https://doi.org/10.35662/unine-thesis-3066

Université de Neuchâtel
Faculté des sciences

The role of oxidative stress in senescence and immune responses of the mosquito *Aedes aegypti*

Thèse présentée à la Faculté des Sciences

pour l'obtention du grade de docteur ès Sciences

par

Alessandro Belli

Membres du jury :

Prof. Jacob Koella, directeur de thèse, Université de Neuchâtel, Suisse

Prof. Thomas Flatt, rapporteur, Université de Fribourg, Suisse

Prof. Daniel Croll, rapporteur, Université de Neuchâtel, Suisse

Soutenue le 7 septembre 2023

unine
Université de Neuchâtel
Faculté des sciences

Rue Emile-Argand 11
CH-2000 Neuchâtel
doctorat.sciences@unine.ch

IMPRIMATUR POUR THESE DE DOCTORAT

La Faculté des sciences de l'Université de Neuchâtel autorise l'impression de la présente thèse soutenue par

Monsieur Alessandro BELLI

Titre:

"The role of oxidative stress in senescence and immune responses of the mosquito *Aedes aegypti*"

sur le rapport des membres du jury composé comme suit :

- Prof. Jacob Koella, directeur de thèse, Université de Neuchâtel, Suisse
- Prof. Daniel Croll, Université de Neuchâtel, Suisse
- Prof. Thomas Flatt, Université de Fribourg, Suisse

Neuchâtel, le 11 septembre 2023

Le Doyen, Prof. R. Bshary

"Dietro non si torna
Non si può tornare giù
Quando ormai si vola
Non si può cadere più"

Vasco Rossi, Gli Angeli (1996)

Acknowledgements

I extend my heartfelt gratitude to Prof. Jacob Koella for providing me with the rare opportunity to pursue my ideas and questions in the field of science. His invaluable scientific advice and mentoring have been instrumental in my journey. Prof. Koella's direct, positive, and critical thinking has been an enduring source of inspiration.

I give special thanks to Alida, Giacomo, Anouk, Gaël, Patrick, Kevin, Tiago and Jon for their enriching discussions, valuable suggestions, constructive feedback, and unwavering support. Our time together, not only in scientific pursuits but also in camaraderie, has been truly memorable.

I am also grateful to Olivier (Pitou) and Alfonso for their valuable and essential help during laboratory work.

I thank Dolores, Cincy, Sandra, Luìs, and all others who have been part of our lab for their insightful comments and assistance, which have contributed significantly to my research.

I wish to express my gratitude to Thomas Flatt for his enlightening discussions during the mid-thesis phase and throughout the final examination. I extend my appreciation to Daniel Croll for his guidance during the final examination, his invaluable advice, and the stimulating discussions we've had.

Last but certainly not least, I would like to express my profound thanks to my family, whose unwavering support has constantly been present across time and space. And to Marie, without whom this thesis would never have been successfully concluded.

Abstract

Oxidative stress arises from an imbalance between the production of free radicals and the body's capacity to counteract them with antioxidants. An excess of free radicals can lead to damage in various cellular components. Initially, free radicals were considered inherently toxic, and given their presence throughout Earth's history, Harman proposed that the accumulation of oxidative damage over time was the fundamental mechanism behind senescence (ageing). However, this theory relied heavily on evidence from studies comparing data across different species. As studies began to manipulate free radical production and antioxidant defences within the same species, contradictory results emerged. Consequently, we adopted an evolutionary approach to explore whether the accumulation of oxidative damage could account for senescence within a species where two populations had been selectively bred for varying lifespans. Regrettably, our experiment did not yield direct support for the free radical theory of ageing. The long-living mosquito lines did not exhibit significant differences in oxidative damage levels compared to the short-living lines. Nevertheless, our investigation revealed that mosquitoes with longer lifespans had lower levels of reactive oxygen species (ROS) and higher levels of antioxidant defences, resulting in a superior redox potential. This suggests that rather than oxidative damage, an individual's ability to maintain the balance between free radical production and antioxidant defences throughout its life may be the primary driver of senescence.

Additionally, recent discoveries have unveiled the beneficial roles of ROS, which are essential for normal cellular metabolism. This paradigm shift led to a reevaluation of the role of free radicals in biology. We now understand that they are involved in several critical biological processes, such as cell proliferation, mobility, signalling, immunity, and more. However, it is crucial to recognize that while ROS play essential roles, they must be maintained within a narrow concentration range for their benefits to manifest. When employing them in their metabolic processes, organisms require effective defences against excessive ROS. Hence, our research aims to elucidate the role of antioxidant defences in the immune response of *Aedes aegypti* mosquitoes against the microsporidia parasite *Vavrai culicis*. Our study supports previous findings that ROS are vital for resistance against the parasite, and we also observed that individuals with higher levels of glutathione have a reduced probability of being infected by the parasite. Therefore, mosquitoes must produce ROS in sufficient quantities to have an effective immune response and possess robust antioxidant defences.

Finally, we investigated whether oxidative stress could be a mechanistic link in trade-offs between various life history traits. We examined whether males' susceptibility to oxidative stress influences the cost of reproduction and whether intrasexual competition drives this trade-off. Our results demonstrated that male *Aedes aegypti* mosquitoes experienced higher mortality rates, reduced tolerance to bacterial infection, and increased oxidative damage when residing in a group dominated by females (24 females / 8 males). This

suggests that oxidative stress is associated with the trade-off between reproduction and survival. However, the primary driver of this trade-off appears to be the act of mating itself, possibly due to the proteins males must transfer to females during copulation.

In conclusion, our thesis emphasizes that merely assessing oxidative damage is insufficient to comprehend the role of oxidative stress in various life history traits. What holds greater significance is the maintenance of a delicate equilibrium between free radical production and antioxidant capacity throughout an organism's lifespan. This equilibrium seems to drive the emergence and evolution of diverse life history traits and trade-offs.

Keywords: *Aedes aegypti*, oxidative stress, free radical theory of ageing, life history theory, host's immune response, longevity, cost of reproduction, *Vavraia culicis.*

Résumé

Le stress oxydatif résulte d'un déséquilibre entre la production de radicaux libres et la capacité du corps à les neutraliser avec des antioxydants. Un excès de radicaux libres peut endommager divers composants cellulaires. Initialement, on considérait que les radicaux libres étaient intrinsèquement toxiques, et compte tenu de leur présence tout au long de l'histoire de la Terre, Harman a proposé que l'accumulation de dommages oxydatifs au fil du temps était le mécanisme fondamental derrière la sénescence (vieillissement). Cependant, cette théorie reposait principalement sur des preuves provenant d'études comparant des données entre différentes espèces. À mesure que les études ont commencé à manipuler la production de radicaux libres et les défenses antioxydantes au sein de la même espèce, des résultats contradictoires ont émergé. Par conséquent, nous avons adopté une approche évolutionniste pour explorer si l'accumulation de dommages oxydatifs pouvait expliquer la sénescence au sein d'une espèce où deux populations avaient été sélectionnées pour des durées de vie variables. Malheureusement, notre expérience n'a pas apporté de soutien direct à la théorie des radicaux libres sur le vieillissement. Les lignées de moustiques avec un plus longue durée de vie n'ont pas montré de différences significatives dans les niveaux de dommages oxydatifs par rapport aux autres lignées. Néanmoins, notre étude a révélé que les moustiques vivant plus longtemps présentaient des niveaux plus faibles de espèces réactives de l'oxygène (ROS) et des niveaux plus élevés de défenses antioxydantes, ce qui se traduisait par un potentiel redox supérieur. Cela suggère que, plutôt que les dommages oxydatifs, la capacité d'un individu à maintenir l'équilibre entre la production de radicaux libres et les défenses antioxydantes tout au long de sa vie pourrait être le principal moteur de la sénescence.

De plus, des découvertes récentes ont dévoilé les rôles bénéfiques des ROS, qui sont essentiels au métabolisme cellulaire normal. Ce changement de paradigme a conduit à une réévaluation du rôle des radicaux libres en biologie. Nous comprenons désormais qu'ils sont impliqués dans plusieurs processus biologiques essentiels, tels que la prolifération cellulaire, la mobilité, la signalisation, l'immunité, et bien d'autres. Cependant, il est crucial de reconnaître que, bien que les ROS jouent des rôles essentiels, ils doivent être maintenus dans une plage de concentration étroite pour que leurs bienfaits se manifestent. Les organismes ont besoin de défenses efficaces contre les ROS excessifs lorsqu'ils les utilisent dans leurs processus métaboliques. Par conséquent, notre recherche vise à élucider le rôle des défenses antioxydantes dans la réponse immunitaire des moustiques *Aedes aegypti* contre le parasite microsporidien Vavrai culicis. Notre étude confirme les découvertes précédentes selon lesquelles les ROS sont essentiels pour la résistance contre le parasite, et nous avons également observé que les individus ayant des niveaux plus élevés de glutathion ont une probabilité réduite d'être infectés par le parasite. Par conséquent, les moustiques doivent non seulement produire des ROS en quantité suffisante pour avoir une réponse immunitaire efficace, mais ils doivent également posséder de solides défenses antioxydantes. Enfin, nous avons examiné si le stress oxydatif pouvait servir de lien mécanique dans les compromis entre divers traits d'histoire de vie. Nous avons

étudié si le coût de la reproduction était influencé par la susceptibilité des mâles au stress oxydatif et si la compétition intrasexuelle alimentait ce compromis. Nos résultats ont montré que les moustiques mâles d'*Aedes aegypti* présentaient des taux de mortalité plus élevés, une tolérance réduite aux infections bactériennes et des dommages oxydatifs accrus lorsqu'ils résidaient dans un groupe dominé par les femelles (24 femelles / 8 mâles). Cela suggère que le stress oxydatif est associé au compromis entre la reproduction et la survie. Cependant, la principale cause de ce compromis semble être l'acte de l'accouplement lui-même, peut-être en raison des protéines que les mâles doivent transférer aux femelles pendant la copulation.

En conclusion, notre thèse met en évidence que l'évaluation des dommages oxydatifs uniquement est insuffisante pour comprendre le rôle du stress oxydatif dans divers traits d'histoire de vie. Ce qui revêt une plus grande importance, c'est le maintien d'un équilibre délicat entre la production de radicaux libres et la capacité antioxydante tout au long de la vie d'un organisme. Cet équilibre semble être le moteur de l'émergence et de l'évolution de divers traits d'histoire de vie et de compromis

Mots-clés : *Aedes aegypti,* Stress oxydatif, Théorie des radicaux libres du vieillissement, Théorie des traits d'histoire de vie, réponse immunitaire de l'hôte, longévité, coût de la reproduction, *Vavraia culicis.*

Contents

Chapter 1

General introduction

1.1. Background

Life history theory was proposed by Stearn (1992) and Roff (1992) [1] and aims to explain the variation in the traits presented by an organism, such as size at birth, growth rates, size and age at maturity, reproductive effort, mortality rate and lifespan [2]. Three points are considered the plausible cause of the evolution of life history traits: (a) traits are shaped by the interaction of extrinsic and intrinsic factors, (b) extrinsic factors are the ecological parameters impacting survival and reproduction, (c) intrinsic factors are the physiological constraint that creates trade-off among life history traits. Thus, the classical life history theory approach aims to understand how the environment affects survival and reproduction, how traits are connected, and their constraints [3]. Additionally, the interaction between traits determines fitness, and the analysis of it provides insight into phenotypic adaptations [4]. Several studies found evidence of the factors influencing survival and reproduction at different ages and stages using this approach.

Moreover, thanks to the development of evolutionary genetic studies, it was possible to understand the constraint between the traits and in all these studies, we reported the existence of trade-offs. Trade-offs exist at three levels: 1) phenotypic level, which is defined as the whole-organism measurements of traits directly connected with reproduction and survival; 2) genotypic level, which is all types of evidence related to genetic (quantitative genetic, Mendelian or molecular genetic); and 3) the intermediate level [5]. This latter level is formed by the mechanisms connecting the genotypic and phenotypic levels. It includes the physiological and development processes under endocrinological control that determine the allocation of resources among the life history traits (reproduction, growth, maintenance and survival) [5].

Probably the most prominent example of a life-history trade-off is the cost of reproduction [5], and it is composed of two major components: costs paid in survival and costs paid in future reproduction [6]. Hence, investment in actual reproduction is thought to be a possible cause of the decline in future survival and reproduction, commonly called ageing. Ageing is defined as the decline of physiological functions over time, and the effect of ageing is reflected in a deterioration in both fecundity and survival [3]. The question of ageing is treated at two different levels: 1) the evolutionary, why has ageing evolved and maintained in the population, and 2) the mechanism by which ageing processes occur during an organism's lifetime.

