**ERC Starting Grant 2024**

**Research Proposal [Part B2]**

**MitoGuardians: Sex-Specific Mitochondrial Regulators of Reproductive Aging: From Toxicant Exposure to Transgenerational Effects**

* Name of the Principal Investigator (PI): **Chen Lesnik**
* Name of the PI’s institution for the project: **University of Haifa**
* Proposal duration in months: **\_**

**Section a. State-of-the-art and objectives**

**Summary:** The trajectory of reproductive aging differs between men and women, leading to distinct fertility challenges, particularly as parenthood is delayed and environmental exposure to toxins becomes more prevalent. This proposal aims to unravel the complex interplay between mitochondrial function and reproductive longevity, focusing on how mitochondrial dysfunction and mitochondrial ROS (mtROS) contribute to the age-related decline in oocyte and sperm quality. Despite the established link between mitochondria and reproductive aging5–9, the specific mitochondrial proteins and pathways that maintain ROS levels in gametes remain poorly understood, especially regarding their sex-specific roles. Additionally, the impact of environmental toxicants on these processes is underexplored. We will address key questions: Could differences in the mitochondrial proteome and regulation of mtROS contribute to the distinct reproductive aging trajectories between sexes? How do environmental toxicants disrupt mitochondrial regulation of ROS, and what are the mechanisms driving sex-specific reproductive aging? Moreover, what are the heritable effects of these disruptions on offspring? Using *C. elegans*, a powerful model organism for studying reproductive aging with dual-sex capabilities, we will: **1) Identify and characterize sex-specific mitochondrial proteins and metabolic pathways essential for maintaining ROS levels in oocytes and sperm for reproductive longevity, 2) Elucidate the cellular and molecular mechanisms by which environmental toxicants disrupt mitochondrial regulation of ROS and induce reproductive aging in a sex-specific manner, and 3) Investigate the sex-specific heritable effects of these disruptions.** These objectives will serve as the foundation for identifying interventions targeting these pathways and mechanisms. The ultimate goal is to extend reproductive longevity and address long-term reproductive risks from environmental exposures, including heritable effects. Ourcomprehensive approach to reproductive aging and environmental toxicity combines sex-specific analysis of mitochondrial function in males and hermaphrodites. Our approach offers a novel perspective on the interplay of mitochondrial function, reproductive aging, and environmental factors, with implications for human fertility.

**State-of-the-art and objectives**

Reproductive aging, the age-related gradual decline in fertility, is a fundamental aspect of human biology affecting women and men. The decline typically begins in women in their mid-30s, approximately 15 years before menopause, and represents one of the earliest phenotypes associated with aging in humans1. At the same time, this process is more gradual in men2. Decreased reproductive ability is predominantly attributed to the quantitative and qualitative deterioration of oocytes in women1 and sperm in men2, which is affected by a range of cellular and environmental factors3. **As the maternal and paternal ages for first childbirth have risen in recent years**4,5**, elucidating the mechanisms that govern reproductive aging in both sexes is crucial.**

While we have yet to fully identify and understand the cellular factors involved in reproductive aging, emerging data highlight organelles, particularly mitochondria, as pivotal regulators of reproductive health6–10. The significance of mitochondria is apparent from their abundant presence within fully-grown oocytes11 and their crucial function in sperm flagella6. Aging is also associated with impaired mitochondrial function and increased ROS, leading to oxidative stress12,13. While not the sole cause of aging, oxidative stress contributes significantly to onset and progression. Sex-specific differences in physiological and biochemical characteristics, driven by biological, environmental, and social factors, result in distinct vulnerabilities to oxidative stress12. Mitochondria are integral to this interplay, affecting metabolism and redox balance12. **These sexual differences highlight the importance of a sex-specific approach in aging research, particularly when studying oxidative stress, gene regulation, and developing targeted interventions for healthy aging.**

