on their consistent and statistically significant differential abundance between selected and unselected sperm fractions, predicted surface localisation, and the availability of validated antibodies suitable for flow cytometry in human tissue. Imaging flow cytometry was employed using the BD FACSDiscovery S8 system to assess marker expression and localisation on sperm from 5 donors. Sperm were sorted following incubation for longevity, with the addition of viability dye prior to fixing. This approach enabled single-cell analysis of fluorescence intensity in both long- and short-lived sperm subpopulations. OLFM4 and CTNG were predicted to have lower fluorescence intensity in long-lived sperm, while AOC3 was selected as a contrasting marker predicted to show increased abundance in selected sperm. The aim was to confirm the membrane localisation of these markers and assess their utility in reliably distinguishing sperm subpopulations, contributing to the development of surface-based biomarkers for sperm quality. Both OLFM4 and CTNG were validated as potential biomarkers of sperm fitness with a consistent trend of higher marker fluorescence in short-lived sperm. In contrast, AOC3 was not found to be a suitable biomarker due to the highly variable trend between donor samples. This work provides an essential step toward developing molecularly guided sperm selection strategies for improving ART outcomes.

5.2 Introduction

One of the largest advances in reproductive medicine was the invention of Assisted Reproductive Technologies (ART). The first of these was in vitro fertilisation (IVF), in which multiple sperm are co-incubated with an oocyte in a culture dish, allowing fertilisation to occur outside the body under controlled laboratory conditions (Steptoe & Edwards, 1978). A second milestone came with the development of Intra-Cytoplasmic Sperm Injection (ICSI), in which a single sperm is selected and injected directly into the cytoplasm of the oocyte using micromanipulation techniques (G. Palermo et al., 1992). Both, IVF and ICSI are now routine clinical practice, together accounting for estimates of around 3–6% of all births in high-income countries and more than 10 million births worldwide since the first IVF child was born (Adamson et al., 2025)

Despite their widespread use, ART procedures remain associated with significant challenges. Even with technical improvements, the chance of a live birth per treatment cycle remains below 30% in most age groups, and miscarriage rates are approximately 20–30%, compared with around 10–15% in natural conceptions (Szamatowicz & Szamatowicz, 2023; van Oosterhout et al., 2022). Women undergoing treatment face substantial physical and emotional burdens, including repeated cycles of ovarian stimulation, invasive oocyte retrieval, and the stress of variable outcomes (Szamatowicz & Szamatowicz, 2023). While much of the focus has been on maternal factors, growing evidence suggests that paternal contributions, including sperm DNA fragmentation, chromosomal abnormalities, and epigenetic alterations, can play a role in early embryo loss and adverse pregnancy outcomes (Alavioon et al., 2021; van Oosterhout et al., 2022). These challenges underscore that ART, while transformative, still has substantial room for improvement in order to increase success rates and reduce the physical and emotional burden on patients.

A key concern is that both IVF and, to an even greater extent, ICSI circumvent many of the natural barriers that normally regulate fertilisation. In vivo, sperm must undergo capacitation, migrate through the female reproductive tract, interact with

the zona pellucida, and complete the acrosome reaction before fusing with the oocyte (Kim et al., 2008). Each of these steps functions as a stringent filter that reduces the probability of fertilisation by sperm carrying DNA damage, chromosomal abnormalities, or compromised morphology (Alavioon et al., 2021). By bypassing these checkpoints, ART relaxes natural selection on sperm quality, which may increase the genetic load passed to offspring. Genetic load refers to the accumulation of deleterious mutations or other heritable defects within a population that reduce overall fitness (van Oosterhout et al., 2022). This relaxed selection may partly explain the elevated miscarriage rates observed in ART pregnancies, as well as raise concerns about the long-term consequences of transmitting hidden defects to the next generation.

Recognising that ART bypasses many of the natural filters of fertilisation raises a fundamental question: which characteristics define a sperm that would normally succeed in passing these barriers, and how do such sperm differ from the rest of the ejaculate? Traditional semen analysis focuses on concentration, motility, and morphology (Nixon et al., 2023). While these parameters are useful for diagnosing severe cases of male infertility, they have limited ability to predict fertilisation, embryo development, or live birth outcomes in ART (Villani et al., 2022). Beyond these basic measures, studies have suggested that traits such as sperm longevity (Alavioon et al., 2017), progressive motility (Kumar & Singh, 2021), and intact DNA integrity (Alahmar et al., 2022) provide better indications of reproductive potential. However, methods for separating sperm on the basis of these traits remain technically challenging.

A number of assays have been developed to enrich for sperm with presumed superior fertilisation potential. The most widely used approaches are density gradient centrifugation and swim-up (Amano et al., 2024), both of which select for sperm with greater motility and reduced levels of cellular debris or dead cells. More recent methods, such as microfluidic devices (Doostabadi et al., 2022), attempt to mimic aspects of the female reproductive tract by favouring sperm with intact membranes or more mature functional profiles. However, none of these

approaches consistently predicts treatment outcomes, and their ability to reduce miscarriage or improve live birth rates remains uncertain.

Given the limitations of current selection methods, one major area of research has focused on identifying reliable biomarkers of sperm quality. The aim is to find molecular or cellular features that distinguish sperm most capable of supporting fertilisation and embryo development from the rest of the population. For example, studies of sperm surface biomarkers such as Heat Shock Protein A2 (HSPA2), Parkinsonism-associated protein DJ-1 (DJ-1), and Serum Amyloid P component (SAP), examined how these markers correlated with sperm quality across 114 donors with either normal or abnormal spermiograms (Amaral, Paiva, et al., 2014). However, these markers were assessed at the level of whole ejaculate populations and were primarily explored as diagnostic tools for distinguishing between fertile and infertile men.

Before candidate biomarkers can be advanced for intensive clinical investigation, they must undergo a structured development process that includes analytical validation to assess their reliability, reproducibility, and specificity for the intended application (McShane et al., 2013). One widely used analytical method is immunohistochemistry (IHC), which relies on antibody-based detection of protein targets in fixed tissue samples (Ramos-Vara et al. 2014). This technique typically uses enzyme-linked or chromogenic systems to visualise antigen–antibody complexes, although fluorescent conjugates may also be employed in immunofluorescence-based approaches.

A more recently developed platform that can be applied to biomarker validation is imaging flow cytometry, which combines high-throughput single-cell fluorescence analysis with microscopic imaging (Harte et al., 2024). This approach allows highly targeted detection using a wide range of antibodies, either through premade antibody–fluorophore conjugates or via secondary antibody labelling strategies. Importantly, imaging flow cytometry can be coupled with flow-assisted cell sorting

(FACS), enabling the non-destructive separation of sperm populations based on biomarker expression for downstream analysis.

Flow cytometry has already been established as a powerful platform for biomarker detection in clinical settings. In reproductive biology for example, sperm aneuploidy testing by Fluorescence In Situ Hybridisation (FISH) coupled with flow cytometric analysis has been used to assess chromosomal abnormalities in men with severe infertility, providing diagnostic information that can guide ART decisions (de Lima Rosa et al., 2023). Although not yet widely standardised, such assays demonstrate the feasibility of applying flow-based methods to sperm biomarker detection. Beyond reproduction, flow cytometry is routinely employed in clinical haematology, where immunophenotyping of leukocytes using antibody-labelled surface markers is a cornerstone of diagnosing and monitoring blood cancers such as leukaemia and lymphoma (Brestoff, 2023). These established examples highlight how flow cytometry–based biomarker assays can move from research to clinical application, supporting its potential utility in the development of sperm-specific biomarkers.

Whereas previous biomarker studies have largely aimed to stratify fertility status across individuals (Blaurock et al., 2022), this study focuses on identifying and separating potentially less-fit sperm within a single ejaculate. This shift in emphasis is motivated by evidence that sperm populations are highly heterogeneous, with only a small proportion typically capable of successful fertilisation under natural conditions (van Oosterhout et al., 2022). By targeting this within-ejaculate variation, it may be possible to exclude sperm carrying DNA damage or other hidden defects and thereby enrich for those with the greatest developmental potential (Alavioon et al., 2021). Such an approach has the potential to improve reproductive outcomes, even in cases where infertility has already been diagnosed, by providing a means to optimise sperm selection beyond conventional semen analysis or bulk biomarker assays.

Following the proteomic profiling of intra-ejaculate variation in sperm fitness presented in Chapter 4, and the identification of 68 candidate surface biomarkers, it became necessary to validate the presence, trends, and localisation of these proteins on live spermatozoa. Proteomic data alone cannot establish whether such markers are accessible at the cell surface, nor whether they exhibit sufficient variability within an ejaculate to permit targeted selection. Because of these constraints, three candidate biomarkers were selected from the shortlist generated in Chapter 4. These markers were prioritised based on both their high median abundance ratios and statistically significant p-values in the proteomic dataset, as well as the commercial availability of antibodies validated for use in human tissues.

The first of these biomarkers chosen for further validation, is Olfactomedin-4 (OLFM4), which was found to have statistically significantly lower abundance in the T4/edge selected samples across all measured donors and mass spectrometry analysis (Chapter 4). OLFM4 (Figure 5-1A) is a glycoprotein that has previously been found in neutrophil granules, intestinal crypts and prostate tissue (Kobayashi et al., 2007). This protein has been previously described as a biomarker for disease severity in conditions such as sepsis, viral infections, and malaria (W. Liu et al., 2010). Similarly, OLFM4 has been described to modulate inflammation and tissue repair by inhibiting the antimicrobial and pro-inflammatory functions of neutrophil cationic proteins (Vandenberghe-Dürr et al., 2024). The ability to regulate immune responses and interact with other surface proteins suggests that it may play a role in cellular viability and stress responses and therefore may be relevant to sperm viability and longevity under selection pressures.