For the evolutionary aspect, Medawar et al. (1952) proposed that the strength of natural selection declines with age, and enough old individuals are irrelevant for evolution [3], [7]. This fact has a significant effect on two types of genes:

a) Genes with positive effects early in life will be positively selected and accumulated in the genome even though they have adverse effects late in life. This effect is described as antagonistic pleiotropy. This effect is antagonistic pleiotropy because these genes affect more traits (pleiotropy) and have opposite (antagonist) effects according to the organism's age.

b) Genes with a deleterious mutation, expressed in only older, rather than younger, age classes, would be maintained and accumulated. This effect is described as mutation accumulation.

Both effects can happen because the selection strength for genes affecting young age is stronger than for genes affecting old age [7]. So, the evolutionary framework seems quite clear: senescence has originated and is maintained because the strength of natural selection decreases with time, and consequently, it cannot get rid of the genes harming the individual at an older age. Additionally, in the evolutionary view, ageing is a by-product of extrinsic mortality, no matter the molecular mechanisms [8]. For instance, an organism could live eternally. However, if even a minimal probability of dying by extrinsic factors exists (and this always exists), it will have a better fitness to invest more in reproduction at the expense of eternal life. Following this principle and the life history theory, we will find that the organism evolves an optimal investment in reproduction to maximize its fitness, even though this will be at the expense of eternal life, leading to ageing and death. Multiple studies tested this idea, such as the one well-known performed by Stearns et al. (2000) [4] with the fruit fly Drosophila melanogaster. They exposed several populations to either high extrinsic mortality (1% probability of surviving one week) or low extrinsic mortality (81% probability of surviving one week). After some generations, they tested the line for longevity, and they found that the population with high extrinsic mortality had a higher mortality rate (one measurement of ageing in population) than the lines under low extrinsic mortality.

Hence, ageing seemed to be well understood on the evolutionary level, but on the opposite, the mechanisms behind this process remain unclear. In various studies, primarily conducted in mammals but with the aim of expanding their applicability to diverse organisms, researchers typically list nine preliminary hallmarks that serve as shared characteristics of ageing. These hallmarks encompass genomic instability, telomere shortening, modifications in epigenetic patterns, decline in proteostasis, disruption in nutrient sensing regulation, impaired mitochondrial function, the emergence of cellular senescence, depletion of stem cell resources, and changes in intercellular communication [9]. One common thread among these hallmarks is the involvement of free radicals, specifically reactive oxygen species, as a mechanistic factor contributing to ageing. This concept aligns with the free radicals theory of ageing, originally proposed by Harman in the 1950s, which posits that ageing results from the damage inflicted by free radicals generated during normal metabolism [10].

Free radicals are defined by chemistry as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. In biology, the most prevalent free radicals contain an oxygen atom and are called reactive oxygen species (ROS) [11].

ROS are molecules formed from diatomic oxygen during a 4-step, 1-electron reduction reaction.

$$O_2(+e^-) \rightarrow O_2^{\bullet -}(+ e^- + 2\,H^+) \rightarrow\ H_2O_2(+ e^-) \rightarrow HO^\bullet + HO^-(+ e^- + 2\,H^+) \rightarrow 2H_2O$$

They are separated into two categories: (a) free oxygen radicals, which contain one or more unpaired electron(s), such as superoxide anion ($O_2^{\bullet -}$) and the hydroxyl radical ($HO^{\bullet}$), (b) non-radical ROS which do not have unpaired electrons, such as hydrogen peroxide (H_2O_2) [11]. The main property of free radicals is their high reactivity, allowing them to react with numerous organic substrates, leading to a chain reaction of radicals' production. For example, the hydroxyl radical ($HO^{\bullet}$) can react with a hydrogen link to carbon in an organic molecule. This reaction stabilized the hydroxyl radical, which became a water molecule. However, the carbon deprived of its hydrogen becomes a carbon-centred radical, which reacts rapidly with diatomic oxygen to produce a peroxyl radical. At this point, the peroxyl radical may react with a new organic substrate that can generate a new radical, and the chain reaction will continue. Often, the biomolecules touched by this reaction lose their normal configuration and biological function. For example, the free radical can attack the polyunsaturated fatty acids (PUFAs), such as the arachidonic acids of the cell membrane. As described above, the lipids lost one hydrogen and gained one oxygen, becoming a lipid peroxyl radical, which reacts with another PUFA to create lipid hydroperoxides. This latter can further react to create bicyclic endoperoxide that, after cleavage, will generate malondialdehyde (MDA) (Fig. 1). Once formed, MDA can either be enzymatically metabolized to produce water and carbonic dioxide or spontaneously react with cellular or tissular protein or DNA to form adducts resulting in biomolecular damages [12]. Due to this correlation between MDA and cellular damage, MDA has been used as a biomarker of oxidative stress in many health problems such as cancer, psychiatry, chronic obstructive pulmonary disease, asthma, or cardiovascular diseases [13].

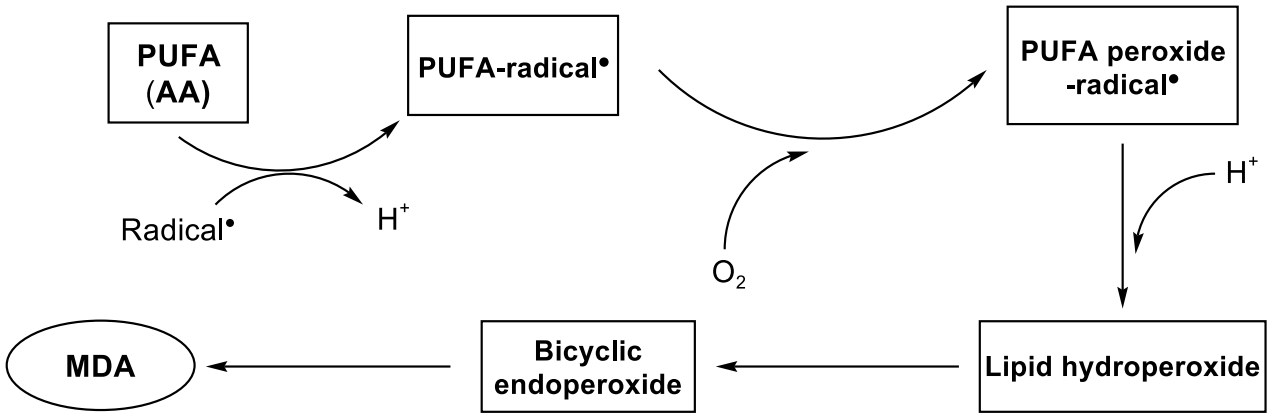

Figure 1: MDA formation and metabolism. MDA can be generated in vivo by the decomposition of arachidonic acid (AA) and larger PUFAs through non-enzymatic processes by bicyclic endoperoxides produced during lipid peroxidation. Adapt from [12].

Thus, ROS can be deleterious for biomolecules and lead to oxidative damage. Unfortunately, ROS are inevitable by-products of all aerobic metabolisms, and they are also intentionally produced by various enzymatic reactions in different cell compartments. The two main production sites are the cytoplasmic membrane NADPH oxidase (NOX) and the enzyme complex of the mitochondrial respiratory chain. NADPH oxidase is the enzyme responsible for the catalyzed reduction of molecular oxygen (O_2) into superoxide anion ($O_2^{\bullet -}$) [14], [15].This reaction can either occur in phagocytic cells to respond to an immune challenge

or in non-phagocytic cells, specifically vascular cells with probably a secondary messenger role [16]. Although NADPH oxidases are enzymes that produce ROS, they are no major source of reactive species within the organism. This role belongs to the mitochondria. During adenosine triphosphate (ATP) biosynthesis, electrons and protons pass through the electron transport chain to one final molecular oxygen, which is reduced to water. Around 1 to 3 % of the total electron may leak either in complex I (NADH-ubiquinone oxidoreductase) or complex III (ubiquinol-cytochrome c oxidoreductase) of the chain and leads to the production of superoxide anion [17]. Although the electron transport chain evolved to be highly efficient and the leak is minimal, the high activity of the mitochondrial respiratory chain in aerobic organisms makes this leak the primary source of ROS production in cells [18]. Other enzymes, such as xanthine oxidase (XO) in the cytosol, can also produce ROS [19].

Excessive ROS production can lead to oxidative damage, reportedly involved in several pathologies [20]. For instance, in metastatic cancer, researchers reported that tumour cells presented a mutated NADPH gene, causing a deficiency in the respiratory complex I and ending up in the overproduction of ROS that helped tumour progression [21], [22]. In the vascular system, high ROS levels can lead to hypertension and promote its pathological process [23]. ROS also negatively affect diabetes by reducing the organism's insulin sensitivity. Finally, the high consumption of molecular oxygen and the high content of polyunsaturated fatty acids, susceptible to peroxidation, of the brain makes this organ exceptionally sensible to oxidative damage, and numerous studies reported a role of ROS in neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) [24].

So ROS are deleterious for biomolecules, and they are involved in several deadly pathologies; however, the "recent" discovery of enzymes such as the NADPH oxidase or the xanthine oxidase, which have the scope of producing these molecules has questioned the vision of ROS as sole cytotoxic compounds. This questioning has pushed researchers to study ROS's roles in organisms more and discovered that they play an essential role in several physiological functions. For example, hydrogen peroxide could be a perfect secondary messenger for cell signalling [25]. Cell signalling or signal transduction is the process by which an extracellular message carried by a first messenger, such as hormones or cytokines, is passed across the plasma membrane into the intracellular components. An essential component of cell signalling is the secondary messenger responsible for carrying the signal from the first messenger, which is extracellular, to the intracellular component to eventually initiate the induction of physiological responses such as gene expression or cell proliferation. To be effective, this secondary messenger must be easily synthesized or destroyed according to an external signal, diffusible and ubiquitous. The fact is that hydrogen peroxide matches all these features, making it an optimal candidate [25]. Another example of ROS's role in physiology is the active induction of DNA demethylation by oxidative stress, an essential process in memory formation [26]. We have already spoken above about the enzyme NADPH oxidase and its function to produce superoxide anion. This enzyme has been found in the membrane of phagocytic cells, where it generates large amounts of $O_2^{\bullet -}$ to fight

invading pathogens. This large production of ROS is called an "oxidative burst" or "respiratory burst". In addition to their cytolytic activity, ROS are involved in several other aspects of the immune system, such as immune cell interaction and activation or suppression [27]. Finally, ROS physiological roles are not limited to the example above; studies reported ROS involvement in regulating cell homeostasis, stem cell proliferation and differentiation, autophagy, cell apoptosis, cell mobility and migration [28].

Nevertheless, ROS homeostasis control is a critical aspect of normal cellular functions. Any disruption of the oxidant-antioxidant balance can shift the ROS effect from beneficial to toxic, leading to an oxidative stress condition and cellular damage [27]. Keeping ROS homeostasis is critical because the disruption of the equilibrium and, consequently, the ROS-related physiological functions have been reported to help the onset of several pathologies as much as the cytotoxic effect of ROS themselves.

Therefore, all organisms have evolved several defensive mechanisms, the antioxidants, to contain the deleterious effects of free radicals and maintain their physiological level. Antioxidants include a wide range of non-enzymatic free radical scavengers. Some are exogenous, such as Vitamin C (ascorbic acid), Vitamin E (α-tocopherol) and carotenoids, acquired mainly through diet. Other are endogenous, such as Glutathione (GSH), uric acid, cysteine, melatonin, and coenzyme Q. Hence, the organism produces them through a series of reactions.

For instance, glutathione was discovered in 1888 [29], and since then, several researchers have studied its biosynthesis pathway, which is now well established (Fig 2). Y-glutamylcysteine (γ-EC) is synthesized from L-glutamate and L-cysteine thanks to the enzyme γ-glutamylcysteine synthetase. Then, glycine is added to the C-terminal end of the γ-EC by the enzyme glutathione synthetase (GS) to form the GSH. Both enzymatic reactions need one ATP each [30]. Once synthesized, GSH is the co-factor, allowing the enzyme glutathione peroxidase (GPx) to transform hydrogen peroxide into water. For each hydrogen peroxide molecule, two molecules of GSH are converted into one molecule of oxidized glutathione (GSSG), which can be recycled into its reduced form by the enzymatic reaction of the glutathione reductase and NADPH [30], [31].