Mitochondria are the primary source of ROS14,15, and accumulating evidence from *C. elegans* to humans indicates a physiological relevance of mtROS levels in the quality of both oocytes16–18 and sperm10,18. Although ROS can function as a signaling molecule19, it is cytotoxic at high concentrations by inducing oxidative stress20. ROS can damage cells generally and mitochondria specifically21–23. Mitochondrial proteins are key targets of oxidative stress, and age-related ROS accumulation is associated with changes in the abundance of mitochondrial proteins across diverse organisms13,16,24,25. During my postdoctoral research in Prof. Coleen Murphy’s lab at Princeton University, I showed a link between low mtROS and a 'youthful' mitochondrial protein profile, high oocyte quality, and enhanced reproductive longevity16. Corroborating evidence across species26,27, including humans26, indicates that maintaining low mitochondrial activity and suppressing ROS production is crucial for oocyte quality. Conversely, mitochondrial dysfunction and the consequent increase in ROS levels associated with aging adversely impact both oocyte23,28 and sperm10,23,29 quality in various species. **Elucidating the age-related changes in the mitochondrial proteome of oocytes and sperm and uncovering the molecular mechanisms linking mtROS to reproductive aging is a critical gap in our understanding. It is unknown if these proteins are sex-specific and contribute to differential aging in male and female gametes.**

**Impact:** Addressing this knowledge gap is essential because uncovering these sex-specific differences may facilitate the development of targeted strategies for extending reproductive longevity.

**Sexual dimorphism in aging and mitochondrial function**

Aging is a fundamental biological process conserved across species, yet its progression and effects exhibit sexual dimorphism. The underlying mechanisms driving these sex-specific differences remain elusive30. Mitochondria, crucial for energy production and cellular metabolism, play a significant role in the aging process. Age-related mitochondrial dysfunction results from intrinsic factors like genetics and extrinsic factors like environmental stressors. These factors affect gene expression related to mitochondrial function and stress response, leading to changes in mitochondrial function. Mitochondrial dysfunction plays a key role in aging across diverse species, from yeast to mammals, indicating conserved mechanisms. Recent studies have expanded our understanding of sex-dimorphic stress responses and mitochondrial maintenance, highlighting the complex interplay between aging, mitochondrial function, and sex-specific biological processes31.

Reproductive aging in human males and females exhibits distinct patterns and mechanisms, reflecting differences in physiology and reproductive strategies. Female fertility sharply declines with age, particularly after age 35, and is characterized by a clear endpoint known as menopause. Menopause typically occurs in the late 40s to early 50s, marking the cessation of ovulation and reproductive capability. In contrast, male reproductive aging occurs gradually without a definitive endpoint32. Whereas males and females rely on mitochondrial function for reproductive health, the mechanisms and implications of mitochondrial dysfunction may differ. Male germ cells undergo continuous replication throughout adulthood33, allowing for frequent turnover of mitochondria. Along this developmental pathway, there is also an increase in mitochondrial abundance and mitochondrial fusion and fission that facilitate the repair of mitochondrial damage34. Recent studies show that paternal age can also impact fertility, possibly through epigenetic changes influenced by sperm mitochondrial function35. During early oogenesis in females, mitochondrial replication increases to ensure mature oocytes have adequate mitochondria11. Once the oocyte reaches maturation, mitochondrial replication typically halts, and the number of mitochondria remains stable unless damaged by aging or environmental factors. However, as oocytes age, mitochondrial function declines, and the overall quality of mitochondria deteriorates, leading to reduced oocyte quality and fertility36. **Divergent aging trajectories of oocytes and sperm raise intriguing questions. What factors drive these distinct pathways? Do mitochondria play a role, and if so, how does it differentially impact reproductive aging in females versus males?**

**mtROS as a biomarker for age-related oocyte quality**

Recent research identifies the pivotal role of mitochondria in determining oocyte and sperm quality and reproductive longevity. In Prof. Coleen Murphy's lab, we discovered a strong link between a ‘youthful’ mitochondrial protein profile, high oocyte quality, and extended reproductive longevity. Of fundamental importance, we found a novel association between low mtROS levels and prolonged reproductive lifespan. By contrast, elevated mtROS is correlated with poor oocyte quality in aged individuals and earlier reproductive decline (Fig. \_)16. Our findings indicate that maintaining low mtROS levels is crucial for preserving oocyte quality as organisms age. This result aligns with that of Rodríguez-Nuevo et al., who suggested that inactivating mitochondrial complex I in early-stage oocytes reduces ROS production and enhances oocyte longevity26. This independent result confirms the importance of mitochondrial function and ROS regulation in human reproductive aging. Age-related increases in ROS levels also negatively impact sperm quality across various species10,23,29.