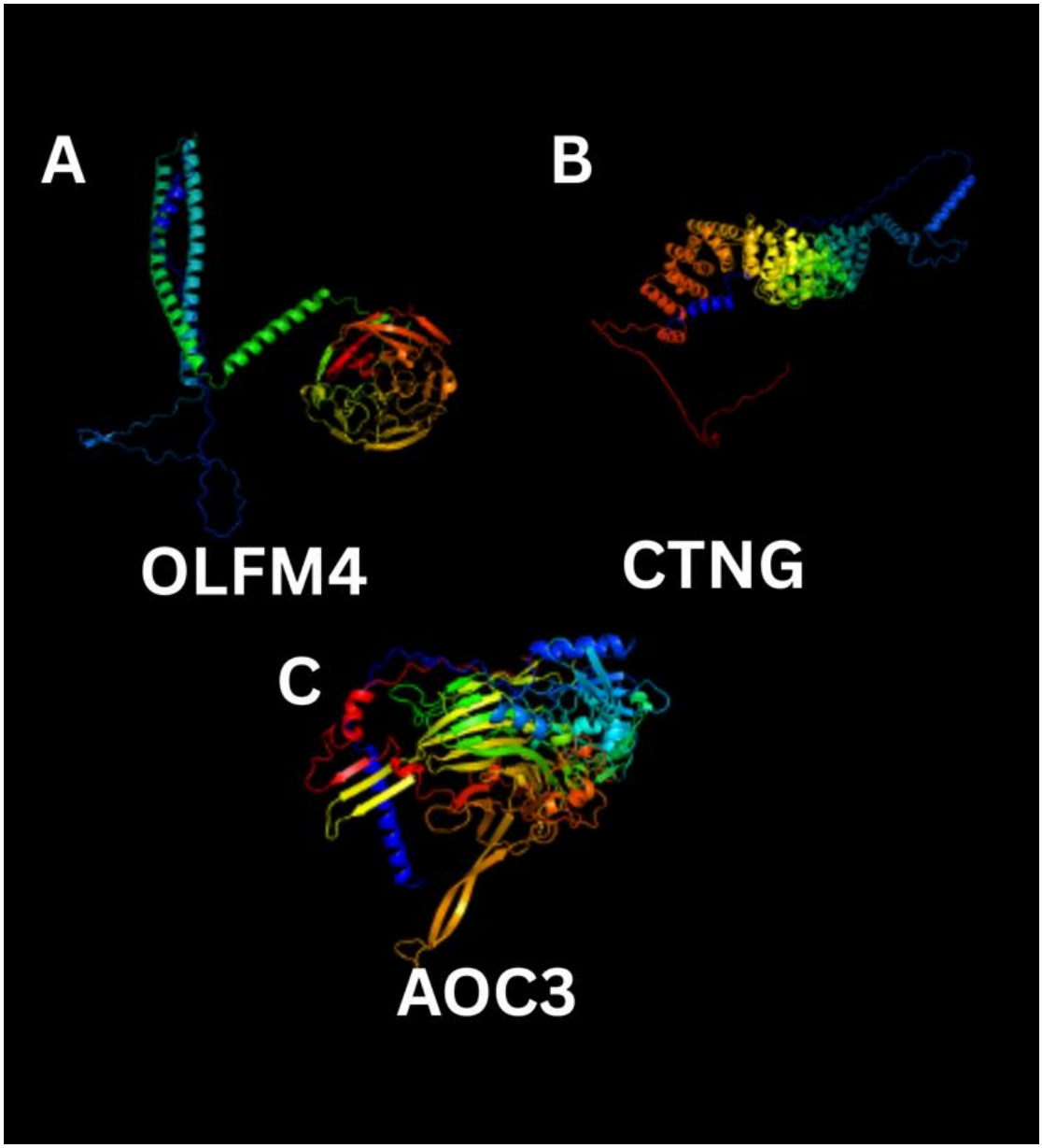


Figure 5-1: Predicted tertiary structures of OLFM4, CTNG, and AOC3. *A–C)* Three-dimensional protein structures visualised in PyMOL using the corresponding Protein Data Bank (PDB) entries retrieved via UniProt. Each structure is colour-coded by protein chain/domain, where each colour represents an individual polypeptide chain or structural domain within the protein. A) OLFM4 (Olfactomedin-4): coloured by chain to illustrate the organisation of the olfactomedin domain relative to the N-terminal region. B) CTNG (Junction plakoglobin / γ-catenin): coloured by chain to highlight the arrangement of its armadillo repeats and domain layout. C) AOC3 (Amine oxidase, copper-containing 3): coloured by chain to visualise the enzyme's dimeric architecture and domain structure.

The second selected candidate biomarker is junction plakoglobin (JUP) also known as γ-catenin (CTNG) (Figure 5-1B). This is a cell adhesion molecule in the armadillo family, homologous to β-catenin, and acts as a critical component of desmosomes and adherens junctions, anchoring cadherins to the cytoskeleton and helping maintain tissue integrity (Ding et al., 2025). Previous research has found that the dysregulation of CTNG is associated with various cancers, and overexpression of CTNG has been linked to increased proliferation, migration, and invasion in oral squamous cell carcinoma (Fang et al., 2020). Whilst the function of CTNG in sperm has not been widely studied, it is likely that the established membrane localisation and role in cell–cell adhesion suggest it may contribute to sperm structural integrity, interactions with Sertoli cells and response to membrane stress or survival.

The final selected candidate protein for validation was Amine Oxidase, Copper Containing 3 (AOC3), also known as vascular adhesion protein-1 (VAP-1). This protein was chosen to investigate potential biomarkers that were found to increase in abundance in T4 and edge selected sperm populations, as a contrast to CTNG and OLFM4. AOC3 (Figure 5-1C) is a membrane-bound surface protein with dual roles of a monoamine oxidase- producing aldehydes, ammonia, and hydrogen peroxide from primary amines, and as an adhesion molecule where it facilitates leukocyte binding and transmigration at inflamed endothelial sites (Boyer et al., 2021). AOC3 has been found to be highly expressed on vascular endothelium, myofibroblasts, and inflammatory tissues, and has been identified as a biomarker for myofibroblasts (Hsia et al., 2016). Whilst, as with OLFM4 and CTNG, AOC3 has not previously been studied in sperm, its consistent membrane localisation and roles in redox biology and cellular adhesion suggest a potential contribution to sperm membrane integrity or oxidative stress response.

To validate these candidates, the BD FACSDiscovery S8 spectral imaging flow cytometry system was employed to examine their surface expression on live spermatozoa. Each marker was assessed using separate antibody staining protocols to ensure specificity, and all samples were processed without cell

permeabilization to maintain membrane integrity and confirm surface localisation. This strategy enabled single-cell resolution of marker expression on both live and dead spermatozoa, allowing direct comparison of fluorescence intensity across sperm longevity subpopulations and supporting the identification of consistent, surface-accessible biomarkers.

Together, OLFM4, CTNG, and AOC3 represent three surface proteins with distinct biological functions and contrasting expression patterns in the proteomic dataset. Their analysis in this chapter provides both validation of proteomic findings and a proof-of-concept for applying spectral imaging flow cytometry to biomarker detection in sperm. This approach represents a key step toward developing clinically relevant tools for sperm selection in ART.

5.3 Methods

5.3.1 Biomarker candidate selection

Based on the 68 shortlisted candidate cell surface biomarkers identified in Chapter 4, a refined selection of OLFM4, CTNG, and AOC3 was chosen for validation. Selection criteria included: (i) predicted surface availability to enable antibody access, (ii) statistical significance and consistent trends in the proteomic dataset, and (iii) the commercial availability of validated antibodies suitable for flow cytometry. Fluorophore-conjugated antibodies were selected where possible, and fluorophore choice together with panel design was optimised using the IntelliPanel™ tool available through FluoroFinder (FluoroFinder, Boulder, CO, USA).

5.3.2 Biomarker titration and experimental design

Fluorophores were selected to minimise spectral overlap with the eFluor™ 520 fixable viability dye (eBioscience, Invitrogen). As the surface abundance of the candidate markers had not been previously quantified, the brightest available fluorophore conjugates were chosen to maximise detection sensitivity. Due to time constraints, a fixation-based method was employed.

Antibody titration was performed for each candidate marker (OLFM4–PE, CTNG–mFluor Violet 450, AOC3–AF594) using both sperm samples and UltraComp eBeads (Thermo Fisher). Frozen sperm samples (T0/mid and T4/edge fractions; 16 × 10^6 cells per fraction) were thawed, counted, and aliquoted at 1 × 10^6 cells in 100 µL of flow staining buffer per tube. For each antibody, three concentrations were tested (5 µL, 7.5 µL, 10 µL), in both T0 and T4 samples, with fully unstained and Fc-blocked controls included. Parallel bead controls were stained under identical conditions to assess non-specific binding.

Samples were incubated at room temperature for 30 min in the dark with gentle vortexing, washed in flow buffer, pelleted at 1,300 rpm for 5 min, and resuspended in 200 µL buffer for storage at 4 °C until acquisition. Titration outcomes were evaluated by assessing fluorescence intensity and background in both sperm and bead controls, with optimal antibody concentrations determined as those providing maximal signal-to-noise ratio without evidence of nonspecific staining.

5.3.3 Sample collection and preparation

Samples were collected from five self-reported healthy donors at the University of East Anglia, UK, under ethics permit no. ETH2324-0997 and giving full consent. Following collection, samples were warmed to 37 °C and measurements of volume and pH were taken. Sperm concentration, motility and velocity parameters were assessed using Computer Assisted Sperm Analysis (CASA) with the motility module of Integrated Semen Analysis System (ISAS) v1 (Poiser, Spain), and the data was captured with an ISAS 782M camera connected to a UB203i microscope with a 10x phase contrast objective and a pre-heated stage. Preheated (37°C) disposable 75 mm x 25 mm glass chamber slides with 20-micron depth chambers (Cytonix, USA) were loaded with 2µl of sample and at least 200 sperm were analysed from at least two fields for one second at 50 frames per second (f/s). Kinematics parameters recorded for each sample included: curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN) = (VSL/ VCL)*100, straightness (STR) = (VSL/ VAP)*100, wobble (WOB) = (VAP/

VCL)*100, amplitude of lateral head displacement (ALH) and beat cross frequency (BCF).

Samples were then aliquoted to approximately 1.5 million cells in 1 mL of human tubal fluid (HTF) medium. Aliquots were incubated for 4 hours at 37 °C with 5% CO_2 prior to antibody staining. Following incubation, sperm samples were reassessed for motility and cell number using CASA, then immediately stained with eFluor™ 520 fixable viability dye (eBioscience, Invitrogen) according to the manufacturer's protocol. After viability staining, cells were fixed in 4% paraformaldehyde (PFA) at 4 °C.

Fixed cells were then blocked using Human Fc Receptor Blocking Solution (eBioscience, Invitrogen) to minimise non-specific antibody binding. Fluorophore-conjugated primary antibodies were subsequently added and incubated under standard conditions. The following antibodies were used: anti-OLFM4 clone 12, conjugated to R-phycoerythrin (PE), at a 1:10 dilution (Novus Biologicals, NBP3-06415PE); anti-CTNG (CTNG/2155R), conjugated to mFluor Violet 450 SE, at 1:10 dilution (Novus Biologicals, NBP3-08485MFV450); and anti-AOC3 (VAP1/AOC3 - TK8-14), conjugated to Alexa Fluor 594, at 1:10 dilution (Bio-Techne, NBP2-81043AF594.). Throughout staining, cells underwent minimal washing steps to preserve cell numbers and were stored overnight at 4 °C prior to flow cytometry analysis.

5.3.4 Flow cytometry

Samples were analysed using the BD FACSDiscover™ S8 Cell Sorter (BD Biosciences), operated in fully spectral mode. No cell sorting was performed. Imaging and fluorescence data were acquired simultaneously for each sample with fixable viability dye (eFluor™ 520) detected using imaging laser B1 to allow for imaging. Events were gated to isolate the sperm population (P1), based on forward and side scatter properties (Figure 5-2). All subsequent data acquisition and analysis were restricted to this P1 population. For each antibody-stained

sample, between 5,000 and 50,000 individual events within the P1 gate were recorded, depending on sample quality and cell availability.