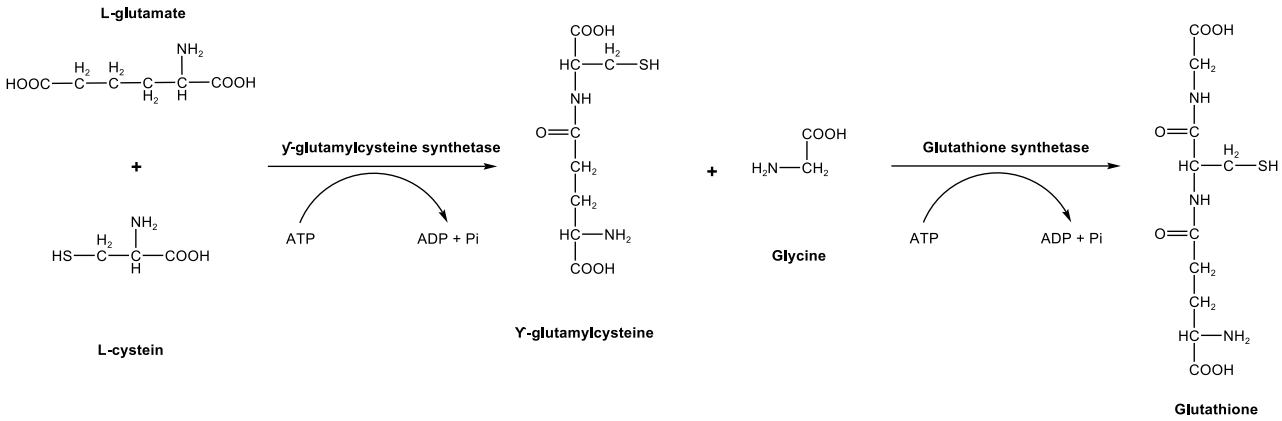

Figure 2 Schematic representation depicting the glutathione biosynthesis pathway from constituent amino acids. Adapt from [30].

In addition to the non-enzymatic antioxidant, the protection against the effect of ROS is carried out by several enzymes, namely the catalase, the glutathione peroxidase and the thioredoxin reductase that control the levels of hydrogen peroxide by converting it into water and molecular oxygen [32], [33]. Furthermore, we have the superoxide dismutase (SOD) that reduces the superoxide anion ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) [34]–[38].

Summarising ROS are either by-products of normal aerobic metabolism or produced to ensure specific physiological functions. Nevertheless, because they react with various biomolecules, their level and effect must be limited to avoid unwanted cellular damage. For this reason, organisms have evolved several mechanisms of defence called antioxidants, and in normal conditions, the organisms are in a situation where oxidants and antioxidants are balanced. However, if either ROS production increases or antioxidant defences decrease, it leads to an oxidative stress condition and cellular damage.

1.2. Thesis Introduction

In this thesis, I used the mosquitoes *Aedes aegypti* and the microsporidian parasite *Vavraia Culicis* to study senescence's evolution and oxidative stress's role in various life history traits.

1.2.1. Experimental evolution of senescence

Natural selection maximizes the fitness of organisms through the evolution of the optimal set of life history traits according to environmental, physiological and genetic constraints. Although the best life history strategy would be to maximize all the fitness components simultaneously (Darwinian demons) [39], it is evident that life history traits, such as lifespan and reproduction, show an inverse relation [40]. To explain the traits expressed, life history theory assumes that the resources are limited, and organisms increase their fitness by replacing costly activities with one another [41]. Often, reproductive activity is considered a priority, and organisms tend to invest more in that, but this can only be reached by reducing the investment of resources devoted to other energetically demanding activities [40]; within this view, the disposable soma theory of ageing was postulated to explain the senescence. This theory assumes that every organism disposes of a limited energetic budget that must be divided between different vital activities. Perpetuating the germ line is crucial in all species, so reproduction often diverts vital resources away from somatic maintenance and protection, accumulating cellular and molecular damages [42].

Moreover, reproduction is often associated with increased metabolic rate, leading to a situation where the organisms have reduced somatic protection coupled with high energy consumption. It has been demonstrated that individuals investing more and earlier in energetically expensive activities associated with reproduction suffer from rapid ageing and shorter lifespans [43]. Thus, it seems that reproduction can shape the evolution of ageing. Ageing is the time-related deterioration of physiological function necessary for the survival or reproduction of an individual [44], and several theories have been proposed to explain it [10]. For instance, Waldorf proposes that a prolonged and mild autoimmune response was the cause of the ageing process [45]. Hayflick postulated the concept that cells had a limited number of possible replications. After that, they will die, which is the cause of ageing in the organisms [46]. All these theories aimed to find a single process that explained the ageing of an entire organism. Thus, this process should be ubiquitous and present since the apparition of living on Earth. For that reason, in the 1950s, D. Harman proposed the free radical theory in which the ageing process of the organism is the result of the free radicals' action. This ageing theory proposed one process that could be modified by genetic or environmental factors, in which free radicals' action produced oxidative damage [10]. Accumulating such damage in cells or tissue leads to the organism's ageing. Harman reviewed this theory in the 1970s after discovering that mitochondria were responsible for the majority of ROS production, and thus, they were the main target of free radical action [47], [48]. But finally, the theory core remains the same. In addition, free radicals matched the requirement to be ubiquitous and are supposed to be the reason life appeared on Earth. So, following the free radical theory of ageing, the

free radicals are produced as by-products during the normal metabolite. Because of their high potential to damage the organism, this latter has evolved an extensive array of defences, the antioxidants, that balance and neutralize ROS production under normal conditions, and no oxidative damage should be produced. However, we have already discussed that ROS production can quickly increase in stressful situations, such as hypoxia or hormone stress [49] or by energetically demanding activities, such as reproduction or immune response [40], [50]. In these situations, we have an imbalance between oxidants and antioxidants, and the organism suffers from an oxidative stress condition. The accumulation of damages, which negatively impacts the organisms' physiological functions with time, will result in the ageing and death of the living being.

With our study, we wanted to test the prediction of the evolutionary theory that senescence is a by-product of the selection of reproductive fitness and that free radicals might be the physiological mechanism of the ageing process.

1.2.2. Effect of sex proportion on the physiology and immunity of male Aedes aegypti

As said before, reproduction cost is the most prominent trade-off in life-history theory. The hypothesis behind this cost posits that reproduction is costly for future survival and reproduction. This cost can be increased by an external factor such as first breeding, predation or parasitism [51], [52]. As organisms are selected to maximize their lifetime fitness, they face a trade-off between their current and future fitness, composed of a combination of their future survival and reproduction. Hence, according to the classical approach, we found that if the adult mortality rate increases in one adult class, the optimal reproductive effort increases before such age [53]. Or if the mortality rate increases at all age, age at maturity decrease, and the optimal reproductive effort increase early in life [53].

However, the physiological mechanisms controlling these trade-offs are more challenging to understand, and it is where studies need to focus their attention. Traditionally, the cost of reproduction is explained by the limited resource allocation. The base idea is the Y model, where resources are acquired and allocated to reproduction or somatic maintenance. Evidence of this trade-off can be seen in the cricket *G. firmus*, where short-winged divert fewer resources to synthesizing triglyceride (used for movement) and more resources to the production of ovarian proteins than long-winged females even though both morphs consumed and assimilated an equivalent amount of food [54] Other studies expanded this vision to include non-energetic aspects of the reallocation, such as the hormonal control of antagonistic traits [55]. For example, a *Drosophila melanogaster* mutant producing less juvenal hormones showed increased longevity compared to the wild-type strain [54]

Finally, recently, it was proposed that oxidative stress could be a possible mechanism explaining the link between traits, and several eco-physiologists embraced this concept and studied it under natural, semi-natural and laboratory conditions. However, the results have often been contradictory, mainly from

correlative studies [56]. Thus, with our study, we wanted to understand the cost of reproduction better, and in particular, we are interested in understanding whether the reproduction cost we observed in males of *Aedes aegypti* survival was mediated by reactive oxygen production and oxidative stress susceptibility.

1.2.3. Genetic correlation between immunity and free radical resistance in Aedes aegypti

Mosquito innate immunity is composed of humoral and cellular components. Hemocytes are the central pillar of the cellular response, whereas several soluble components, such as pattern-recognition receptors (PRRs), antimicrobial peptides (AMPs), oxygen/nitrogen reactive species or components of the phenoloxidase cascade, form the humoral responses in the hemolymph [57], [58]. However, this separation line is somewhat subjective, given that hemocytes produce several humoral responses, while many humoral molecules play essential roles in regulating hemocyte activities [59]–[64]. For this reason, I will further explain the different mechanisms without classifying them into one of the two immune components.

The initial step of the immune response is recognition, which the PRRs perform in the hemolymph. These receptors scout the hemocoel for any pathogen-associated molecular patterns (PAMPs) [57], [65]. Once they recognize PAMPs, they start a signalling cascade to activate specific immune responses and neutralize the pathogen. For instance, the TEP1 family of PRRs has been reported to enhance phagocytosis. This role has been experimentally reported in several invertebrates such *as D . melanogaster, An. gambiae*, and *Ae. Aegypti* [66].

Hence, we have already introduced the second step of the immune response, which is signalling, which consists of passing the signal from the extracellular matrix to the cytoplasm of the immune cells to activate the constitutive effector mechanisms such as melanization, AMPs or ROS to neutralize the pathogen. The signalling is carried out by several families of receptors expressed on the membrane surface of the cells [57], [65]. One of these families that has been well studied is the Toll-like receptors (TLRs), induced by gram-positive bacteria and fungi and activating the cellular responses and the production of AMPs. This pathway has been reported to significantly influence regulating the resistance against the dengue virus in *Ae. Aegypti* [67]. Another example is the IMD pathway, which has been reported to be activated by bacteria and *Plasmodium*, and interestingly, the activation of this pathway seems to be regulated by the interaction with the endogenous bacterial flora of the mosquito midgut. It has been reported that mosquitoes carrying specific bacteria strains in their midgut were less susceptible to dengue virus, thanks to the induction of AMPs by the midgut microbiota [68].

So, in our immune response timeline, we have, first, pathogen recognition and, second, signalling. The next step is the activation of the effector mechanisms to control and neutralize the infectious agent, such as AMPs, which are small peptides, often positively charged and produced in the hemocytes, fat bodies and epithelial cells in response to the recognition of specific PAMPs by the PRRs. Like the defensins induced by bacteria,

both Gram-positive and Gram-negative are the predominant immune-inducible peptides in mosquitoes [69], [70]. Another important effector mechanism in mosquitoes regulated by PRRs is phagocytosis, which neutralizes and removes microorganisms such as bacteria, yeast, and *Plasmodium*. These pathogens can also be neutralized by melanization, another critical effector of the immune response, but also involves the formation of the egg chorion or wound healing. The detection of PAMPs triggers a series of enzymatic and non-enzymatic reactions in forming a thick and dark proteinaceous complex around the pathogen. Thus, melanization kills the pathogens by isolating them from the surrounding environment, denying any exchange of nutrients or oxygen [57], [65].

Additionally, the production of melanin, the material constituting the coat surrounding the pathogen, and its intermediates created an oxidative stress environment. This environment helps to kill the pathogen. However, it can also harm the mosquito cells, causing damage and adverse effects. Finally, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are two essential effector components of the mosquito immune response. Several studies reported their roles in bacterial and plasmodial infections. It has been proved that a refractory strain of *An. Gambiae* to *Plasmodium* infection showed an increased level of ROS when challenged with the pathogen [71]. Another study found a reduction of the melanization of *Plasmodium* after the oral administration of antioxidants to mosquitoes [72]. This evidence revealed the fundamental role of ROS for mosquitoes to mount an effective immune response. However, the benefits of using ROS in immune responses are related to the organism's capacity to deal with its potential autoimmune effects. For this reason, mosquitoes dispose of a wide range of defence mechanisms, the antioxidants presented in the previous sections.

All these mechanisms are energetically costly, and according to the life history theory, immunity should have evolved in the context of cost and benefits. One cost of mounting an immune response can be the increase of the metabolic rate up to 28%, which, on the one hand, demands energy but can dramatically increase the production of toxic by-products such as ROS [73]. As we have seen before, ROS can be beneficial for immune responses. However, the expression "the dose makes the poison" is more than true with ROS. Excessing these molecules can cause an imbalance in physiology, leading to an oxidative stress status and an accumulation of cellular damage that can lead to organism death.

For this reason, with our study, we aimed to understand whether susceptibility to oxidative stress could be a mechanistic link between immune responses and other traits and better understand the role of antioxidants in immune responses. In addition, until now, we have discussed only the mechanisms of parasite resistance. However, it has been suggested that organisms have two immune strategies to deal with an infection. The first is resistance, so try to reduce the infection load. The second is to tolerate the infection, and instead of reducing the parasite load, it could reduce the negative effect of the infection on its fitness and the effect of being resistant to the parasite. Studying tolerance requires precise fitness measurements.