**is These insights provide the foundation for our hypothesis that germline-specific mtROS can serve as a biomarker for assessing oocyte and sperm quality as well as reproductive aging.**

**Objective 1 will validate mtROS as a marker for reproductive aging.**

**Sex differences and environmental factors**

Sex differences significantly impact health, influencing disease prevalence, risk factors, progression, and treatment responses. **Despite sexual differences, a substantial knowledge gap persists regarding the role of environmental factors in these differences.** González Zarzar et al, for instance, used sex-stratified phenomic environment-wide association studies (EWAS) using National Health and Nutrition Examination Survey (NHANES) data. They identified environmental-phenotype associations, revealing potential biological pathways with significant sex-based differences. These

disparities may stem from varying exposure levels or biological mechanisms37. Males and females are vulnerable to the damaging effects of environmental exposures on their reproductive systems, particularly through impacts on mitochondrial function in oocytes and sperm. Testicular and sperm damage are often a result of oxidative stress, compounded by a decline in antioxidant activity and mitochondrial dysfunction with age35. Exposure to pollutants, toxins, and endocrine-disrupting chemicals, such as heavy metals, pesticides, and industrial chemicals, is linked to increased ROS production, impaired sperm production, reduced sperm quality, and elevated DNA damage in sperm cells35,38. Similarly, oxidative stress due to lifestyle factors such as chemical exposure is implicated in the aging of ovarian cells and reduced reproductive potential in females36. **Despite this knowledge, no large-scale study has simultaneously examined the differential impact of environmental exposures on age-related mitochondrial function, ROS, and reproductive aging in both sexes.** To our knowledge, no comprehensive screen investigating potential reproductive toxicities and their effects on age-related mitochondrial quality in oocytes and sperm has been conducted. **Which contaminants disrupt mitochondrial regulation of ROS in the germline? What is their effect on reproductive longevity, and what are the mechanisms? Do they have a selective or similar effect on exposed males versus females?**

Objective 2 will focus on these questions and discover contaminants affecting sex-specific reproductive aging.

Environmental toxicants significantly impact mammalian reproduction, inducing transgenerational effects via epigenetic alterations. **Which toxicants that disrupt germline mitochondrial function also cause reproductive toxicity in unexposed generations? Is transmission maternal, paternal, or both? How many generations are affected? What molecular mechanisms drive these persistent changes in germ cells?**

Objective 3 will answer these questions by identifying toxicants that lead to transgenerational and sex-specific effects?

***C. elegans* as a model for sex-specific reproductive aging, reproductive toxicity, and transgenerational effects**

mtROS can be elevated by factors, including endogenous factors and environmental stressors, such as exposure to high concentrations of environmental toxicants23. Notably, these environmental stressors can not only impair reproductive health in males and females39,40 but also induce heritable effects in progeny39,41,42. The nematode *C. elegans* has emerged as a valuable model organism for assessing reproductive aging, the toxicity of various environmental pollutants, and the underlying cellular mechanisms. Thus, *C. elegans* is a powerful model43 for studying aging in general, and particularly reproductive aging44.

*C. elegans* has a short lifespan, rapid reproductive cycle, and ease of genetic manipulation, enabling high-throughput studies and facilitating the study of various experimental conditions and genetic perturbations. Thus, the model is excellent for studying the causal relationship between cellular pathways and reproductive aging. A major advantage of using *C. elegans* to study reproductive aging is that it exists naturally in two sexes - males and hermaphrodites, with mutants available that can fully convert them into fertile females45. Despite having vastly different reproductive chronologies and strategies, there is a remarkable conservation of cellular and molecular components governing oocyte quality and reproductive aging from worms to humans46–48. Like women, *C. elegans* adult hermaphrodites undergo reproductive aging due to a decline in oocyte quality47. Women and *C. elegans* hermaphrodites have similar reproductive aging profiles, comprising comparable proportions of their respective lifespans46,47. Given that reproductive aging in *C. elegans* is regulated by highly conserved genes and pathways governing oocyte quality with age49, it is a powerful organism for studying age-related reproductive decline in a biologically meaningful context. *C. elegans* offers unique advantages for oocyte biochemical analysis. Unlike the paucity of samples and technical constraints of human oocytes6,17, *C. elegans* hermaphrodites produce hundreds of oocytes throughout their reproductive span, providing an abundant and accessible source for studying oocyte biology50. Critically for this project, the model enables the study of sperm aging. Several studies find that some sperm features do not change with age and reproductive aging in males may be related to mating behavior51,52. However, these findings cannot fully explain the well-established correlation between increasing paternal age and detrimental outcomes such as increased embryonic death and decreased production of fertilized eggs51,53. This indicates other factors affecting sperm quality. **Furthermore, altering endogenous metabolic processes can impact sperm aging54,55, which strongly emphasizes the need to study the metabolic pathways and cellular mechanisms that may contribute to age-related sperm deterioration.**