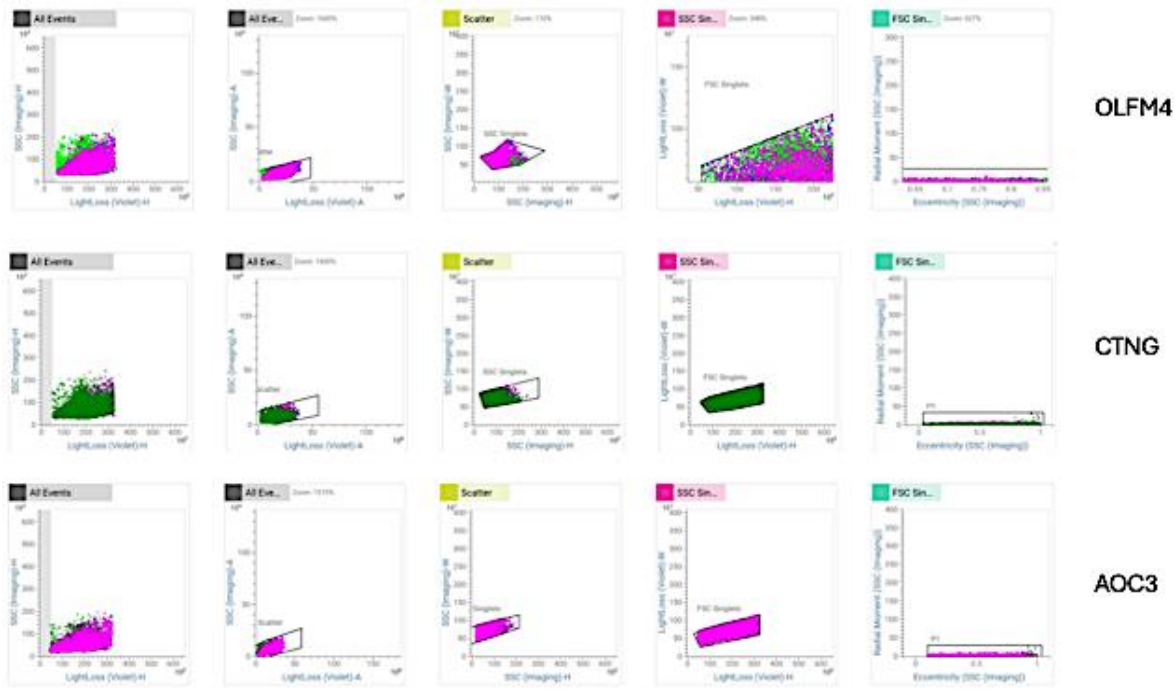


Figure 5-1: Gating strategy used for imaging flow cytometry acquisition. Graphs generated using BD FACSChorus software illustrating the gating strategy used during sample acquisition on the BD FACSDiscovery S8 imaging flow cytometer. Gating was performed to identify a P1 population of single sperm cells based on forward and side scatter properties, and to exclude debris and aggregates. Gates were validated using the images generated on the S8.

5.3.5 Data analysis and statistical modelling

Spectral unmixing was performed using BD FACSChorus™ software (BD Biosciences), based on single-stained controls (fluorescence minus one) for each fluorophore-conjugated antibody and the fixable viability dye. An unstained sperm sample was included in experiment set to control for cellular autofluorescence during unmixing. Compensation and unmixing matrices were applied prior to further analysis.

Flow cytometry image files were merged with the primary fluorescence data using BD CellView™ Image Extractor. The resulting datasets were processed in R

v4.4.3 (R Core Team, 2017). Data were normalised using the CytoNorm package (Van Gassen et al., 2020), and quality control was performed using PeacoQC (Emmaneel et al., 2022) to identify and remove anomalous events. Gated and cleaned data were subsequently analysed in FlowJo v 10.10.0 (BD Life Sciences – FlowJo, LLC, Ashland, OR, USA. https://www.flowjo.com), with all events restricted to the P1 sperm population (see Figure 5-2 for gating strategy).

Statistical modelling of marker expression in live and dead cell populations was conducted in R v4.4.3 (R Core Team, 2017), with residual diagnostics simulated using the DHARMa package (Hartig et al., 2024) to evaluate model fit and distribution assumptions..

5.4 Results

5.4.1 OLFM4

The potential biomarker identified as both consistently and significantly reduced in higher fitness sperm, and likely to be expressed on the cell surface, was OLFM4**.** Five samples were stained with both eFluor 520 (fixable viability dye) and an anti-OLFM4 antibody conjugated to the fluorophore PE. During data acquisition, doublets and aggregates were excluded through gating, ensuring that only single-cell events within the P1 population were analysed (Figure 5-2).

Following data acquisition, gating was initially applied using one representative sample (GD219) to separate live and dead cells. The eFluor 520 signal enabled clear resolution between these populations. A distinct band of cells located between the live and dead gates was observed; these likely represent apoptotic cells at the time of fixation, exhibiting intermediate staining intensity.

To define OLFM4 marker positivity, a heatmap colour scale was applied to the OLFM4 signal, and a fluorescence intensity threshold of 10^5 was selected to distinguish OLFM4-positive cells. Cells with a fluorescence intensity above this

value were classified as OLFM4 positive (Figure 5-3). While this cut-off was not formally validated, it allowed for clear discrimination of marker-positive cells within the observed fluorescence range. Across all donors, approximately 40–50% of spermatozoa were classified as dead, with the remaining 30–55% viable at the time of analysis. OLFM4 staining was detected in both live and dead populations, though the proportion of positive cells varied between donors (Table 5-1). Percentages do not sum to 100% because a small proportion of events could not be confidently classified as live or dead due to ambiguous or low-intensity fluorescence signals and were therefore excluded from the summary. In most samples, 10–25% of dead sperm and 7–20% of live sperm were OLFM4-positive, with GD411 showing the highest proportion of live OLFM4+ cells (22.5%) and GD413 the lowest (6.6%).

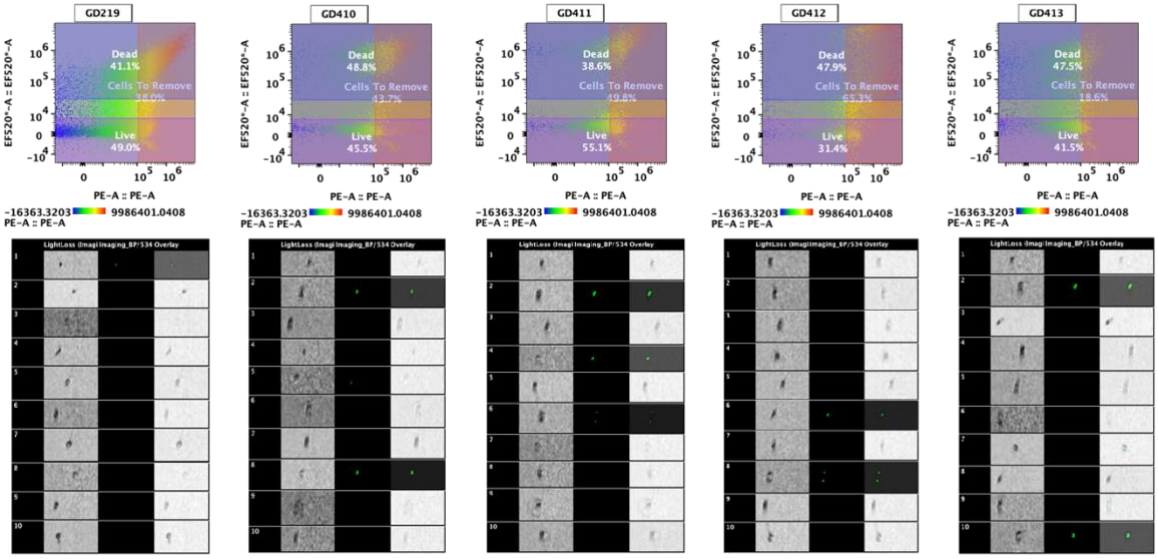


Figure 5-2: OLFM4 staining results across donors. Biexponential flow cytometry plots displaying OLFM4 staining on spermatozoa from each donor, with eFluor 520 viability staining on the y-axis and OLFM4-PE fluorescence on the x-axis. Each plot represents a single donor sample. The first ten cell images from each sample, captured using the BD FACSDiscovery S8 imaging system, are shown below the corresponding plot to illustrate representative staining patterns and cell morphology. Gating was applied to distinguish live and dead sperm cells, and to assess OLFM4 surface expression under non-permeabilised conditions. Three gating populations, live cells, dead cells, and OLFM4-positive cells, were applied for all samples to support standardised comparison across donors.

Table 5-1: Proportion of cells within live/dead gates and above the threshold of 10^5 (OLFM4 Positive). Flow-cytometry–derived cell counts and percentages for each OLFM4-stained sample. “Total Dead” and “Total Live” correspond to events falling within the viability gates; cells that fell between gates and could not be classified were therefore excluded from the dataset. “Dead + Positive” and “Live + Positive” represent the subsets within each viability gate that were OLFM4-positive. Percentages are shown relative to P1 (all confirmed singlet sperm cells). “Total cell count” reflects all recorded events within P1.

Sample	Total Dead	Total Dead (%P1)	Dead + Positive	Dead + Positive (% P1)	Total Live	Total Live (% P1)	Live + Positive	Live + Positive (% P1)	Total cell count
OLFM4 GD219	18733	41.1	11124	24.4	22374	49	5211	11.4	45630
OLFM4 GD410	4881	48.8	2931	29.3	4548	45.5	1134	11.3	10000
OLFM4 GD411	3860	38.6	2434	24.3	5507	55.1	2253	22.5	10000
OLFM4 GD412	4794	47.9	4123	41.2	3139	31.4	1354	13.5	10000
OLFM4 GD413	4752	47.5	1064	10.6	4151	41.5	660	6.6	10000

Across all five samples, the proportion of OLFM4-positive cells was significantly higher in the dead population compared to the live population. This was tested using a binomial generalised linear mixed model with the proportion of OLFM4-positive cells as the response variable, live/dead status as a fixed effect, and sample ID as a random intercept (Table 5-2 ; z = -67.67, estimate = -0.888, $p < 0.01$). Model diagnostics performed using the DHARMa package (Hartig et al., 2024) indicated no evidence of overdispersion, zero-inflation, or model misspecification.

Table 5-2: Results from a generalised linear mixed-effects model (GLMM) comparing OLFM4 expression on live versus dead sperm cells.

Dependent variable:	Marker Positive (Proportion)
Intercept (Dead Cells)	-1.098
z = -5.033	***p < 0.01***
Live Cells vs Dead	-0.888
z = -67.674	***p < 0.01***
Observations	10
Log Likelihood	-549.810
Akaike Inf. Crit.	1,105.620
Bayesian Inf. Crit.	1,106.528
Note:	**p < 0.1; **p < 0.05; **p < 0.01*

These findings further support the proteomic results presented in Chapter 4, where OLFM4 was found to be significantly more abundant in lower-fitness sperm. Taken together, this strengthens the case for OLFM4 as a potential negative biomarker of sperm quality.