However, these measurements are often not practical in many animal models. So they are replaced by health-based proxies, incorporating metrics such as longevity loss, anaemia, fecundity loss or mass loss [74]–[76]. Then, tolerance is calculated using a reaction norm approach, specifically the slope of the relationship between the fitness or the health-based proxies and the parasite burden [77]. It has been proposed that tolerance should be divided in two: 1) tissue-specific tolerance (t_{ts}), which limits fitness losses by reducing tissue damage during infection [78] and 2) behavioural tolerance (t_{beh}), which by keeping normal, fitness-enhancing behaviour, try to limit the fitness losses [79]. Nowadays, research in the field of animal tolerance to the parasite is a rapidly developing area. However, the immune mechanisms regulating this immune response remain mainly unknown. It has been suggested that tissue-specific tolerance relies on mechanisms reducing the damages caused by the parasite and by the host's immune system [78]. Therefore, we are interested in understanding whether tissue-specific tolerance is correlated with antioxidant defences, which are one of the possible organism's defences against cellular damage.

1.3. Experimental system

1.3.1. The mosquito Aedes aegypti

We used the UGAL strain of Ae aegypti for all the experiments reported in this thesis. This strain was established in the 1970s but is undocumented [80]. Patrick Guérin (University of Neuchâtel) kindly gave it to our laboratory. The mosquito *Aedes aegypti* is the primary vector of yellow fever [81] and several arboviruses such as dengue, chikungunya fever and Zika virus [82], [83]. *Ae. aegypti* is globally distributed in tropical and subtropical regions, where its larvae grow easily in various natural and artificial containers holding clean, fresh water [84]. Its holometabolous life cycle includes four stages: egg, larva, pupae and adult (Fig. 3). The only non-aquatic stage is the adults; only females require a blood meal to produce and lay the eggs. Its ecology has been studied intensively and is known in detail [85], and its whole genome has been published [86]. The possibility to stock eggs for an extended period and the need for a single mating for the females to lay eggs throughout their life are two aspects that make this species suitable for work in the laboratory and on experimental evolution of life history traits [87], [88]. Additionally, we can place the eggs under a partial vacuum, which results in a synchronized hatching of the larvae, assuring that we have individuals of the same age in our experiments [85].

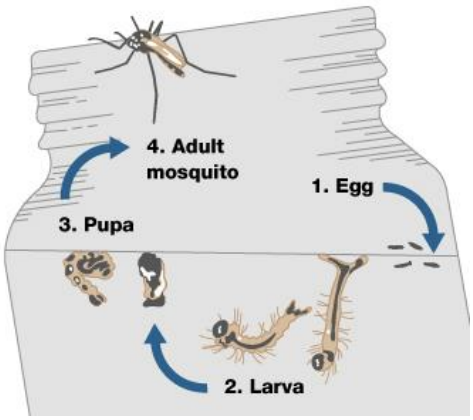

Figure 3: Life cycle of Aedes aegypti Life cycle of the mosquito. The mosquito's development comprises four separate and distinct stages: egg, larva, pupa, and adult. The juvenile stages and aquatic stages are the larva and pupa. The adult is the only non-aquatic stage. The mosquito species Aedes aegypti require a blood meal to lay eggs. Image source [89]

1.3.2. Vavraia culicis

The group Microsporidia belong to the kingdom of fungus, and it is composed of a variety of species of single-cell intracellular parasite with a broad range of hosts. They are widespread in arthropods (almost half of the species described are parasites of insects). The species infecting mosquitoes have been studied for more than a century on topics varying from biological control to the effect on the development of mosquito-borne diseases [90]. They are an optimal model system for fundamental and applied research because they are easily manipulated in the laboratory. For all the experiments reported in this thesis, we used the

microsporidian parasite *Vavraia culicis*, initially derived from *Ae. albopictus* in Florida and obtained by J.J. Becnel (USDA, Gainesville, USA).

It has been isolated initially from *Culex pipiens* field populations and then associated as a natural parasite of several genera of mosquitoes, including *Aedes aegypti* [91], [92]. Thus, *Vavraia culicis* is a common parasite of mosquitoes; the natural prevalence ranges from 1% to 54%, depending on the mosquito species and geographical location [93].

It is a multisporous species and an obligate intracellular parasite. All stages are uninucleated; only one stage can infect new larval hosts orally (Fig. 4). Once ingested, the spores infect the gut and the epithelial cells and then spread to the fat body and wing muscles [93], [94]. It has been reported that *Vavraia culicis* can evade most of the mosquito's immune responses probably because its development within the host involves few intercellular movements. The spores are the only stage of the parasite that can survive outside the host cells, and they are transmitted in two ways. First, horizontally among larvae when infected larvae die or release spores in the environment [93], [95]. In the second way of transmission, the larvae survive the infections without clearing them and normally develop into adults. The spores are released in the environment at the host's death or in the females; the parasites migrate to the ovaries, adhere to the eggs' surface, and are released during oviposition. In this way, they can infect newly hatched larvae, known to feed on eggshells [93]. In stressful situations such as food restriction or high spore density, it was reported that the parasite significantly increased its virulence to favour the spores' horizontal transmission [96].

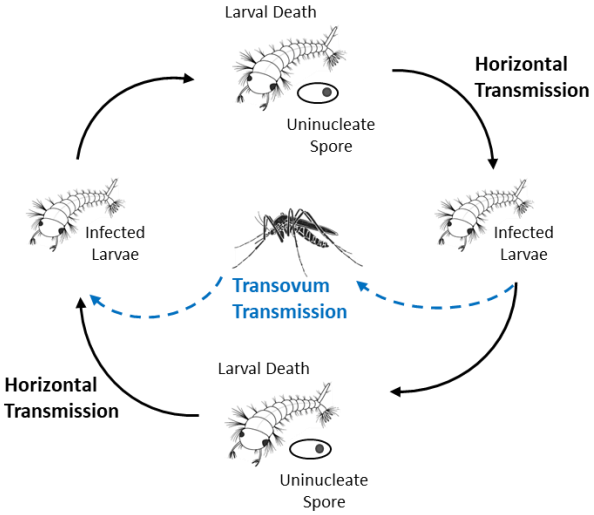

Figure 4: Life cycle of Vavraia Culicis. Larvae are infected from the injection of spores (solid lines) released either by dead larvae (horizontal transmission) or dead adults (not shown in the figure). So, infected larvae could die to continue spreading the spores or survive and develop into adults. Adults could die in the aquatic environment and release the spores to infect new larvae (not shown in the figure) or in females; the parasite can adhere to the eggs' surface and be released with them to infected newly hatched larvae (transovum transmission, dashed lines) (Images Source: Vecteezy.com)

1.4. Research aim

This PhD aims to investigate the role of free radicals in the physiology of the mosquito *Aedes aegypti*. We wanted to understand better oxidative stress's influence on some mosquito life-history traits, such as resistance and tolerance to parasites, reproduction and somatic maintenance. Additionally, with an evolutionary experimental approach, we aimed to assess whether the evolution of the ageing process could be attributed to different management of oxidative stress. The thesis is based on three main projects reported in the following chapters:

Chapter 2

Aim to determine if there is a genetic correlation between the immune responses (tolerance or resistance to parasites) and the antioxidant defences. Reactive oxygen species are a fundamental part of the insect immune response but need a closed control system. Thus, we aim to investigate whether the difference in immune responses reported between individuals could be associated with the physiological antioxidant defences.

Chapter 3

Oxidative stress may be the link between reproduction and survival in males. Classically, the mechanistic link between reproduction and other life-history traits is energy. However, susceptibility to oxidative stress has been proposed as an alternative. We aimed to investigate that possibility.

Chapter 4

Test theoretical predictions about the evolution of senescence when reproductive fitness is manipulated. Investigate the role of oxidative stress on the evolution of the ageing process and if it can be the physiological mechanism.

Chapter 2

Genetic correlation between immunity and free radical resistance in *Aedes aegypti*

Belli Alessandro[a] and Jacob C. Koella[a]

[a] Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland

2.1. Abstract

Mosquitoes have long been acknowledged as organisms where reactive oxygen species (ROS) play a dual role, akin to a double-edged sword. While ROS are recognized as potent toxic by-products of aerobic metabolism, recent studies have illuminated their indispensable role in various biological functions, with immunity taking center stage. Specifically, immune cells employ dedicated enzymes to generate ROS in response to infections. However, the beneficial effects of ROS can rapidly transform into toxicity unless the delicate equilibrium between their production and the detoxifying action of antioxidants is carefully maintained. This paper explores the intricate relationship between mosquito immune responses, ROS, and antioxidant defences. We propose the intriguing possibility that it is not solely the quantity of ROS but the equilibrium between ROS and antioxidants that ultimately shapes the immune responses of these vectors. Our findings, based on investigations in *Aedes aegypti*, reveal that resistance to the microsporidian pathogen *Vavraia culicis* is genetically determined and unaffected by maternal influences. Strikingly, resistance exhibited a positive correlation with glutathione's overall physiological levels and oxidised glutathione levels in mosquitoes. These results affirm the role of ROS in the immune response while emphasizing the organism's need for sufficient defences against ROS toxicity to enhance resistance against parasites. Remarkably, our resistant mosquitoes displayed no signs of oxidative damage, suggesting their ability to induce ROS production only when required. This finely tuned balance underscores the intricacies of mosquito immunity.

2.2. Introduction

Oxidative stress describes the imbalance between free radicals and antioxidant defences. Free radicals are highly reactive [97] and rapidly oxidize other molecules, starting a chain reaction that damages lipids, proteins, nucleic acids and carbohydrates. Because of these oxidative damages, molecules gradually lose their ability to function, with potentially catastrophic consequences for the organism. Free radicals are often produced as by-products of essential biochemical processes [98]. Aerobic respiration, for example, produces peroxide and superoxide anions (that is, reactive oxygen species (ROS)) when mitochondria reduce oxygen to water to produce ATP [28]. The deleterious effects of such free radicals are blocked by antioxidants, which are non-enzymatic free radical scavengers such as vitamin C, vitamin E, cysteine, uric acid and glutathione (GSH), and enzymes such as superoxide dismutase (SOD), catalase, GSH-peroxidase and GSH-reductase peroxide [34]–[36].

Free radicals, however, are not only harmful by-products of essential processes. Instead, ROS are also essential signalling molecules in physiological processes such as cellular homeostasis, stem cell proliferation and differentiation, cell motility and migration, autophagy, apoptosis and ageing, and they are part of immune responses. Thus, regulating the production and defence against ROS is a delicate balance that enables their biological functions without suffering too much oxidative damage [28].

Essential biological functions that often involve ROS are a defence against parasitic infections through resistance (i.e., reducing the pathogen load) and tolerance (i.e. reducing the damage induced by the pathogen). Resistance of many insects, for example, is helped by the secretion of ROS in the hemolymph or midgut, which kills parasites, bacteria, malaria and other parasites, and the phagocytosis of pathogens, during which ROS are used to kill the pathogen inside the phagosome. Tolerance is less clearly linked to ROS. However, during an immune response, an organism undergoes several metabolic changes, including anorexia, depletion of glycogen energy reserves, and increased oxygen and energy consumption [99]–[102], all of which increase the probability of producing toxic by-products such as ROS. More tolerant individuals may be more able to compensate for the damages inflicted by the immune response and thus maintain better health when the pathogen load is equal [103]. If the ideas described above are essential, we expect clear correlational patterns between uninfected individuals' oxidative stress markers, resistance and tolerance. Thus, individuals who produce more ROS should be able to use it in their immune response and, therefore, be more resistant to infection, and individuals with more antioxidant defences should be better protected against an efficient immune response and be more tolerant. We tested these ideas with the mosquito *Aedes aegypti* and one of its parasites, the microsporidian *Vavraia culicis*. In particular, we were interested in the genetic correlations to understand whether correlations with oxidative stress markers would constrain the evolution of resistance and tolerance.

2.3. Material and Methods

2.3.1. Ades aegypti

The mosquito *Aedes aegypti* is a vector of arboviruses like those causing yellow fever [81], dengue [85], Chikungunya or Zika [82]. It is ubiquitous in tropical and subtropical regions. We used the UGAL strain of the mosquito (obtained from Patrick Guérin, University of Neuchâtel), which we had maintained in our standard laboratory conditions for many years at 26.5 °C, 70% humidity, and 12:12 hours light-dark photoperiod with access to 10% sucrose solution.

2.3.2. Vavraia culicis

As a parasite, we used the microsporidian *Vavraia culicis* (obtained from J.J Becnel, USDA, Gainesville, USA). *Vavraia culicis* parasitises many *Aedes, Culex, Anopheles, Culiseta, Ochlerotatus* and *Orthopodomyia* mosquitoes species. It produces a single spore type, which is used for transmission. When larvae have ingested spores, the parasite replicates intensively within its host before producing the next generation of spores, which are released to the environment when infected larvae die, with the faeces of infected individuals, or when infected females survive to become adults and lay their eggs into water [93]. Our laboratory stock has been maintained for ten years, alternating generations on *Ae. aegypti* or *Anopheles* gambiae, during which larvae were infected with spores obtained from homogenized adults at least ten days old.