King DE et al. conducted whole-worm respirometry experiments on different *C. elegans* strains at the young adult stage, including wild-type (hermaphrodites) and male-enriched strains, including *fog-2*. They reported no significant differences in oxygen consumption rates (OCR) between wild-type N2 worms and male-enriched strains56. We found that mitochondrial proteins from young wild-type and *daf-2* mutant worms are similar, whereas aged wild-type worms show distinct differences in their mitochondrial proteome, indicating that mitochondrial changes become more pronounced with age. Given these findings, we tested whether mitochondrial function also exhibits more robust differences between males and hermaphrodites with age. **As a proof of concept, we conducted a pilot small-scale respirometry experiment comparing OCR between day-5 *fog-2* males and hermaphrodites**. To minimize the impact of embryos on measurements, we treated worms with serotonin to enhance egg-laying. All measurements were normalized to mean worm length to account for size differences. **Significantly, maximum respiration is significantly lower in day-5 *fog-2* hermaphrodites compared to age-matched males (Fig.\_\_).** We will repeat and optimize the experiment; however, these preliminary results are highly promising.

*C. elegans* has also been studies for various inherited effects of environmental stressors57 and the effects of different environmental toxicants on reproductive parameters, including brood size, egg production, embryo viability, germline apoptosis, and gene expression41. Environmental pollutants can induce transgenerational toxicity in *C. elegans*, with different cellular mechanisms regulating these effects. These effects include activation of oxidative stress, damage to reproductive systems, induction of UPRmt, and decreased mitochondrial membrane potential58. *C. elegans* is an excellent model for studying these effects due to its short life cycle and similarities to other organisms in toxicity mechanisms. **Whereas *C. elegans* has been widely used to study reproductive toxicity, the specific effects of environmental contaminants on reproductive longevity and the potential involvement of mitochondrial impairment in both sexes offer an invaluable opportunity for novel insights.**

This proposal aims to leverage advanced techniques in molecular biology, toxicology, and reproductive biology to dissect the complex interplay between environmental contaminants, mitochondrial dysfunction, and reproductive aging in both sexes.

Our goal of identifying sex-specific mitochondrial regulators of reproductive aging and assessing how contaminants affect oocytes, sperm, and transgenerational reproductive health will be achieved through the following objectives:

1. Identify sex-specific mitochondrial proteins and pathways regulating ROS in oocytes and sperm for reproductive longevity
2. Elucidate how environmental toxicants disrupt mtROS regulation and promote sex-specific reproductive aging

3) Investigate the sex-specific transgenerational effects of these disruptions

**Our unique approach to the study of reproductive aging and environmental toxicity**

Our research proposal presents a unique, comprehensive approach to studying reproductive aging by combining a multi-level analysis from organelles to whole organisms with a strong focus on sex-specific differences. Our proposal leverages the genetic tractability of the dual-sex system of *C. elegans*, which has hermaphrodites and males and a short lifespan. Our study emphasizes mitochondrial function and ROS levels as key factors in reproductive aging. It introduces a novel high-throughput screening method using the MitoTimer reporter to assess chemical impacts on mtROS levels in aged male and female germlines. A key advantage of this approach is the ability to simultaneously test a large number of compounds and assess their effects on males and hermaphrodites/females in parallel. Unlike previous studies, we will specifically examine aged reproduction and integrate environmental factors. We will explore how toxicants affect mitochondrial function during reproductive aging and investigate transgenerational effects. The comprehensive methodology combines genetic, biochemical, molecular, cellular, and high-throughput techniques, including tissue-specific analyses. The approach offers an innovative strategy for unraveling the complexities of reproductive aging, with significant implications for understanding and addressing age-related fertility decline in humans. The knowledge gained will enable substantial advances in elucidating the complex interplay between mitochondrial function, environmental factors, and reproductive aging in both sexes.