5.4.2 CTNG

The second candidate biomarker selected for validation was CTNG**.** The same five donor samples were stained with eFluor™ 520 and an anti-CTNG antibody conjugated to mFluor Violet 450. Doublets and aggregates were similarly excluded during acquisition, and only single-cell events within the P1 sperm population were retained for analysis (Figure 5-4). Following the same method as was applied to the OLFM4 stained cells, gating to distinguish live and dead cells was initially defined using sample GD219 as a template with the eFluor 520 signal again providing clear separation. As with OLFM4, a distinct population of apoptotic cells was observed between the live and dead gates.

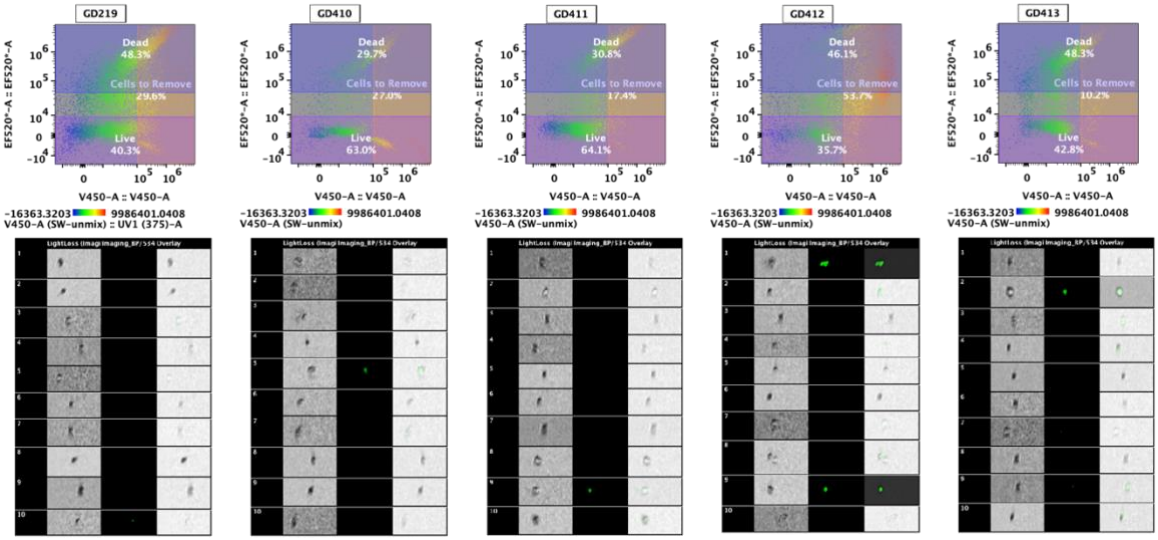


Figure 5-3: CTNG staining results across donors. Biexponential flow cytometry plots displaying CTNG staining on spermatozoa from each donor, with eFluor 520 viability staining on the y-axis and CTNG-V450 fluorescence on the x-axis. Each plot represents a single donor sample. The first ten cell images from each sample, captured using the BD FACSDiscovery S8 imaging system, are shown below the corresponding plot to illustrate representative staining patterns and cell morphology. Gating was applied to distinguish live and dead sperm cells, and to assess CTNG surface expression under non-permeabilised conditions. Three gating populations, live cells, dead cells, and CTNG-positive cells, were applied for all samples to support standardised comparison across donors.

A fluorescence intensity threshold of 10^5 was selected for CTNG positivity, based on visual inspection of the heatmap colour scale. Cells with intensities above this value were classified as CTNG-positive (Figure 5-4). This threshold was not formally validated but allowed for clear visual discrimination across the dataset. Across donors, 30–50% of spermatozoa were non-viable at the time of staining, with the remaining 35–65% classified as live (Table 5-3). CTNG staining was predominantly detected in dead cells, with 6–38% of non-viable sperm positive for CTNG across samples. In contrast, the live fraction generally exhibited very low CTNG positivity (<5%), except for donor GD410, which displayed a notably higher proportion of live CTNG+ sperm (13.4%). Overall, CTNG expression appeared largely restricted to dead sperm populations.

Table 5-3: Proportion of cells within live/dead gates and above the threshold of 10^5 (CTNG Positive). Flow-cytometry–derived cell counts and percentages for each CTNG-stained sample. “Total Dead” and “Total Live” correspond to events falling within the viability gates; cells that fell between gates and could not be classified were therefore excluded from the dataset. “Dead + Positive” and “Live + Positive” represent the subsets within each viability gate that were CTNG- positive. Percentages are shown relative to P1 (all confirmed singlet sperm cells). “Total cell count” reflects all recorded events within P1.

Sample	Total Dead	Total Dead (%P1)	Dead + Positive	Dead + Positive (% P1)	Total Live	Total Live (% P1)	Live + Positive	Live + Positive (% P1)	Total cell count
CTNG GD219	12149	48.3	5858	23.3	10146	40.3	1111	4.41	25165
CTNG GD410	1703	29.7	704	12.3	3607	63	767	13.4	5729
CTNG GD411	3080	30.8	1441	14.4	6410	64.1	217	2.17	10000
CTNG GD412	4613	46.1	3815	38.1	3571	35.7	624	6.24	10000
CTNG GD413	4831	48.3	662	6.62	4282	42.8	254	2.54	10000

Across all five samples, the proportion of CTNG-positive cells was higher in the dead population compared to the live population, as assessed by a binomial generalised linear mixed model with live/dead status as a fixed effect and sample ID as a random intercept (Table 5-4; z= -76.786, estimate = -1.657, $p < 0.01$). The

effect was significant, with a $p < 0.001$. DHARMa diagnostic plots indicated a well-fitted model with no violations of model assumptions.

Table 5-4: Generalised linear model (GLMM) results comparing CTNG expression on live versus dead sperm cells.

Dependent variable:	Marker Positive (Proportion)
Intercept (Dead Cells)	-1.505
z = -5.339	***p < 0.01***
Live Cells vs Dead	-1.657
z = -76.786	***p < 0.01***
Observations	10
Log Likelihood	-679.799
Akaike Inf. Crit.	1,365.598
Bayesian Inf. Crit.	1,366.505
Note:	**p < 0.1*; ***p < 0.05*; ***p < 0.01*

These findings are also in line with the proteomic data from Chapter 4, where CTNG was more abundant in lower-fitness sperm. Therefore, CTNG can be considered a strong second candidate to act as a biomarker for lower sperm fitness within an ejaculate.

5.4.3 AOC3

The third candidate biomarker selected for validation was amine oxidase copper-containing 3 (AOC3), which was identified in Chapter 4 as being significantly more abundant in higher-fitness sperm and predicted to localise to the cell surface. This candidate was selected due to its potential to function with inverse action in selection compared to OLFM4 and CTNG. The same five donor samples were stained with eFluor™ 520 and an anti-AOC3 antibody conjugated to Alexa Fluor 594 and gated for single-cell events within the P1 sperm population (Figure 5-2).

Live and dead cells were gated using the same approach as described for OLFM4 and CTNG, with eFluor 520 providing clear separation. A population of cells with intermediate staining was again observed and excluded from further analysis.

A fluorescence intensity threshold of around $10^{4.5}$ was selected based on visual inspection of the heatmap colour scale to distinguish AOC3-positive cells (Figure 5-5). Across all donors, 55–75% of spermatozoa were non-viable, with 23–33% classified as live (Table 5-5). AOC3 staining was detected in both live and dead populations, though the proportion of positive cells varied between donors. Among dead sperm, AOC3 positivity ranged from 1–28%, whereas in live cells, 2–13% were AOC3-positive. Donor GD219 displayed the highest proportion of live AOC3+ sperm (13%), while GD411 exhibited minimal staining in both live and dead fractions (<2%).

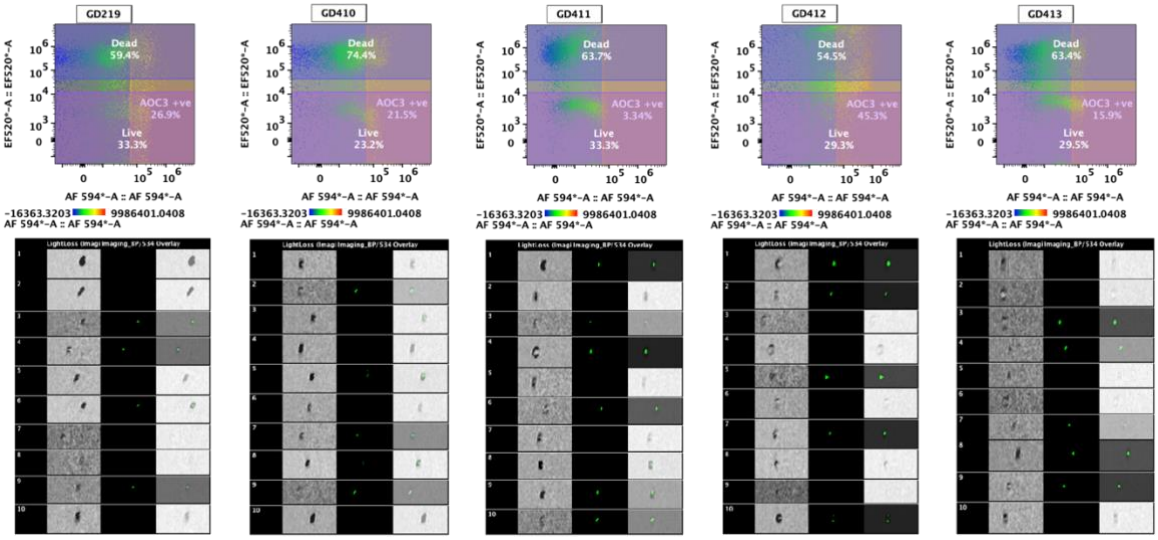


Figure 5-4: AOC3 staining results across donors. Biexponential flow cytometry plots displaying AOC3 staining on spermatozoa from each donor, with eFluor 520 viability staining on the y-axis and AOC3-AF594 fluorescence on the x-axis. Each plot represents a single donor sample. The first ten cell images from each sample, captured using the BD FACSDiscovery S8 imaging system, are shown below the corresponding plot to illustrate representative staining patterns and cell morphology. Gating was applied to distinguish live and dead sperm cells, and to assess AOC3 surface expression under non-permeabilised conditions.

Table 5-5: Proportion of cells within live/dead gates and above the threshold of 10^5 (AOC3 Positive). Flow-cytometry–derived cell counts and percentages for each AOC3-stained sample. “Total Dead” and “Total Live” correspond to events falling within the viability gates; cells that fell between gates and could not be classified were therefore excluded from the dataset. “Dead + Positive” and “Live + Positive” represent the subsets within each viability gate that were AOC3-positive. Percentages are shown relative to P1 (all confirmed singlet sperm cells). “Total cell count” reflects all recorded events within P1.