2.3.3. Half-sib families

We estimated genetic variances with a half-sib design [104], where each male was mated with two females. Genetic correlations were estimated with the correlations of family means, although this tends to overestimate the correlations, for we could not measure each trait on each individual.

To generate the half-sib families, we hatched uninfected eggs of the colony under reduced atmospheric pressure and reared larvae individually in 12-well tissue-culture plates containing 3 mL of deionized water. They were fed daily with TetraMin™ fish food (day 0 (day of hatching): 0.06 mg/larva, day 1: 0.08 mg, day 2: 0.16 mg, day 3: 0.32 mg, day 4: 0.64 mg, from day five onwards: 0.32 mg). Each pupa was placed into a 50-mL falcon tube with bed netting for emergence. Three days after emergence, two females and one male were transferred into a 180-mL plastic cup covered with mosquito netting. The male was removed three days later, and the females were separated. Females were allowed to blood feed on AB's arms for 8 minutes and to lay eggs on a filter paper for six days. The filter paper with the eggs was removed and stored in a petri dish in the same laboratory conditions as the colony. The cycle of blood-feeding and egg-collecting was repeated for four weeks.

2.3.4. Main experiment

We haphazardly chose ten half-sib families for which both full-sib families had enough eggs for the experiment. The eggs were rehydrated in deionized water and let hatch synchronously under reduced air pressure. Unfortunately, one family did not have enough viable eggs. Thus, we continue the experiment with nine families. The larvae were reared individually in 12-well tissue-culture plates containing 3 mL of deionized water, with each plate containing six individuals of two full-sib families sharing a father. The larvae were fed TetraMin™ fish food according to the regime described above. Four days after hatching, half of each family's individuals were exposed to 20'000 spores of *Vavraia culicis*. The number of spores and their vitality were checked on the day of infection with a counting chamber (Neubauer improved bright-line, 0.0025 mm2, depth 0.100mm) and a phase-contrast microscope (Zeiss Axio Lab.A1).

Pupae were individually transferred to Falcon tubes, and the emerging adults were provided with a cotton ball soaked with a 10% sugar solution. Males and females were separated, and the two-day-old females were moved individually to a plastic cup containing one two-day-old male from our colony. Each pair was allowed to mate for seventy-two hours. The pairs in which one individual died were discarded from the experiment. The females were grouped in a 1.5L glass jar covered with bed-netting by the mother and treatment (infection or no infection with *V. culicis*) for the blood meal (8 minutes on AB's arm). After the blood meal, the females were supplied with a cotton ball soaked with a 10% sugar solution. Twenty-four hours later, they were moved individually to plastic cups containing moist filter paper for laying eggs.

We counted the eggs laid by each female, and the survival was checked daily. Seven days after the blood meal, the surviving females were killed at -20°C and stored in a 2 mL Eppendorf tube at -80°C for further analyses. The mosquitoes that had died earlier were not used for further analysis.

In the infected mosquitoes, we measured spore load by adding 0.1 mL of deionized water to the dead mosquitoes, homogenizing them with a TissueLyser LT – QIAGEN, and counting the spores using a counting chamber and a phase-contrast microscope.

2.3.5. Oxidative stress assay

All uninfected females were used to assay the oxidative stress markers. On the day of glutathione assay, the wings and legs of each mosquito were removed, the rest of the body was weighed to the nearest 0.001 mg, placed into a 2 mL tube containing a 5mm steel bead and 120 µL of PBS, homogenised with a tissue lyser for 4 minutes at 45 Hz and centrifuged for 10 minutes at 10'000 rpm at 4 °C. Each homogenate was split into three aliquots and stored at -80 °C.

Glutathione peroxidase quenches cellular ROS by oxidizing the glutathione in glutathione disulphide, so the level of oxidized glutathione is a measure of the ROS detoxified by the cells, and the proportion of the

oxidized form over the total glutathione is a measure of the oxidative balance of the cells [105], [106]. To determine the glutathione levels in the reduced (GSH) and oxidized (GSSG) forms, we extracted glutathione on the day we homogenized the mosquitoes. We added 100 µL miliQ water, 5 µL formic acid 1.25% and 5 µL glutathione ethyl ester 1.25 µg/mL (GSHee; Sigma Aldrich, USA) to one of the aliquots (15 µL) of each homogenate, mixed with a vortex for 10s, and centrifuged the solution for 15 minutes at 15'000 RPM and 4°C. We then removed 115 µL of the extract and placed them in a syringe with a PTFE hydrophilic filter (pore size 0.22 µm), and gently blew it into a glass HPLC vial to ensure no particles were present in the samples. The two forms of glutathione were quantified with UHPLC-MS/MS following Rojas Mora et al. (2016) [107] and were added to obtain the total glutathione.

Superoxide dismutase is part of the antioxidant defence mechanisms of organisms. It was quantified with the Cayman Superoxide Dismutase Assay Kit (Cayman Chemical, USA) with minor modifications. In particular, the mosquito's homogenate was diluted 1 in 8 parts PBS. Samples were run in duplicates with an average within-plate coefficient of variation (CV) of 2.59%, and 35 samples were assayed in several plates, showing inter-plate repeatability of r = 0.92.

Malondialdehyde (MDA) is one of the final products of the peroxidation of polyunsaturated fatty acids in cells. Since its production releases free radicals, it is often used as an oxidative stress marker. We quantified MDA following the procedure described by Mendonça et al. (2017) [108] with minor modifications. 40 µL of NaOH 1.2 M and 10µL of mosquito homogenates were mixed and incubated for 30 minutes at 60°C for the protein hydrolysis. The samples were cooled at 4°C for 2 minutes, and 10 µL of the internal standard (d2-MDA 30 µM in 0.1 M HCl) was added. So that the proteins precipitated, we added 142 µL of trichloroacetic acid 20%, mixed, sonicated, and then centrifugated the samples for 5min at 9300 rcf. 180 µL of the supernatant were transferred to a 1.5 mL microcentrifuge tube. We added 18 µL of 2,4 dinitrophenylhydrazine 5mM and incubated the samples for 10 minutes at room temperature with gentle agitation to derive the MDA. 22 µL of NaOH 10 M were used to alkalize the sample. We added 250 µL of a solution of toluene and cyclohexane (1:1 v/v) and transferred the supernatant to a 1.5 mL microcentrifuge tube for the two phase-to-phase extractions. The recovered organic phase was evaporated in a SpeedVac ® at 35°C, and the pellet was reconstituted in methanol 50%. The extracts were finally filtered with a 22 µm PTFE filter (BGB, Germany) into an HPLC vial before being analyzed with an HPLC-MS. A standard curve, ranging from 0 to 40 µg/mL, was done with MDA tetrabutylammonium salt (Sigma-Aldrich Inc. St-Louis, Missouri), and all the standards went through the extraction protocol together with the samples.

2.3.6. Statistical analyses

All analyses were done with the software R (version 4.2.2). We assessed significance with the function Anova() of the car library, using type III sums of squares if interactions were significant and type II otherwise.

The variances among mothers included genetic variances and maternal effects; the variances among fathers were considered to reflect the genetic variances.

2.1.1.1. Resistance

We defined the absence of spores as the resistance of a mosquito and the proportion of uninfected individuals as the resistance of a family. We analyzed resistance with a generalized linear model mixed effect (GLMER) with a binomial distribution of errors, where the explanatory variables were the father and the mother nested within the father. Since mother and father were considered random factors, we analysed three models (one with mother and father, one with only mother and only father) and tested for significance by comparing the models using the function Anova().

2.1.1.2. Tolerance

We defined tolerance as the residual of the regression of fecundity on parasite load using all infected individuals. We analysed these residuals with a linear mixed effect model (LMER) with a Gaussian error distribution, where the explanatory variables were the father and mother (see above) and the average fecundity of the uninfected individuals of the full-sib family. We chose to analyse only fecundity tolerance instead of survival tolerance or both because *V. culicis* is known to cause a low mortality rate, which was also confirmed in our study, and thus, only a few individuals died during our study. Consequently, the data on longevity tolerance had low statistical power.

2.1.1.3. Oxidative stress markers

We analysed each marker of oxidative stress (Total GSH, GSSG, SOD and MDA) with a linear mixed effect model (LMER) with a Gaussian error distribution, where the explanatory variables were the father and mother (see above). Only uninfected individuals were tested for oxidative stress, so we did not include the infection status.

2.1.1.4. Correlations

We used the function cor. test() to calculate the correlations with the means of each full-sib family and the means for each half-sib family.

2.4. Results

2.4.1. Resistance and tolerance

Fathers significantly influenced the proportion of uninfected offspring (χ_2 = 8.0137, df=1, p<0.001, variance explained 93.2% (1.568/1.683)); conversely, the mother's identity didn't (χ_2 = 8.0137, Df=1, p = 0.349, variance explained 6.8% (0.115/1.683)) (Fig. 5).

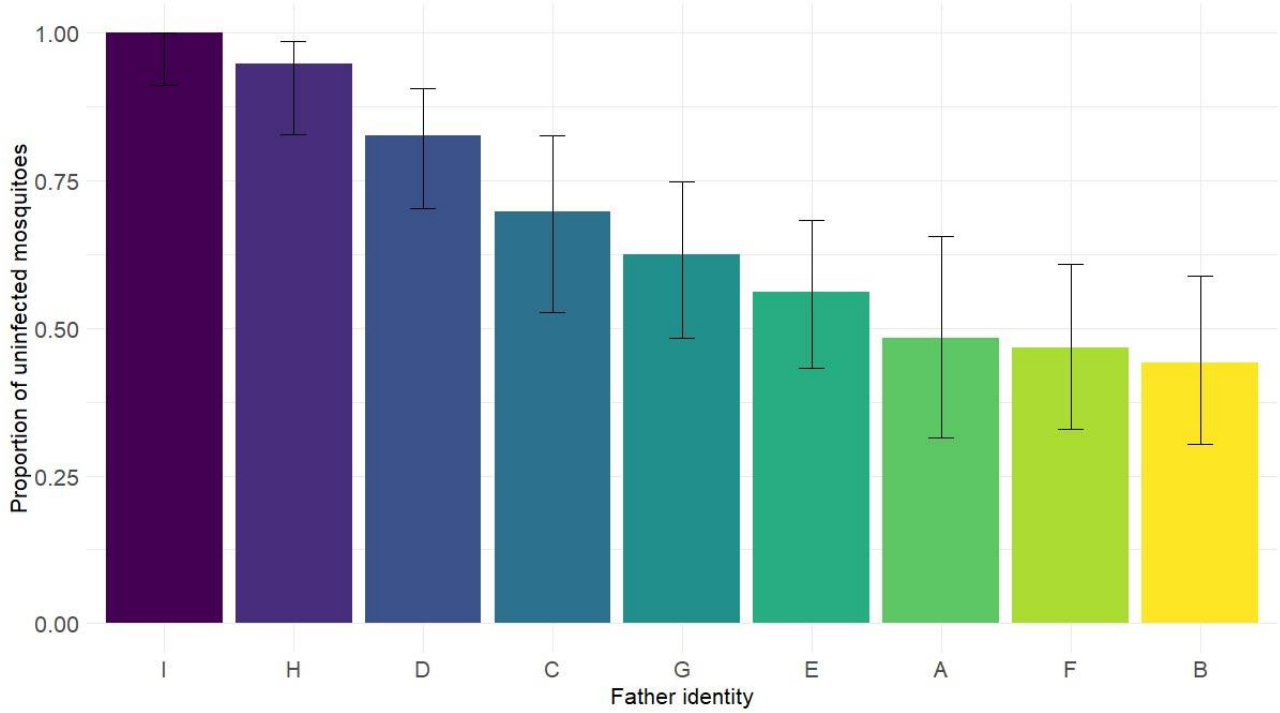

Figure 5: Proportion of uninfected mosquitoes for each half-sib family. The bars indicated the family's proportion, and the vertical lines indicated the 95% confidence intervals.

For tolerance, neither the father's identity (χ_2 = 0.033, df=1, p = 0.856, variance explained 8.7% (30.057/343.532)) nor the mother's identity (χ_2 = 0.067, df=1, p = 0.796, variance explained 1.8% (6.327/343.532)) has a significant influence.

2.4.2. Oxidative stress markers

Father's identity did not significantly influence either the physiological level of total glutathione (χ_2 = 0, df=1, p = 1, variance explained 0% (0/3610.2)) or the physiological level of superoxide dismutase (χ_2 = 0.8, df=1, p = 0.38, variance explained 2.0% (0.001/0.068)), or the level of lipid peroxidation (MDA) (χ_2 = 0, df=1, p = 1, variance explained 0% (0/0.025)) or the physiological level of oxidized glutathione (χ_2 = 2.2, df=1, p = 0.14, variance explained 9.6% (10.08/104.95).