Sample	Total Dead	Total Dead (%P1)	Dead + Positive	Dead + Positive (% P1)	Total Live	Total Live (% P1)	Live + Positive	Live + Positive (% P1)	Total cells
AOC3 GD219	5944	59.4	1222	12.2	3334	33.3	1299	13	10000
AOC3 GD410	7442	74.4	1159	11.6	2322	23.2	947	9.47	10000
AOC3 GD411	6366	63.7	149	1.49	3326	33.3	176	1.76	10000
AOC3 GD412	5451	54.5	2780	27.8	2934	29.3	1120	11.2	10000
AOC3 GD413	6336	63.4	703	7.03	2953	29.5	820	8.2	10000

Whilst statistical modelling using a **binomial generalised linear mixed model** indicated a significant effect of live/dead status on AOC3 expression (Table 5-*6; z* =-17.040, *estimate = -0.364, p* < 0.01), with a higher proportion of AOC3-positive cells observed in the dead population, this trend was not consistent across all five samples. In some individuals, the proportion of AOC3-positive cells was nearly identical between live and dead populations, while in others the trend was opposite to that predicted based on the proteomic data from Chapter 4.

Table 5-6: Results from a generalised linear mixed effects model (GLMM) comparing AOC3 expression on live versus dead sperm cells.

Dependent variable:	Marker Positive (Proportion)
Intercept (Dead Cells)	-2.248
z = -5.505	***p < 0.01***
Live Cells vs Dead	-0.364
z = -17.040	***p < 0.01***
Observations	10
Log Likelihood	-379.681
Akaike Inf. Crit.	765.363
Bayesian Inf. Crit.	766.270
Note:	**p < 0.1; **p < 0.05*; ***p < 0.01*

These findings suggest that while AOC3 may exhibit differential expression in bulk proteomic datasets, its surface expression in individual sperm cells does not follow a consistent live/dead pattern. As such, AOC3 does not appear to be a reliable marker of sperm viability or quality in this context and may not be suitable for use as a single-cell biomarker.

5.5 Discussion

One of the fundamental requirements for biomarker validation is the determination of cellular localisation. For all three markers tested here, the expected binding site for the antibodies was the sperm cell surface. The presence of these biomarkers on the cell surface was confirmed, as cells were not permeabilised prior to staining and the expected fluorescence signals were observed. This was further supported by the fluorescence minus one (FMO) controls, which were used to ensure the correct spectral unmixing had been applied and indicated that the detected signals were unlikely to result from non-specific binding. All three markers were also detected on both live and dead cells. This suggests that cell structure was well preserved following fixation, and that antibody binding was maintained across both populations. Together, these results support the conclusion that the staining patterns observed accurately reflect true marker localisation on the sperm surface.

Whilst the results for both OLFM4 and CTNG matched the expected surface binding patterns predicted by the proteomics data, the pattern observed for AOC3 did not align with these predictions. The proteomics results for AOC3 had already indicated variability between donors, both in abundance trends between T0 and T4 and in whether AOC3 was detectable at all. This suggests that AOC3 abundance, particularly at the cell surface, varies substantially among individuals. The biomarker validation results further support this, demonstrating high variability in AOC3 expression across the five donors tested. Such inconsistency indicates that AOC3 is not a suitable biomarker for sperm fitness assessment.

In contrast, despite natural donor-to-donor variation, OLFM4 and CTNG showed consistent surface binding patterns across all donors. This stability supports their potential as reliable biomarkers of sperm fitness, as the expected biological variability among individuals did not disrupt the overall expression patterns of these markers.

Whilst this study represents an early-stage investigation, it provides an important step toward bridging the gap between descriptive sperm proteomics and functional sperm selection. Previous biomarker studies in reproductive biology have primarily sought to differentiate between fertile and infertile individuals (Amaral, Paiva, et al., 2014; Castillo et al., 2019), identifying proteins such as AKAP4, SEMG1, and HSPA2 as indicators of general sperm quality (Amaral, Paiva, et al., 2014). However, these approaches treat the ejaculate as a homogenous population. In contrast, the present work targets within-ejaculate variation, building on evidence that sperm populations exhibit functional heterogeneity even within a single sample (Alavioon et al., 2021; van Oosterhout et al., 2022). By identifying and validating biomarkers linked to sperm longevity and structural integrity, this study shifts focus from diagnostic classification toward the selective enrichment of viable sperm for assisted conception.

The potential applications of such biomarkers lie primarily in sperm selection for ART. Current ART procedures such as IVF and ICSI rely on relatively crude selection strategies, swim-up, density gradient centrifugation, or simple

morphological assessment, that do not account for molecular indicators of sperm competence (Nixon et al., 2023). Biomarker-guided selection could theoretically enable the exclusion of sperm carrying DNA damage, oxidative stress markers, or defective membrane proteins before fertilisation occurs, thereby reducing miscarriage risk and improving embryo quality (van Oosterhout et al., 2022). However, these methods have not translated into routine clinical use due to variable specificity and technical demands. The present validation of OLFM4 and CTNG adds to this growing body of evidence suggesting that molecular-level selection is feasible when coupled to modern imaging and sorting technologies. However, the routine use of FACS or spectral flow cytometry in a clinical setting remains limited by cost, infrastructure, and the technical complexity of sample preparation and instrument operation. In addition, regulatory constraints around the manipulation of human gametes currently restrict the use of such techniques to research contexts rather than clinical fertilisation procedures.

In the longer term, the most viable translational pathway may involve adapting these validated biomarkers for use with non-cytometric selection platforms. Antibodies or ligands targeting surface markers such as OLFM4 or CTNG could be immobilised onto microfluidic filters, magnetic bead systems, or size-exclusion matrices to selectively capture or enrich viable sperm expressing the desired molecular profile. Alternative approaches using biotinylated antibodies and streptavidin-coated columns have already been demonstrated for cell-surface sample separation in other contexts such as separating metastatic tumour cells from other cancer cells (Karhemo et al., 2012), suggesting that similar principles could be applied to sperm. Such antibody-based filtration systems could bridge the gap between laboratory-scale validation and clinically deployable sperm selection tools, offering a practical route for translation into ART workflows.

Before clinical translation can be considered, several limitations must be addressed. Firstly, although OLFM4 and CTNG have been validated as sperm surface markers and consistent trends have been observed across two analytical platforms, the sample size remains limited to nine donors. Future studies involving

a larger and more diverse donor population, including individuals with varied fertility outcomes, will be essential. Secondly, this study employed fixed sperm, which may potentially alter protein architecture; given that live sperm are required for fertilisation in ART, replication of these findings using live, non-fixed sperm is critical. Co-localisation studies would also be beneficial to determine the spatial distribution of these markers relative to each other and to key fertilisation proteins such as IZUMO1, to understand any potential for antibodies binding these markers to interfere with fertilisation. Finally, prior to clinical application, it would be necessary to assess both the fertilisation and developmental competence of sperm selected by these biomarkers, to ensure no detrimental effects and to verify whether "higher fitness" sperm confer tangible benefits to offspring health.

Another limitation on the validation of the candidate biomarkers identified in Chapter 4 was the availability of antibodies to the identified proteins. As flow cytometry has traditionally been applied primarily to immunological research, non-immunologically relevant antibodies are less common. Whilst it is possible to create new antibodies if there is a lack of commercially available options, the time and expense required to both produce these and validate that they are binding the intended target often renders this approach impractical. In future, recombinant antibody libraries and phage-display technologies may offer a route to rapidly generate high-specificity reagents for sperm surface proteins. Integration of antibody-based sorting with microfluidic sperm handling devices may also provide a clinically scalable path forward, allowing for biomarker-based enrichment without high-speed sorting of live gametes.

In conclusion, this study validates OLFM4 and CTNG as sperm surface biomarkers that are enriched in sperm cells failing to survive longevity selection, as initially predicted by TMT mass spectrometry. In contrast, AOC3 was found to be an unsuitable biomarker due to its inconsistency across donors and with proteomics predictions. These findings provide a promising foundation for the development of novel sperm selection strategies in ART; however, substantial further research is required before clinical implementation can be realised.

6 General Discussion

Sexual reproduction is widespread yet inefficient because it halves genetic contribution per parent, requires costly mate search and mating behaviours, and entails the production and maintenance of specialised gametes and reproductive tissues (Gibson et al., 2017; Lively & Morran, 2014). A defining feature of many sexually reproducing eukaryotes is a brief haploid phase embedded within an otherwise diploid life cycle, creating the potential for selection to act directly on phenotypes expressed by haploid genotypes before fertilisation (Immler, 2019; Mable & Otto, 1998). This process, termed haploid selection, was long considered minor in animals because gametes were thought to be largely transcriptionally silent (Joseph & Kirkpatrick, 2004), gene products were believed to be equalised through intercellular bridges (Dym & Fawcett, 1971; Greenbaum et al., 2011; Weber & Russell, 1987), and in some taxa fertilisation can occur even with anucleate sperm (Lindsley & Grell, 1969), all of which were thought to limit haploid individuality and opportunity for selection (Caldwell & Handel, 1991; Joseph & Kirkpatrick, 2004).

Recent work has challenged this view by demonstrating post-meiotic gene expression and incomplete sharing among spermatids, measurable genotype–phenotype associations within ejaculates, and offspring consequences of selecting specific sperm phenotypes, establishing haploid selection as a plausible and consequential evolutionary force (Alavioon et al., 2017, 2019; Bhutani et al., 2021). In humans, within-ejaculate genomic shifts consistent with haploid selection have been reported (Marcu et al., 2024), directly precipitating the questions addressed in this thesis. Building on these findings, recent reviews argue that recognising haploid-stage filtering has implications for both evolutionary theory and clinical practice, including assisted reproductive technologies (ART) (Alavioon et al., 2021; van Oosterhout et al., 2022).

Against this backdrop, the overarching goal of the thesis was to discover sperm biomarkers with clinical potential to separate higher-fitness from lower-fitness cells

within an ejaculate using approaches that are realistic for translation into ART workflows. This goal was developed into four connected research aims:

Aim 1. Determine how dynamically the testicular proteome responds to a defined selective pressure via environmental variation, and whether such perturbations leave molecular “legacy” signatures with potential functional relevance for sperm production. Motivated by prior evidence that environmental challenges can have lasting effects on sperm and offspring (Irish et al., 2024), I used prolonged mild thermal stress in zebrafish to quantify pathway-level proteome remodelling in testes (Chapter 2).

Aim 2. Assess whether comparable proteomic signatures are detectable in mature sperm produced by the same testes analysed in Aim 1. Due to technical constraints that precluded statistically significant comparison of heat-treated and control sperm proteomes, this aim was reframed to generate a high-coverage global sperm proteome as a cross-species reference and as a molecular baseline for subsequent within-ejaculate work in humans (Chapter 3).

Aim 3. Test whether established functional selection methods that enrich for higher-quality human sperm, namely swim-up and methyl-cellulose migration, produce measurable proteomic differences among sperm from the same ejaculate (Chapter 4), and evaluate whether any of these differences include surface-exposed molecules that could serve as candidate biomarkers of sperm fitness (Marcu et al., 2024).

Aim 4. Validate shortlisted biomarkers of sperm quality at single-cell resolution and assess translational feasibility (Chapter 5). Spectral imaging flow cytometry on non-permeabilised human sperm, with fluorescence minus one controls and viability gating, was used to confirm surface localisation and accessibility of candidate biomarkers and to test whether marker intensity reflects sperm longevity and survival.