In contrast, mother's identity is significant in determining the physiological level of total glutathione (χ_2 = 32.8, df=1, p < 0.001, variance explained 25.9% (935.9/3609.9)), the level of lipid peroxidation (MDA) (χ_2 = 28.5, df=1, p < 0.001, variance explained 21.6% (0.005/0.025)) but not the physiological level of superoxide

dismutase (χ_2 = 0, df=1, p = 1, variance explained 0% (0/0.068))) and physiological level of oxidized glutathione (χ_2 = 1.74, df=1, p = 0.19, variance explained 4.7% (4.92/104.95)).

2.4.3. Correlation

In half-sib families, we found that the resistance is positively correlated with the total amount of glutathione (r (7)= 0.717, p = 0.037). No other oxidative stress markers were significantly correlated with resistance (SOD: r (7)= -0.416, p= 0.270; MDA: r(7)= 0.117, p= 0.780). However, the physiological level of oxidized glutathione showed a slightly non-significant positive correlation with resistance GSSG: r (7)= 0.617 p= 0.086) (Fig. 6).

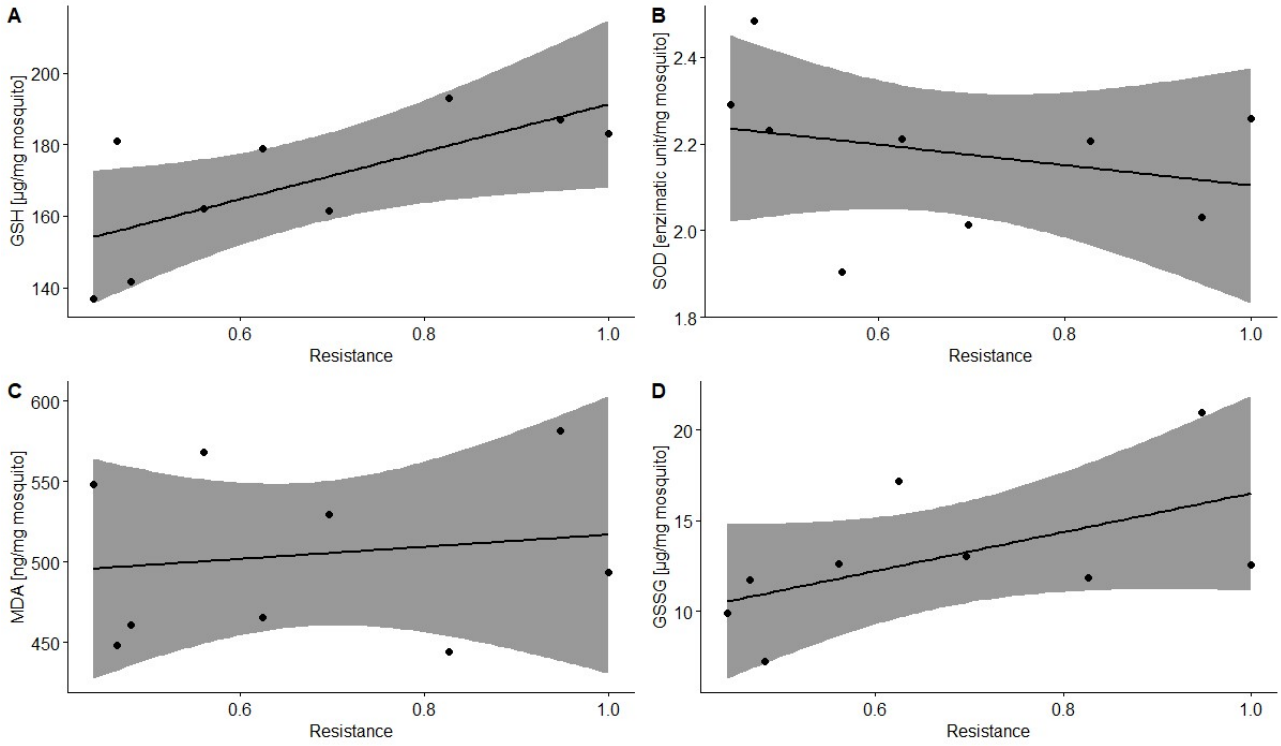

Figure 6: Correlation in the half-sib families between the resistance (mean proportion of uninfected individual per family) and A) the physiological level of total glutathione, B) the physiological level of superoxide dismutase, C) the physiological level of malondialdehyde and D) the physiological level of oxidized glutathione. Symbols indicate the mean for each family, and the grey area indicates the 95% confidence interval.

For tolerance, none of the four oxidative stress markers showed a significant correlation with this particular immune strategy (GSH: r (7)= 0.433, p=0.250; SOD: r(7)= -0.033, p= 0.948; MDA: r(7)= -0.383, p= 0.313, GSSG: r(7)= 0.300, p= 0.437)(Fig. 7).

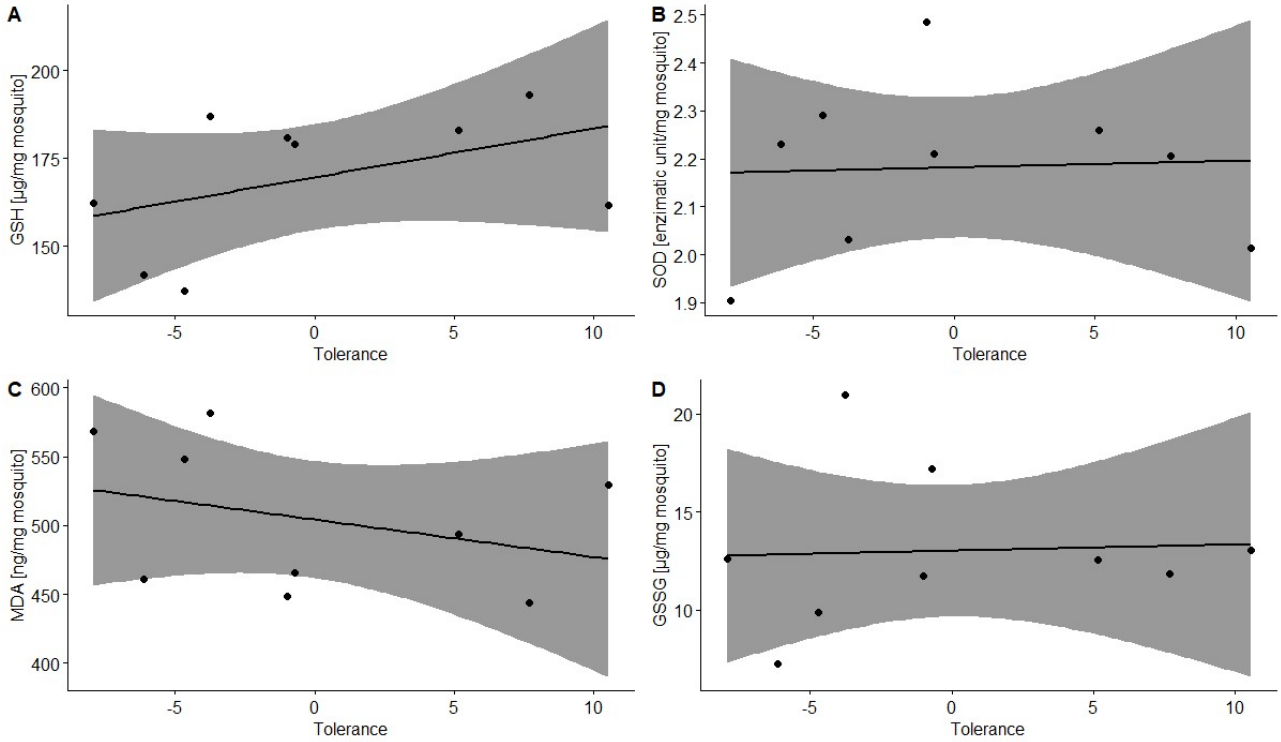

Figure 7: Correlation in the half-sib families between the tolerance (mean residual of the regression of fecundity on parasite load per family) and A) the physiological level of total glutathione, B) the physiological level of superoxide dismutase, C) the physiological level of malondialdehyde and D) the physiological level of oxidized glutathione. Symbols indicate the mean for each family, and the grey area indicates the 95% confidence interval.

2.5. Discussion

The results of our study provide some new evidence for a correlation between resistance to parasites and the physiological level of reactive oxygen species (ROS) in insects. We found that our family's resistance was positively correlated with the physiological level of glutathione (GSH) and also tended to be positively correlated with the level of oxidized glutathione (GSSG). Additionally, we found a maternal effect on the offspring's physiological level of GSH and malondialdehyde (MDA).

Our data showed indirectly that mosquitoes with higher resistance to the microsporidia parasite *V. culicis* also had to cope with higher basal levels of ROS. Unfortunately, our study did not directly measure the level of ROS, but a higher concentration of GSSG generally implies that the individual is coping with more ROS. This observation aligns with the growing research suggesting that ROS are essential components of the insect immune system. ROS production at the site of infections has been suggested to participate in several immune responses, such as encapsulation, metallization or direct cytotoxic effect on pathogens [71], [109], [110]. Interestingly, we found that our mosquitoes do not need to be in a constant oxidative stress status, as found by Kumar et al. (2003) [71], to benefit from the positive effects of ROS on immunity thus probably, the increased resistance derived from a higher capacity to produce ROS when infected, as suggested by the studies of Nappi et al. (1995 & 1998) [109], [110], through specific enzymes such as NADPH oxidase (NOX).

However, ROS might have more than a mere cytotoxic effect. They have been reported to interact directly in activating other immune responses. For example, the fat body's production of antimicrobial peptides (AMPs) is the core of the systemic immune response of insects [111]. For example, this immune response has been confirmed to be the main immune pathway used in the resistance against the microsporidian parasite *V. culicis,* which, even though it can evade a large number of mosquito immune responses, cannot avoid significantly enhancing the expression of genes related to the production of the AMPs defensin [94], [112]–[114]. Two distinct signalling pathways mainly control AMP gene expression, the Toll pathway and the Imd pathway, which include homologs of the NF-κB pathway [115]–[119].

NF-κB is a family of transcription factors which play a central role in inflammation and immunity [120], [121]. These proteins regulate the expression of hundreds of genes involved in cell growth, differentiation, development, and apoptosis and have been reported to interact with ROS and influence several pathways. For example, hydrogen peroxide (H_2O_2) has been reported to affect the phosphorylation of IκBα, an NF-κB inhibitor. The alternative H_2O_2-mediate phosphorylation of IκBα leads to the degradation of this factor, which, consequently, stops the expression of NF-κB, enhancing the expression of other genes, such as the AMPs genes [122]. So, higher physiological levels of ROS might be beneficial for directly killing the pathogen and activating other immune responses, such as AMPs. The correlation between ROS levels and insect parasite resistance significantly impacts our understanding of insect immunity. It highlights the importance of the redox balance in maintaining a robust immune response while minimizing potential damage to host

tissues. In our study, we detected a positive correlation between GSH and resistance. Without this control of the level of ROS, the organism could suffer autoimmunity, first due to ROS cytotoxicity and second due to erroneous activation of the immune responses.

Finally, our study showed that resistance has a genetic variance, and parental effects influence antioxidant defences. In particular, our study found that glutathione level is influenced by the mother, outlining a maternal effect on the offspring. The maternal-effect is the situation where the phenotype of the offspring reflects the environment experienced by the mother [123]. Unfortunately, our study did not test the mother's oxidative stress. Thus, we could not compare the oxidative stress status between offspring and mothers. However, as mothers were fed several times, we could hypothesize that they develop different oxidative profiles depending on their genetics and potentially the maternal effects they "received". So the phenotype we found in the offspring could reflect the costs of oxidative stress the mothers have experienced, as a meta-analysis study has reported for rats, where offspring of mothers fed with high fat died, which is known to cause overproduction of ROS, suffered from more oxidative damages than the offspring of control mothers [124].

Nevertheless, the oxidative profile of offspring could also arise from maternal priming to protect their offspring from the possible environment they could experience [125]. However, nowadays, few studies investigate how oxidative stress is transmitted from mother to offspring. Understanding this mechanism will allow us to find which ROS sources are more significant for long-term offspring survival and reproduction [126].