6.1 Evidence of the Effect of Environmental Stress on Sperm Production

In Chapter 2, tandem mass tag (TMT) proteomics was used to profile zebrafish (*Danio rerio*) testes following prolonges mild heat exposure and identified 5,415 proteins. Of these, 377 were significantly differentially abundant between heat-treated and control groups, with 133 increased and 244 decreased in heat-stressed testes. Enrichment analyses highlighted depletion of cilium movement, axonemal dynein complex, microtubule-based process, motile cilium, and oxidative phosphorylation, alongside increased abundance of proteostasis factors including HSP70 family proteins and SERPINH1/HSP47. A gamma generalised linear model showed that shifts in testis mass during exposure were not explained by body mass alone, indicating intrinsic germline responses rather than general condition effects. Together, these patterns provide protein-level evidence for reduced spermatogenesis with a concurrent shift toward cellular protection and repair.

Thermal stress is a pervasive disruptor of male reproduction in ectotherms, and comparable tissue-level outcomes are reported across teleosts. In medaka (*Oryzias latipes*), elevated temperature increases TUNEL-positive germ cells, and elevates HSP70 and HSP90, consistent with apoptosis and proteotoxic stress (Furukawa et al., 2019). In tilapia (*Oreochromis niloticus*), heat reduces spermatogonial proliferation and contracts elongating spermatid pools, with mitochondrial dysfunction and unfolded-protein-response activation that mirror the proteostasis and energy-metabolism shifts observed here (Alvarenga & França, 2009; Jin et al., 2019). In salmonids including *Salmo salar* and *Salmo trutta*, warm-water anomalies impair spermatogenesis, lower milt quality, and reduce sperm motility in parallel with altered antioxidant enzymes and ATP metabolism in testes and sperm (Lahnsteiner, 2012; Pankhurst & Munday, 2011). The depletion of dyneins, radial-spoke and intraflagellar-transport components together with increased chaperones observed in Chapter 2 matches these histological and functional outcomes, indicating that temperature primarily targets the flagellar

biogenesis toolkit and energy homeostasis needed to produce competent, motile sperm.

Mechanistically, convergent pathways recur across models. Proteotoxic stress activates HSF1-driven chaperone induction that stabilises misfolded proteins; mitochondrial dysfunction elevates reactive oxygen species and compromises ATP supply, membranes and DNA; and microtubule-based assembly defects impair manchette dynamics and axoneme formation essential for spermiogenesis (Boni, 2019; Houston et al., 2018; Robinson et al., 2023; Widlak & Vydra, 2017). The enriched and depleted proteins reported in Chapter 2 map onto this triad, indicating a coordinated pause in spermatid maturation with activation of protective programs. Environmentally induced “legacy” signatures can persist into mature sperm, including altered protein cargo and small RNAs with effects on early development, which provides a plausible conduit from testis stress to embryo-level consequences and frames testis-proteome plasticity as an upstream driver of within-ejaculate sperm (Godden et al., 2025).

Morphological responses in tissue structure and size are often subtle early and become pronounced with sustained stress across taxa. In the red flour beetle (*Tribolium castaneum*), testis mass modelled against body mass shows that sub-lethal chronic warming produces a treatment-specific reduction in testis volume independent of somatic growth, consistent with an intrinsic testicular response trajectory under heat stress (Cole et al., 2025). Comparable heat sensitivity is evident in parasitoid wasps such as *Nasonia vitripennis*, where elevated developmental temperature reduces testis size and sperm reserves with downstream losses in male fertility (Nguyen et al., 2013), and in fruit flies (*Drosophila melanogaster*), where sustained heat induces testicular atrophy, meiotic arrest, and depletion of elongating spermatids before whole-body condition declines (David et al., 2005; Rohmer et al., 2004). Taken together, these data support interpreting the present combination of modest mass change and strong proteomic reprogramming as an early or partially reversible phase in which functional disruption precedes gross anatomical change.

These results support three broad conclusions. The zebrafish testis proteome is highly dynamic under heat, shifting coordinated functional enrichment rather than isolated proteins. Biologically meaningful disruption of spermatogenesis can occur without large organ-size changes, implying that proteome-level readouts capture earlier phases of dysfunction. Selective depletion of axonemal and ciliary components provides a plausible molecular mechanism for the reduced spermatogenic output observed in heat-stressed testes.

Future work should resolve these responses by cell type and time: histology and immunostaining to localise stage-specific arrests; sorting enriched germ-cell fractions for targeted proteomics or single-cell transcriptomics; and time-course and recovery designs to discriminate reversible programs from irreversible loss. Coupling testis proteomics directly to CASA-based motility, ATP measurements, and fertilisation assays for the testes measured would link environment to tissue and gamete function, while ecologically realistic warming and heatwave regimes would connect laboratory findings to climate-related risk in wild and cultured fish populations.

6.2 Evidence of Phenotypic Variation Between Ejaculates

In Chapter 3, a high-depth zebrafish sperm proteome was generated. Across three pooled sperm samples from independent male groups, 5,410 proteins were confidently identified and 4,168 were quantified using TMT-multiplexed nanoLC–Orbitrap MS/MS. This represents one of the most complete teleost sperm proteomes to date and provides a baseline catalogue for functional and comparative studies of male fertility.

Depth and cross-species context support the completeness of this dataset. The zebrafish catalogue corresponds to roughly twenty percent of predicted protein-coding genes in the GRCz11 reference, which is unusually broad for a mature haploid cell type. Published sperm proteomes in other species are typically

smaller, for example about 2,850 proteins in epididymal sperm from house mouse (*Mus musculus*) using earlier discovery pipelines (Baker, Hetherington, Reeves, & Aitken, 2008), 348 in common carp (*Cyprinus carpio*) with 2D-gel workflows (Dietrich et al., 2014), and 744 in koala (*Phascolarctos cinereus*) from limited material (Skerrett-Byrne et al., 2021). Several factors likely explain the larger catalogue here, including higher input and pooling per TMT channel, modern Orbitrap acquisition, and well-curated zebrafish databases that improve peptide-to-protein assignment.

The functional composition aligns with known sperm biology. Network analysis of the most abundant proteins highlighted multiple histone variants together with chromatin-associated factors, consistent with histone retention and lack of protamine usage in zebrafish sperm chromatin organisation (González-Rojo et al., 2018b). Cellular-component enrichment showed strong mitochondrial signals and abundant axonemal and dynein components, matching the energetic and structural demands of motility in teleost sperm and the reliance on oxidative phosphorylation and glycolysis to power the flagellum (Inaba, 2011). Biological-process terms such as organelle organisation, cellular component localisation, and protein localisation suggest that mature ejaculated sperm retain a subset of late spermatogenic and maturation proteins, a pattern also reported in human and livestock datasets that differentiate fertile from subfertile males and high- from low-motility samples (Amaral, Paiva, et al., 2014; D'Amours et al., 2019b; Guo et al., 2019; Selvam et al., 2019).

With respect to between-ejaculate differences, the present design maximised proteome depth by analysing pooled material, which strengthens coverage but limits direct inference on inter-male variance because dispersion cannot be estimated per individual. Nevertheless, the magnitude of variation observed in the zebrafish sperm proteome, together with literature showing substantial variation among males in motility, morphology, metabolism, and fertilisation outcomes, indicate that ejaculate-level proteomic differences are likely to be biologically

meaningful and potentially predictive of fertility phenotypes across individuals and species (Amaral, Castillo, et al., 2014; Cannarella et al., 2020).

Future work should retain depth while restoring individual resolution. Practical options remain limited with current technology however, designs that sample pre- and post-exposure within the same male will help control baseline differences, and gentle, detergent-free processing can preserve surface proteins relevant to selection and downstream biomarker discovery for ART translation.

6.3 Evidence of Within-Ejaculate Sperm Variation

In Chapter 4, selected sperm pools from human ejaculates were fractionated to test whether functional heterogeneity among sperm from the same male is reflected in molecular differences. Two complementary assays were used: a methyl-cellulose migration assay that enriches for sperm capable of sustained progression through a viscous medium, and a swim-up that enriches for progressively motile, longer-lived sperm. Tandem mass tag (TMT) proteomics across five donors identified 4,616 proteins, of which 1,178 showed consistent differential abundance between selected and unselected fractions within each assay. Gene ontology analyses converged on the same broad picture across all donors and assays: selected sperm pools were enriched for axonemal, dynein and mitochondrial enrichments and for process terms related to cilium movement, sperm motility and membrane lipid remodelling, whereas unselected sperm pools were enriched for protein folding, RNA splicing and spliceosomal components, consistent with incomplete spermiogenesis and a stress-response state. At the single-protein level, OLFM4 was consistently lower in selected sperm across donors, whereas several adhesion and stress-signalling proteins were higher in unselected fractions.

These patterns align with extensive functional evidence that sperm from a single male are not equivalent and that subtle performance differences bias fertilisation. In externally fertilising Atlantic salmon (*Salmo salar*), within-ejaculate variation in

velocity predicts competitive fertilisation success and drives differences in embryo development and size (Gage et al., 2004). In domestic fowl (*Gallus gallus domesticus*), a simple sperm mobility assay predicts both competitive and non-competitive fertility, linking progressive motility to paternity outcomes (Froman et al., 2002). In mice, variation in morphology and kinematics among sperm from the same male influences the probability of reaching the oocyte in vivo (Firman & Simmons, 2010). Together with zebrafish experiments showing that selecting long-lived sperm within an ejaculate improves embryo viability and adult reproductive performance (Alavioon et al., 2017, 2019), these data indicate that performance-based enrichment maps onto fitness-relevant outcomes across taxa.

To assess the technical repeatability of the TMT proteomics workflow, one human sperm sample analysed in TMT Set 2 was re-run independently in TMT Set 3. Comparison of protein identifications between experiments showed extremely high concordance: 7,414 proteins were shared between the 7,498 proteins detected in Set 2 and the 7,486 proteins detected in Set 3, corresponding to 98.9% and 99.0% overlap respectively. This level of agreement is consistent with published estimates of Orbitrap–TMT technical repeatability, in which replicate injections or repeated multiplexed analyses typically show >90–95% overlap in identified proteins and tightly correlated reporter-ion intensities (Casey et al., 2017). These results confirm that the depth and composition of the human sperm proteome reported here are not artefacts of run-to-run variation but reflect a stable molecular profile of mature human sperm.

The proteomic signatures observed here are also consistent with comparative sperm proteomics in humans and livestock. Studies differentiating fertile from subfertile men, or high- from low-motility ejaculates, repeatedly report higher abundance of axonemal motors, radial-spoke proteins and energy-metabolism enzymes in better-performing samples, together with increased chaperones and RNA-processing machinery in poorer samples (Amaral, Castillo, et al., 2014). In boar (*Sus scrofa*), high-fertility ejaculates show enrichment of glycolytic and mitochondrial proteins that support sustained flagellar beating, whereas low-fertility samples are enriched for proteins associated with stress responses and defective

maturation (Noguchi et al., 2015). In cattle (*Bos taurus*), quantitative proteomics links sperm freezability and field fertility to dynein heavy chains, outer-dense fibre proteins and oxidative phosphorylation, again tying energetic and axonemal capacity to functional competence (D'Amours et al., 2019). The same proteomic signatures emerge in the selected human fractions here, suggesting that the molecular correlates of "better" sperm are conserved.