In conclusion, our study provides compelling evidence for a correlation between parasite resistance and the physiological level of insect reactive oxygen species. These findings deepen our understanding of insect immunity and offer insights into potential strategies for parasite control. Further research is needed to unravel ROS-mediated defence's underlying mechanisms and trade-offs. Additionally, we provide evidence that genetic factors contribute to the observed variation in ROS levels among individuals. Genetic variation in the expression or activity of enzymes involved in ROS production and scavenging can influence the overall ROS capacity of an insect. Furthermore, environmental factors, including diet, temperature, and exposure to pathogens, can modulate ROS levels and impact the insect's ability to resist parasites. Understanding these factors and their interplay is crucial for comprehending the complex dynamics of insect-parasite interactions.

Chapter 3

Experimental evolution of senescence

Belli Alessandro[a] and Jacob C. Koella[a]

[a] Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland

3.1. Abstract

The Free Radical Theory of Aging (FRTA) posits that the rate at which we accumulate oxidative damage influences our ageing process and longevity. However, recent studies have faced challenges finding robust evidence to support this theory. In our research, we employed an evolutionary approach to extend the lifespan of *Aedes aegypti* mosquitoes. This was achieved by manipulating their reproductive patterns, allowing only late reproducers to contribute to the next generation. This well-established method successfully increased the longevity of our mosquito lines.

Additionally, we explored the hypothesis that oxidative damage induced by an immune response could alter the trajectory of senescence. We exposed some mosquito lines to the microsporidia *Vavraia culicis* to investigate this. This exposure led to the selection of mosquitoes that were both more resistant (less prone to carrying spores) and more tolerant (less affected by the harmful effects of the infection on longevity) when exposed to the parasite. Surprisingly, the evolution of these immune strategies did not directly impact the evolution of senescence. More broadly, all the long-living mosquito lines did not exhibit a reduced accumulation of oxidative damage, thus failing to provide strong support for the FRTA. However, we made an intriguing observation—the levels of oxidized glutathione and the proportion of oxidized glutathione relative to the total in the long-living lines were lower than in the other lines. This suggests that the selection process improved the potential redox state of these lines.

These findings imply that reactive oxygen species (ROS) may play a role in senescence but likely not through oxidative damage. Instead, maintaining the balance between ROS production and antioxidant defences is probably more crucial in slowing senescence and increasing longevity.

3.2. Introduction

Senescence is the time-related decline of an organism's physiological functions necessary for survival and fertility, and it has long been a subject of study in evolutionary biology. In fact, from the evolutionary point of view, researchers seem to agree that senescence is maintained in the population because the strength of natural selection is not constant over an individual's lifespan. It has been proposed that natural selection's strength decreases with age, allowing the expression of deleterious traits at an older age, which causes the organism's ageing. These traits are not purged from the population because they benefit early-life reproductive fitness, and as the selection strength for younger classes is stronger than for older classes, it is impossible to avoid the deleterious effect on late-life fitness. Thus, we understand the evolutionary reasons for the senescence process. However, despite that and the ubiquity of ageing across species, our understanding of the underlying mechanisms driving it remains elusive. The Free Radical Theory of Ageing (FRTA) is among the most popular theories. This theory, proposed by Harman in the 1950s [10], suggested that highly reactive species (free radicals) damage cellular components, and the accumulation of such damage with time is the cause of the ageing process. Free radicals, such as reactive oxygen species (ROS) or nitric oxide, are by-products of aerobic metabolism. They are supposed to damage the macromolecular components of cells that accumulate in tissues, leading to functional deterioration and death. [34], [127]–[129]. This theory has been supported by observations that ROS production increases incessantly with age in various tissues in different species, which correlates with macromolecular oxidative damage in older individuals [130]. Some recent studies provided evidence indicating that several enzymes mediate the increase in ROS production with age. This increase results in an overproduction of free radicals in aged cells and organisms correlated with a diminution of their biological functions [131]–[133]. Furthermore, the comparisons of mitochondrial ROS production among species with different maximum lifespans show a negative correlation between the age-associated accumulation of oxidative damage rate and their physiological ROS generation [134], [135]. Regardless of those correlative studies showing a link between free radicals and ageing, there is no clear evidence yet showing a causality effect between ROS management and species' lifespan differences.

Furthermore, recent studies added a layer of complexity to our understanding of senescence's mechanisms by suggesting that ROS have a role in several biological processes. Moderate levels of ROS are critical for healthy cell function, as they function as signalling molecules to regulate many physiological processes, particularly cellular responses to stresses like hypoxia, starvation or infection by pathogens [136]. Moreover, ROS regulates innate immune responses against viruses, bacteria and parasites and kills pathogens directly through an oxidative burst mediated by NADPH oxidases in phagosomes [137]. Finally, organisms can evolve mechanisms that buffer against oxidative stress and damage and mechanisms that regulate the generation of ROS as a response to metabolism. Pigeons and rats, for example, have similar sizes and metabolic rates,

but pigeons live 35 years, whereas rats only live four years. Indeed, pigeons generate ROS in their brain, heart and kidneys about half as fast as rats, and they have more antioxidant defences than rats [134].

These ideas lead to evolutionary predictions about the interplay between ROS, immune response, and senescence. Evolution for less senescence (that is, longer life or later reproduction) should be linked to slower production of ROS or buffering the damaging impact of ROS, and frequent infection during the evolutionary process should increase the production of ROS and thus modify the link between senescence and oxidative stress.

Experimental evolution can provide a powerful tool to test such ideas by manipulating organisms' reproductive patterns and environmental constraints and letting us observe the long-term consequences of senescence-related traits. For example, manipulating the reproductive patterns of *D. melanogaster* made it possible to detect how higher early reproductive fitness comes at the expense of lifespan and reproductive fitness late in life and starvation resistance [138]. Genetic analysis of those *D. melanogaste*r populations showed that long-living lines had more copies of immune genes than non-selected lines. Nevertheless, they had a reduced expression of some immune components, such as AMPs, in late life but an increased tolerance to infection, while their resistance was decreased over their entire lifespan [139]. So, it seemed that to live longer, those populations needed to better control the immune system over their lifespan and reduce the autoimmune effect at an older age. These results give us some insights into how senescence and immunity interplay. However, the experiment did not directly include a selection for immune traits in those studies. For this reason, in our study, we planned to manipulate the reproductive patterns of organisms and expositing them to parasitic infection to observe the evolution of senescence and immunity in parallel.

In this study, we focus on *Aedes aegypti* mosquitoes, which are known vectors for various human diseases, including dengue fever, Zika virus, and yellow fever, to explore the effects of early or late reproduction on markers of oxidative stress in mosquitoes infected or not with the microsporidian *Vavraia culicis*. This approach allows us to investigate whether ROS-mediated damage is a driving force behind senescence, whether it is an adaptive response to pathogenic combat threats, and whether the immune response, particularly the production of ROS, can affect the mosquito's reproductive strategy and longevity.

3.3. Material and Methods

3.3.1. Aedes aegypti

The mosquito *Aedes aegypti* is the main vector of yellow fever [81], dengue [140], Chikungunya fever and Zika virus [82]. It is ubiquitous in tropical and subtropical regions. Its larvae grow easily in natural and artificial containers holding clean, fresh water [84]. We used the UGAL strain of the mosquito *Ae. aegypti* (obtained from P. Guérin, University of Neuchâtel, Switzerland), which we had maintained since 2012 at 26.5 °C, 70% humidity, and 12:12 hours light-dark photoperiod with constant access to 6% sucrose solution.

3.3.2. Vavraia culicis

As a parasite, we used the microsporidian *Vavraia culicis* (obtained from J.J Becnel, USDA, Gainesville, USA). *Vavraia culicis* was discovered in *Aedes albopictus* but was found to parasitise many species of *Aedes, Culex, Anopheles, Culiseta, Ochlerotatus* and *Orthopodomyia.* It produces a single spore type, which is used for transmission. When larvae have ingested spores, the parasite replicates intensively within its how before producing the next generation of spores, which in natural situations are released to the environment when infected larvae die, with the faeces of infected individuals, or when infected females survive to become adults and lay their eggs into the water. Our laboratory stock has been maintained for ten years with alternating generations on *Ae. aegypti* or *Anopheles* gambiae, during which larvae were infected with spores obtained from homogenised adults at least ten days old.

3.3.3. General design

We maintained mosquitoes for ten generations in a factorial design with four evolutionary pressures: with mosquitoes forced to reproduce early (three or four days after emergence) or late (when two-thirds of the mosquitoes had died) and with larvae being exposed to parasitic spores or not. We then tested whether the design changed the longevity and resistance against *V. culicis,* and we measured four parameters underlying oxidative stress (two of which were oxidative assay stress and damage and two assays defence against oxidative stress).

3.3.4. Evolution

To initiate the evolutionary experiment, we hatched eggs from our colony and split the larvae into 16 lines, four lines for each of the four evolutionary pressures (early vs late egg laying and uninfected vs. infected with *V. culicis*). The lines were maintained separately for the next ten generations, and each new generation was started with 500 larvae. The eggs were hatched synchronously under reduced atmospheric pressure. For five days, the larvae of each line were split into ten Petri dishes (8 cm diameter) containing 30 mL deionised water; after that, the larvae were moved to two trays containing 800 mL deionised water. The larvae were fed daily with TetraMin™ fish food according to the standard in our lab (day of hatching: 0.04 mg/larva, day

2: 0.053 mg, day 3: 0.107 mg, half of that if infected), day 4: 0.213 mg, day 5: 0.64 mg, day 6 and later: 0.32 mg). The pupae were moved into a 180 mL plastic cup and placed into a cage (30cm x 30cm x 30cm) for emergence. The adults were supplied with a cup containing 6% sucrose solution, which was changed weekly. We exposed each group of 50 larvae for the infected lines to one million spores forty-eight hours after hatching. The number of spores and their vitality were checked on the day of infection with a counting chamber and a phase-contrast microscope. The females of all the lines were given access to their first blood meal 13 days after hatching. The late-laying lines were given access to further blood meals every seven days until 66 % of the mosquitoes had died. All blood meals were on AB's arms and lasted for eight minutes. The mosquitoes were allowed to lay eggs on filter paper for 6 days. The filter papers with the eggs used for the next generation were stored in a petri dish inside the insectary. We continued the early-laying lines with only the eggs of the first clutch and the late-laying lines with only the eggs of the last clutch.

3.3.5. Impact of evolution

All of the following experiments were done with the eggs produced by the mosquitoes after ten generations of evolution and with larvae that had been reared individually in 12-well tissue-culture plates containing 3 mL of deionised water. To confirm that the evolutionary experiment impacted life histories and resistance to infection, we reared 240 larvae of each treatment's three lines. (The fourth, randomly selected line was omitted, for it was logistically impossible to rear more than the 2880 mosquitoes required for 12 lines). The larvae were fed daily with the quantities mentioned above. Forty-eight hours after hatching, half of the larvae of each line were exposed to 20'000 spores of *V. culicis*. The pupae were placed individually into a 180 mL plastic cup covered with bed netting, and adults were supplied every two days with a cotton ball soaked with a 6% sucrose solution. The mosquitoes were checked daily, and dead mosquitoes were frozen for further analysis. They were later homogenised in 1,5 ml Eppendorf tubes containing 0.1ml deionised water, and the number of spores in 0.1 µl was counted with a counting chamber (Neubauer improved bright-line, 0.0025 mm^2, depth 0.1 mm) and a phase-contrast microscope.

3.3.6. Oxidative stress

To obtain the baseline of oxidative stress markers, we analysed oxidative stress only in uninfected mosquitoes. We hatched 120 larvae for each selection line (a total of 1920 larvae) under reduced air pressure and reared them individually in 12-well tissue-culture plates filled with 3 mL of deionised water and fed with the daily amounts mentioned above. Pupae were individually transferred to Falcon tubes. The adults were given a cotton ball soaked with a 6 % sugar solution every two days. Two days after emergence, the males were discarded, and the females were moved individually into a 180 mL plastic cup covered with mosquito netting. Five or 15 days after emergence, the mosquitoes were killed at -80 °C and then stored in 2 mL

Eppendorf tubes at -80 °C. We estimated oxidative stress with a combination of three assays that quantify glutathione (GSH), malondialdehyde (MDA) and superoxide dismutase (SOD).

On the day of the glutathione assay, the wings and legs of each mosquito were removed. The rest of the body was weighed to the nearest 0.01 mg with a microbalance and then placed into a 2 mL tube containing a 5mm steel bead and 120 µL of PBS and homogenised with a tissue lyser for 4 minutes at 45 Hz and centrifuged for 10 minutes at 10'000 rpm at 4 °C. Each homogenate was split into three aliquots and stored at -80 °C.