The methyl-cellulose assay adds a useful dimension by mimicking viscous microenvironments. Traversing viscous media requires specific membrane biophysical properties and metabolic tuning, including lipid-domain remodelling, cholesterol efflux and efficient ATP supply, which are reflected in the enrichment of lipid-handling and catalytic-regulation terms in the methyl-cellulose–selected fractions (Suarez & Pacey, 2006). By contrast, swim-up exerts a simpler kinematic filter, and the dominant enrichments for sperm motility and cilium movement in swim-up–selected fractions match this expectation (Magdanz et al., 2019). The convergence across assays on mitochondrial and axonemal enrichment, despite their different mechanical challenges, strengthens the inference that core motility and energy pathways underpin within-ejaculate performance differences.

Markers of immaturity were a consistent feature of unselected fractions. Enrichment for RNA splicing, spliceosomal components and protein folding points to persistence of earlier spermatogenic machinery, which is frequently observed in asthenozoospermic ejaculates and in ejaculates with retained cytoplasmic droplets and defective chromatin condensation (Amaral et al., 2014; Saraswat et al., 2017). Cellular-component and molecular-function terms such as focal adhesion, collagen binding and cell–substrate junction suggest a propensity for inappropriate adhesion or agglutination that can impede progressive movement, a phenomenon reported in subfertile human samples and linked to altered surface composition and antisperm factors (Barratt et al., 2011). Against this backdrop, the consistently lower abundance of OLFM4 in selected fractions is notable. OLFM4 is a glycoprotein implicated in cell–cell interactions and immune modulation in neutrophils and epithelia; elevated levels in unselected fractions may reflect a

stress-response or adhesive state that is incompatible with sustained motility and longevity, making OLFM4 a plausible negative marker of functional competence pending direct functional tests.

The broader literature on sperm selection technologies helps situate these findings. Classical enrichment by swim-up or density gradients improves motility and reduces debris but does not reliably remove sperm with DNA damage or epigenetic defects, and clinical gains are inconsistent (Said & Land, 2011). Microfluidic devices and viscous-media filters can reduce DNA fragmentation and oxidative damage and improve blastocyst quality in some studies, but outcomes vary and standardisation is limited (Beckham et al., 2018; Doostabadi et al., 2022). Ligand-based approaches such as zona-pellucida binding or annexin-V magnetic sorting enrich for sperm with lower DNA fragmentation and better morphology, again with mixed translation to live-birth rates (Huszar et al., 2007). The present proteomic contrasts provide a molecular rationale for why some modalities succeed in specific contexts and argue for combining physical selection with molecular readouts to target genuinely “functional” sperm.

The data presented in Chapter 4 therefore does two things. Firstly, provide molecular evidence that subpopulations within a single human ejaculate differ in ways that map onto fitness-relevant traits such as motility, longevity and membrane competence. Secondly, they deliver a shortlist of surface-accessible candidates, including OLFM4 and CTNG, that can be developed into non-destructive enrichment tools more compatible with clinical workflows than high-speed sorting. The immediate next steps are to increase cohort size, obtain paired methyl-cellulose and swim-up fractions from the same ejaculate to partition assay-specific from donor-specific effects, and couple TMT depth to functional readouts such as CASA kinematics, oxidative status, DNA integrity and time-to-blastocyst in appropriate model systems. Ultimately, integrating performance-based selection with biomarker-guided capture will allow tests of whether enriching for specific molecular states translates into higher embryo viability and live-birth rates, the key clinical end points.

6.4 Validation of Biomarkers of Sperm Fitness

In Chapter 5, imaging spectral flow cytometry was used to validate three surface candidate sperm quality biomarkers from Chapter 4, namely OLFM4, CTNG/JUP and AOC3, on non-permeabilised human sperm. Five donor ejaculates were incubated for xeelction on longevity, stained with a fixable viability dye (eFluor 520), and probed in separate panels with fluorophore-conjugated antibodies (OLFM4–PE, CTNG–mFluor Violet 450, AOC3–Alexa Fluor 594). Data were acquired on the BD FACSDiscover S8 in fully spectral mode with simultaneous bright field imaging. Spectral unmixing used single-stained and fluorescence-minus-one controls, gates isolated single sperm (P1), and viability gates separated live and dead cells. At single cell resolution, OLFM4 and CTNG signals localised to the sperm surface and were consistently higher in the short-lived fraction across donors, whereas AOC3 showed marked inter-individual variability and no consistent association with longevity. Binomial generalised linear mixed models confirmed the enrichment of OLFM4-positive and CTNG-positive events among dead relative to live cells. Together these results verify surface availability for OLFM4 and CTNG, link their abundance to within-ejaculate fitness proxies, and indicate that AOC3 is unsuitable as a robust marker in this context.

Discovery proteomics can nominate dozens of putative sperm markers, but clinical or evolutionary relevance hinges on three questions: is the epitope truly on the cell surface in intact sperm, does its single-cell distribution track functional heterogeneity within an ejaculate, and is the pattern reproducible across men. Imaging spectral flow cytometry addresses exactly this translation gap by quantifying marker localisation and intensity in tens of thousands of individual, non-permeabilised sperm while simultaneously recording morphology and viability, thereby converting bulk trends into cell-resolved evidence that can be coupled to performance phenotypes such as longevity or motility (Cannarella et al., 2020; Intasqui et al., 2018).

Viewed against established sperm surface biology, the two validated candidates here, OLFM4 and CTNG/JUP, fall into a class of markers that appear enriched on short-lived, low-fitness cells and thus operate as negative selection cues rather than positive tags for elite sperm. That logic mirrors prior work where surface composition predicts function: for example, the IZUMO1–JUNO pair is essential for gamete fusion and quantitative or spatial differences in IZUMO1 bear on fertilisation competence (Bianchi et al., 2014) and the mucin-like glycoprotein DEFB126 facilitates passage through cervical mucus, where common human deletions reduce paternity odds despite normal semen parameters (Tollner et al., 2011). Beyond proteins, deficits in PLCζ, the sperm oocyte-activation factor, are linked to fertilisation failure in both human and mouse, underscoring how single molecules can set a hard ceiling on embryo development even when motility is adequate (Kashir, 2020). In that landscape,the association of OLFM4 with cell–cell interactions and inflammatory signalling, and the role of CTNG/JUP in junctional architecture, make their enrichment on short-lived cells biologically plausible as indicators of stress-biased or adhesion-prone surface states that are incompatible with sustained progression through the female tract.

Methodologically, imaging flow confers specific advantages for sperm. It permits visual verification of gating and dye localisation in a cell type with unusual size, shape and intrinsic autofluorescence, and it quantifies heterogeneous, non-Gaussian marker distributions that bulk assays average away. Using non-permeabilised cells ensures that signals correspond to surface-available epitopes, a prerequisite for downstream affinity capture or depletion in workflows that are more compatible with IVF/ICSI than high-speed sorting of live gametes (Intasqui et al., 2018).

Important caveats remain. Fixation can alter epitope accessibility for a subset of targets; sample size was modest; and associations were made to longevity rather than to fertilisation and embryo development. The immediate path forward is clear: extend to live, non-fixed preparations using gentle antibody formats, increase donor numbers, pair pre-enrichment modalities within the same ejaculate to test

assay-specific robustness, and couple staining with orthogonal functional readouts in the same cells, such as DNA-integrity assays, mitochondrial potential and capacitation markers, before moving to embryo-level endpoints in suitable animal models or prospective clinical cohorts (Codina et al., 2015; McShane et al., 2013). If these steps confirm consistency, OLFM4 and CTNG/JUP are well positioned as depletion targets for immuno-microfluidic filters or bead-based capture to remove low-fitness sperm, complementing existing motility-based selection and addressing a long-standing gap between “moving” and “functionally competent” sperm.

6.5 Molecular evidence supporting haploid selection

A central aim of this thesis was to test whether molecular heterogeneity among sperm within a single ejaculate provides a substrate for selection acting at the haploid stage. The human proteomics data showed reproducible differences between selected (higher longevity and motility) and unselected subpopulations across donors. Selected sperm were enriched for proteins and pathways consistent with superior performance, including enrichments linked to axonemal function, energy metabolism, and oxidative stress resilience, whereas unselected sperm showed signatures of incomplete maturation, RNA processing, and inappropriate adhesion. These patterns are consistent with selection acting on phenotypes expressed during the haploid phase rather than a homogeneous output of the diploid male genotype (Immler, 2019; Sutter & Immler, 2020).

Single-cell validation with spectral imaging flow cytometry confirmed cell surface localisation and within-ejaculate trends for two candidates. OLFM4 and CTNG were consistently more abundant on short-lived, low-quality sperm and reduced on long-lived, higher-quality cells, aligning with the discovery proteomes. These findings confirm that at least some quality-linked molecular features are externally accessible on intact sperm and measurable at single-cell resolution, which is a prerequisite for functional selection or clinical screening.

Direct one-to-one correspondence between proteomic and genomic signals is not expected, because proteins integrate post-meiotic gene regulation, selective protein retention, and epigenetic programming. Nevertheless, the overall picture aligns with emerging genomic and transcriptomic evidence for allele-biased expression and genotype–phenotype coupling among haploid sperm within a male, supporting the biological plausibility of haploid selection in animals (Bhutani et al., 2021; Marcu et al., 2024).

Taken together, these findings indicate that sperm are not biologically equivalent. Functionally relevant molecular variation exists within ejaculates, can be detected on the cell surface, and tracks phenotypes that matter for fertilisation potential. This supports a model in which selection can act among haploid gametes prior to fusion and provides a molecular basis for future causal tests in models where embryo and offspring outcomes can be measured (Alavioon et al., 2017, 2019).

6.6 Cross-species translation and model choice

Direct experimental tests of whether specific sperm produce healthier embryos or offspring cannot be performed in humans for ethical and legal reasons, which makes model organisms essential for connecting sperm phenotype to developmental outcomes (Hoo et al., 2016; Meyers, 2018). Zebrafish is an effective discovery platform because external fertilisation, high fecundity, and optical access to embryos allow controlled selection regimes and prospective tests of viability that are not possible in humans. Extensive genomic resources, transgenesis, CRISPR, and single-cell methods enable causal interrogation of pathways highlighted by proteomics, while waterborne exposures make temperature and contaminant studies ecologically realistic for aquatic systems (Hoo et al., 2016).

Important physiological differences limit direct one-to-one translation. Teleost sperm are activated by external osmolality rather than undergoing mammalian-style capacitation in an oviduct, many teleosts lack a classical acrosome, and

chromatin packaging relies more on histones and species-specific basic proteins than on protamines, all of which shift baseline proteomes and selection filters relative to humans (Immler, 2019; Suarez & Pacey, 2006). Testis organisation, thermal biology, and post-testicular maturation also differ across taxa, which must be considered when inferring clinical relevance.

A tiered strategy mitigates these gaps. Discovery is performed in zebrafish to identify conserved axes of sperm performance such as mitochondrial function, redox homeostasis, and axonemal integrity. Orthologue mapping and targeted assays are then applied to human ejaculates to confirm cell surface availability and within-ejaculate trends using spectral imaging flow cytometry. Where female-tract biology and acrosome function are critical, mammalian models such as mouse for mechanism and bovine or porcine systems for ART-relevant handling provide complementary validation. Nonhuman primates are reserved for late-stage translational questions where justified (Stouffer & Woodruff, 2017b; Suarez & Pacey, 2006).

This triangulation treats zebrafish as a hypothesis generator, mammals as mechanistic validators, and human ejaculates as the final clinical filter. Conserved features that recur across taxa are most likely to generalise, whereas model-specific signals guide species-tailored applications (Hoo et al., 2016; Immler, 2019).

6.7 Broader Implications

6.7.1 Evolutionary significance

These results support an updated view of sexual reproduction as a multi-level selection process. Evidence for functionally meaningful molecular heterogeneity within ejaculates, coupled with single-cell validation of fitness-linked cell surface features, is consistent with selection acting among haploid gametes prior to fertilisation. This complements classic frameworks of anisogamy and the biphasic life cycle by showing that the brief haploid phase can express phenotypes with

consequences for fertilisation and potentially for offspring performance (Immler, 2019; Mable & Otto, 1998; Parker et al., 1972).

The data also refine models of sperm competition. Traditional models emphasise numbers and motility while assuming within-male homogeneity. The present findings indicate that internal competition within an ejaculate can favour sperm that express specific molecular traits such as enhanced membrane integrity, oxidative stress resistance, and robust axonemal architecture, adding a purifying component to selection before zygote formation (Lüpold & Pitnick, 2018; Sutter & Immler, 2020). This provides empirical grounding for theoretical models that incorporate allele-biased expression, post-meiotic regulation, and selective protein retention as mechanisms enabling haploid selection in “diploid” organisms (Bhutani et al., 2021; Immler, 2019).

6.7.2 Relevance to human fertility and ART

Clinically, these results argue for moving beyond morphology and gross motility toward molecularly informed, functionally validated sperm assessment. Conventional selection methods such as swim-up and density gradients enrich for movement but do not reliably exclude sperm with DNA damage, epigenetic abnormalities, or membrane defects, and they remain poor predictors of embryo viability or live birth (Said & Land, 2011). The identification of cell surface markers that track within-ejaculate longevity and survival, combined with spectral imaging flow cytometry on non-permeabilised cells, provides proof of concept for minimally invasive screening and enrichment of higher-fitness sperm that is compatible with ART workflows (Oseguera-López et al., 2019).

Near-term translational steps include larger multi-centre validations of OLFM4 and CTNG trends across clinical cohorts, development of rapid cell surface assays or microfluidic capture using antibodies against validated targets, and prospective studies that relate marker-guided selection to fertilisation, embryo quality, implantation, and live birth. Such tools are especially pertinent for unexplained infertility and repeated ART failure, where standard semen parameters lack

predictive power. A molecularly informed selection paradigm has the potential to improve outcomes while reducing the emotional and financial burden of repeated cycles (Barratt et al., 2011; Said & Land, 2011).

6.8 Critical reflections and limitations

While this thesis advances understanding of sperm heterogeneity, haploid selection, and male fertility, several limitations should be acknowledged to contextualise the inferences and to guide next steps. These relate to sample size, pooling, reagent constraints, experimental design, and generalisability.

First, sample sizes in the human work were modest, often five or fewer donors per experiment. Mixed-effects models and within-ejaculate contrasts helped control for inter-individual variability, but statistical power and external validity remain constrained. Donor-specific patterns, such as variability in AOC3, underline the need for larger cohorts to distinguish stable biological heterogeneity from sampling noise.

Second, pooled materials were required for high-depth proteomics in zebrafish testes and sperm. Pooling enabled robust protein identification and quantification but averages away individual-level structure, limiting direct linkage between organismal traits and molecular profiles. Tank replicates and standardised pooling mitigate this to an extent, yet one-to-one genotype–phenotype mapping was not possible.

Third, reagent availability and specimen handling bounded the scope of single-cell validation. Only candidates with reliable, surface-accessible antibodies could be tested, and several promising targets were excluded due to lack of suitable reagents for non-permeabilised sperm. The use of fixation in imaging flow cytometry safeguarded morphology and gating but may alter epitope conformation or membrane accessibility, potentially biasing signal magnitude.

Fourth, the thesis establishes associations between molecular signatures and sperm fitness proxies such as longevity and motility, rather than causal links to fertilisation or developmental outcomes. Direct tests of fertilisation success and offspring phenotypes are not feasible in humans and were beyond scope in zebrafish. Future functional assays in tractable models will be required to confirm whether depleting or enriching specific surface-defined subpopulations shifts embryo viability or offspring performance.

Finally, cross-species inference should be cautious. Core pathways related to oxidative stress, metabolism, and flagellar function appear conserved between zebrafish and humans, but species differences in spermatogenesis, maturation environments, and reproductive strategy may shape effect sizes and marker performance. Clinical translation will require validation in human cohorts and under clinically relevant workflows.

Despite these constraints, the combination of multi-species designs, complementary proteomic and single-cell readouts, and convergent within-ejaculate patterns provides a strong evidential base. Stating these limitations delineates the bounds of inference and clarifies priorities for future work: larger human cohorts, unpooled designs where feasible, expanded reagent development for live-cell assays, and prospective tests of biomarker-guided enrichment against fertilisation and embryo outcomes.

6.9 Future directions

Building on the findings of this thesis, a number of promising research directions emerge that could clarify unresolved mechanisms, validate functional relevance, and translate molecular discoveries into clinical tools. While the evidence presented here establishes key associations between sperm phenotypes, molecular profiles, and functional potential, several areas require deeper investigation to fully understanled the biological implications and therapeutic opportunities.

A primary next step involves the functional validation of candidate sperm biomarkers in model organisms. While this thesis demonstrated consistent associations between certain surface proteins and sperm fitness traits such as motility and longevity, causality has not yet been established. *In vivo* fertilisation experiments using model organisms such as zebrafish or mice could directly test whether sperm expressing or lacking specific markers differ in fertilisation success or influence downstream embryo viability and offspring outcomes. Controlled breeding experiments, where sperm are sorted based on biomarker expression before fertilisation, would allow a more definitive assessment of how molecular variation translates to reproductive performance.

Another important direction involves improving the resolution of sperm phenotyping through single-cell or live-cell molecular profiling. While high-throughput proteomics has revealed biologically meaningful variation, it is inherently a bulk technique and therefore obscures cell-level heterogeneity. Recent advances in single-cell mass spectrometry and live-cell barcoding could allow researchers to examine molecular differences between individual sperm cells with greater precision. Coupling live-cell sorting techniques to downstream omics analyses, such as transcriptomics or targeted proteomics, would also offer the opportunity to study dynamic processes such as capacitation, acrosome reaction, and mitochondrial activation in real time.

In human reproductive research, longitudinal outcome studies are needed to evaluate the clinical relevance of the molecular traits identified here. While this thesis focused on associations with sperm motility and viability, future studies should examine whether these markers predict fertilisation success, embryo development, implantation rates, or live birth outcomes in ART contexts. Cohort studies involving patients undergoing IVF or ICSI could provide valuable real-world data linking molecular biomarkers to treatment success. Such studies would also offer insight into inter-individual variability, a recurring theme throughout this thesis, and help identify which molecular features are robust across populations.

The development of non-invasive sperm selection technologies also represents a key translational goal. Building on the flow cytometry work presented in this thesis, novel technologies could be designed to sort or enrich for sperm lacking markers associated with low fitness. These technologies must preserve cell viability and functionality for use in ART, but may include microfluidic devices, optical imaging-based sorters, or label-free selection methods that exploit biophysical differences such as refractive index or dielectric properties. The integration of such tools into fertility clinics would mark a major shift from morphology-based to molecularly informed sperm selection.

Another important area for exploration is the genetic and epigenetic basis of sperm heterogeneity. Although this thesis inferred that functional variation may be shaped by haploid gene expression or selective protein retention, the specific genetic mechanisms remain unclear. Technologies such as single-cell RNA sequencing, allele-specific expression analysis, and methylation profiling could reveal how genetic variants or epigenetic marks influence the molecular phenotype of individual sperm cells. This could also provide insight into how environmental factors, such as stress or toxin exposure, shape gamete quality across developmental time.

Finally, the broader implications of sperm heterogeneity for offspring health warrant further investigation. Emerging evidence suggests that sperm molecular quality may influence not only fertilisation but also embryonic development and long-term health outcomes. Whether sperm surface markers or proteomic profiles can predict disease risk, developmental robustness, or transgenerational effects remains an open and compelling question.

Together, these future directions outline a clear path from the discovery of sperm heterogeneity to the mechanistic understanding and clinical application of this phenomenon. Interdisciplinary research combining molecular biology, reproductive physiology, evolutionary theory, and clinical practice will be essential to advancing

this field. The continued exploration of sperm phenotypes at the molecular level offers an opportunity to deepen our understanding of sexual reproduction while improving reproductive outcomes for patients.

6.10 Conclusions

This thesis demonstrates that sperm fitness is molecularly encoded, phenotypically expressed, and subject to selection. Through a combination of high-resolution proteomics, sperm subpopulation analysis, and single-cell validation, the work provides strong evidence that sperm are not functionally equivalent and that their phenotypic variation has biological and potentially clinical significance. Variation occurs not only between males, but also within individual ejaculates, and this variation can be traced to differences in protein expression that relate to key fitness traits such as motility and longevity.

The findings support and refine evolutionary models of gamete competition and suggest that selection can act at the haploid stage of the reproductive cycle, challenging long-held assumptions about the passivity of sperm cells. This work also lays the foundation for new clinical approaches to diagnosing and treating male infertility. By identifying functionally relevant molecular markers, it offers a path forward for improving sperm selection methods in ART and better predicting fertilisation outcomes.

More broadly, this thesis underscores the value of applying evolutionary theory to clinical fertility problems. It shows that understanding the origins and consequences of sperm heterogeneity is not only a matter of basic science, but a practical necessity for enhancing human reproductive health. This work sets the stage for future efforts to harness molecular variation in the service of more effective, personalised fertility care.

Appendix

Appendix 1: The Zebrafish Sperm Proteome Published Manuscript.

Please see supplied PDF file “The Zebrafish Sperm Proteome 2024.pdf”

Supplementary Table 1: The complete zebrafish testes proteome.

Please see supplied Excel file “Supplementary Table 1.xlsx”

Supplementary Table 2: Proteins with significantly different abundance between heat-treated and control testes

Please see supplied Excel file “Supplementary Table 2.xlsx”

Supplementary Table 3: GO terms for proteins significantly less abundant in heat treated testes

Please see supplied Excel file “Supplementary Table 3.xlsx”

Supplementary Table 4: GO terms for proteins significantly more abundant in heat treated testes

Please see supplied Excel file “Supplementary Table 4.xlsx”

Supplementary Table 5: The zebrafish sperm proteome

Please see supplied Excel file “Supplementary Table 5.xlsx”

Supplementary Table 6: The human sperm proteome

Please see supplied Excel file “Supplementary Table 6.xlsx”

Supplementary Table 7: Biomarker shortlist

Please see supplied Excel file “Supplementary Table 7.xlsx”

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