Glutathione peroxidase quenches cellular ROS by oxidising the glutathione in glutathione disulphide, so the proportion of oxidised glutathione over the total glutathione is a measure of the oxidative balance of the cells [105], [141]. To determine the glutathione levels in the reduced (GSH) and oxidised (GSSG) forms, we extracted glutathione on the day we homogenised the mosquitoes. We added 100 µL miliQ water, 5 µL formic acid 1.25% and 5 µL glutathione ethyl ester 1.25 µg/mL (GSHee; Sigma Aldrich, USA) to one of the aliquots (15 µL) of each homogenate, mixed with a vortex for 10s, and centrifuged the solution for 15 minutes at 15'000 RPM and 4°C. We then removed 115 µL of the extract and placed them in a syringe with a PTFE hydrophilic filter (pore size 0.22 µm), and gently blew it into a glass HPLC vial to ensure no particles were present in the samples. The two forms of glutathione were quantified with UHPLC-MS/MS following Rojas Mora et al. (2016) [107] and were added to obtain the total glutathione necessary to calculate the proportion of oxidised glutathione.

Superoxide dismutase is part of the antioxidant defence mechanisms of organisms. It was quantified with the Cayman Superoxide Dismutase Assay Kit (Cayman Chemical, USA) with minor modifications. In particular, the mosquito's homogenate was diluted 1 in 8 parts PBS before using it as described in the kit protocol. Samples were run in duplicates with an average within-plate coefficient of variation (CV) of 2.9%, and 21 samples were assayed in different plates, showing inter-plate repeatability of r = 0.90.

Malondialdehyde (MDA) is one of the final products of the peroxidation of polyunsaturated fatty acids in cells. Since its production releases free radicals, it is often used as an oxidative stress marker. We quantified MDA following the procedure described by Mendonça et al. (2017) [108] with minor modifications. 40 µL of NaOH 1.2 M and 10µL of mosquito homogenates were mixed and incubated for 30 minutes at 60°C for the protein hydrolysis. The samples were cooled at 4°C for 2 minutes, and 10 µL of the internal standard (d2-MDA 30 µM in 0.1 M HCl) was added. So that the proteins precipitated, we added 142 µL of trichloroacetic acid 20% and mixed, sonicated and then centrifugated the samples for 5min at 9300 cf. 180 µL of the supernatant were transferred to a 1.5 mL microcentrifuge tube. We added 18 µL of 2,4 dinitrophenylhydrazine 5mM and incubated the samples for 10 minutes at room temperature with gentle agitation to derive the MDA. 22 µL of NaOH 10 M were used to alkalise the sample. We added 250 µL of a solution of toluene and cyclohexane (1:1 v/v) and transferred the supernatant to a 1.5 mL microcentrifuge tube for the two phase-to-phase extractions. The recovered organic phase was evaporated in a SpeedVac ®

at 35°C, and the pellet was reconstituted in methanol 50%. The extracts were finally filtered with a 22 µm PTFE filter (BGB, Germany) into an HPLC vial before being analysed with an HPLC-MS. A standard curve, ranging from 0 to 40 µg/mL, was done with MDA tetrabutylammonium salt (Sigma-Aldrich Inc. St-Louis, Missouri), and all the standards went through the extraction protocol together with the samples.

3.3.7. Statistical analysis

All analyses were done with the software R (version 4.2.2). We assessed significance with the function Anova() of the car library, using type III ANOVA if interactions were significant and type II ANOVA otherwise, and we evaluated pairwise comparisons with the functions emmean() and pairs() of the emmeans library.

To test whether evolution affected the resistance to infection, we classified the mosquitoes exposed to *Vavraia* as infected or uninfected. We analysed the infection status with a generalised linear model mixed effect (function glmer() of the mle4 library) with a binomial distribution of errors, where the explanatory variables were the reproduction time during evolution, the exposure to parasite during evolution, their interaction, and the individual's age at death. The lines were included as random factors. Since age at death had little impact on resistance ($\chi 2 = 0.37$, df=1, p=0.542), we omitted it from the results below. To test whether evolution had affected the longevity of mosquitoes and their tolerance to infection, we analysed age at death with a mixed-effect Cox model (coxme() of the coxme library), where the explanatory variables were the reproduction time during evolution, the exposure to the parasite during evolution, the exposure of the tested mosquitoes to *Vavraia, sex and* their interactions. The lines were included as random factors. To test the impact of evolution on the oxidative stress markers, we analysed the logarithm in base ten of the ratio of oxidised to total GSH, the logarithm in base ten of MDA, the power of two of SOD and the GSH with a linear mixed effect model (function lmer() of the mle4 library), where the explanatory variables were the reproduction time during evolution, the exposure to the parasite evolution, the age of the test and their interactions. Age was considered explanatory for the oxidative stress markers because we killed all the individuals at 5- or 15-days post-emergence. The lines were included as random factors.

3.4. Results

3.4.1. Longevity and parasite resistance

Evolving mosquitoes in the presence of spores decreased the probability that exposed mosquitoes became infected from 57% (unexposed evolution) to 33% (exposed evolution) ($\chi2 = 4.81$, df=1, p=0.028) (Fig.8). The timing of reproduction during evolution had no significant impact on resistance ($\chi2 = 0.68$, df=1, p=0.410), and neither the interaction between timing of reproduction and exposure during evolution nor sex (including its interactions) affected resistance ($\chi2 < 2.95$, df=1, p>0.086)

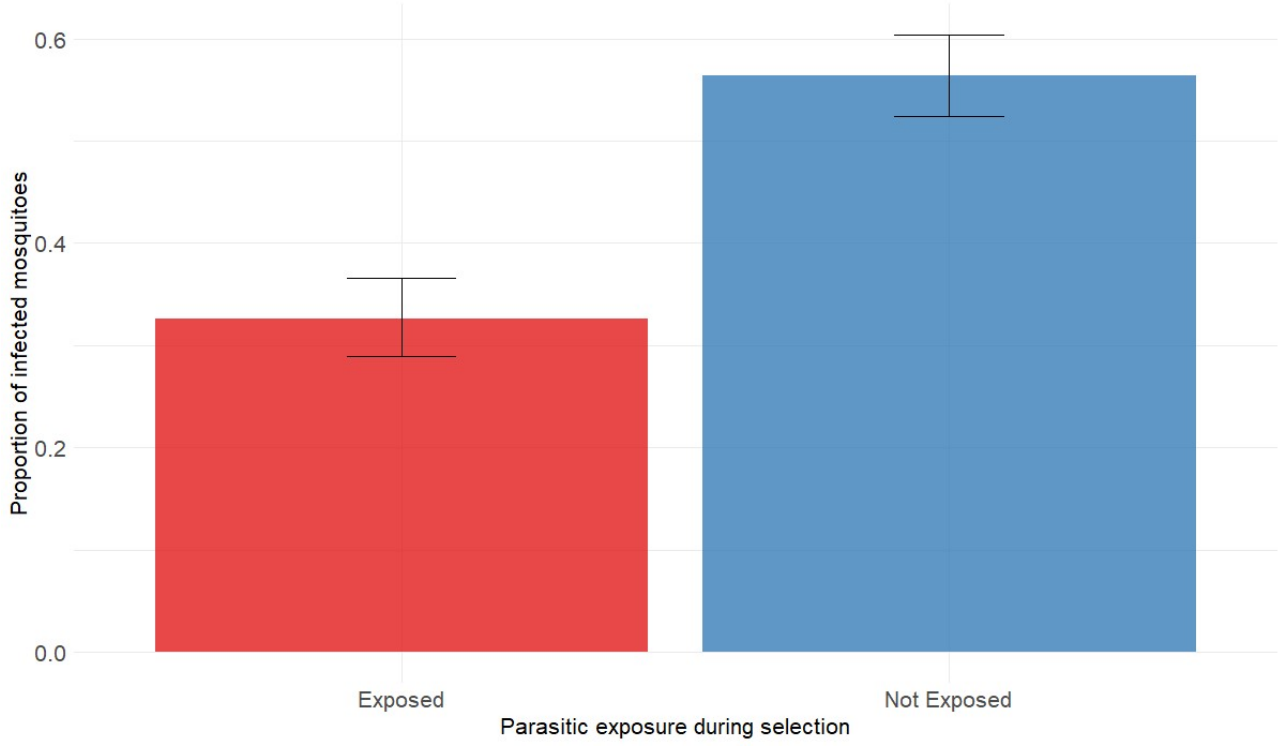

Figure 8: Mosquitoes' resistance after exposure or exposure to V. culicis during ten generations of evolution. The bars show the proportions of mosquitoes harbouring spores upon their death, and the vertical lines indicate the 95% confidence intervals.

Mosquitoes lived from 4 days to 75 days, with an average of 35.2 days. Males lived longer (36.4 days) than females (34.0 days) ($\chi2=22.2$, df=1, $p<0.001$). Mosquitoes that had evolved under parasite pressure lived longer (36.6 days) than those that evolved without parasites (33.8 days) ($\chi2=6.9$, df=1, $p=0.009$), and those evolving with late reproduction lived longer (36.5 days) than those evolving with early reproduction (33.8 days) ($\chi2=9.1$, df=1, $p=0.003$). The impact of the evolution of reproductive timing was more remarkable in females (difference between late and early: 3.66 days) than in males (1.58 days) ($\chi2=9.1$, df=1, $p=0.003$) (Fig. 9).

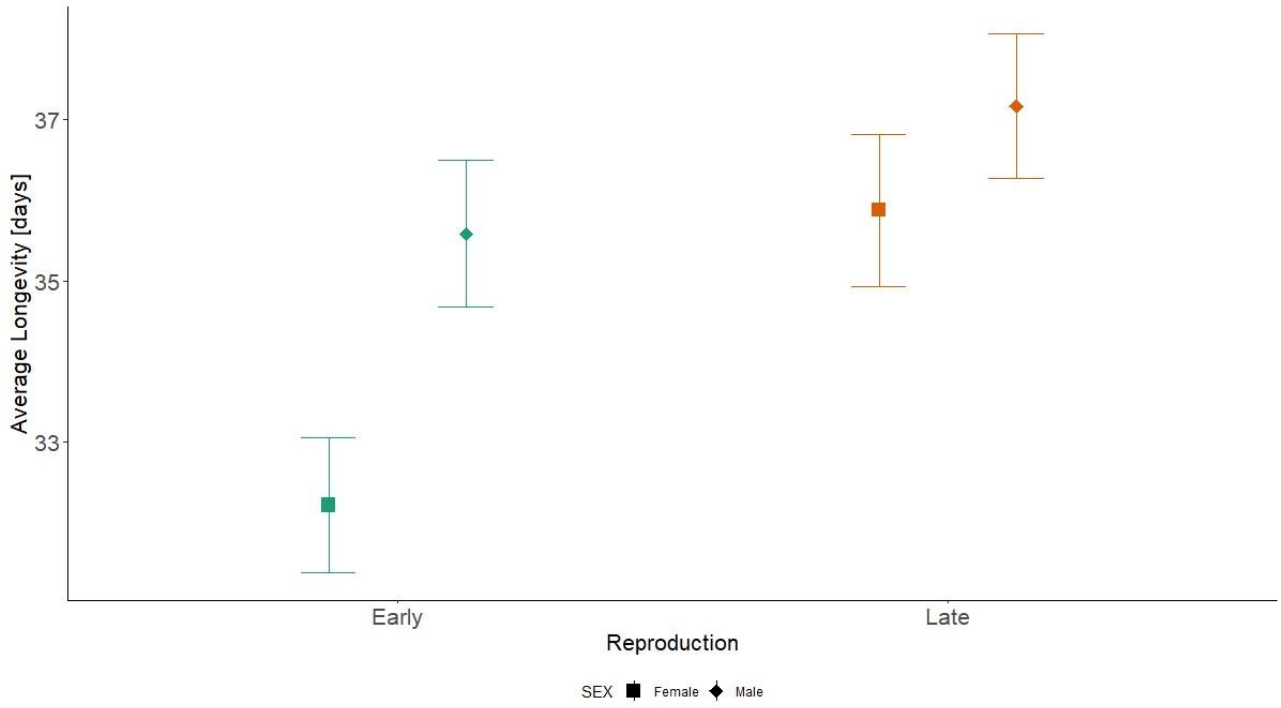

Figure 9: Average longevity (days after pupation) after ten generations being selected for late reproduction (orange) or early reproduction (green). The squares showed the data for females, and the circles showed the data for males. The vertical lines indicate the 95% confidence interval.

Mosquitoes infected with *V. culicis* died earlier (34.1 days) than uninfected ones (36.5 days) ($\chi2=27.8$, df=1, $p<0.001$), and the impact of exposure was stronger if the mosquitoes had not evolved with parasites (difference between uninfected and infected: 3.47 days) than if they had evolved under parasite pressure (0.67 days) ($\chi2=19.9$, df=1, $p<0.001$). None of the other interactions had a significant effect ($\chi2<2.3$, df=1, $p>0.131$) (Fig. 10).

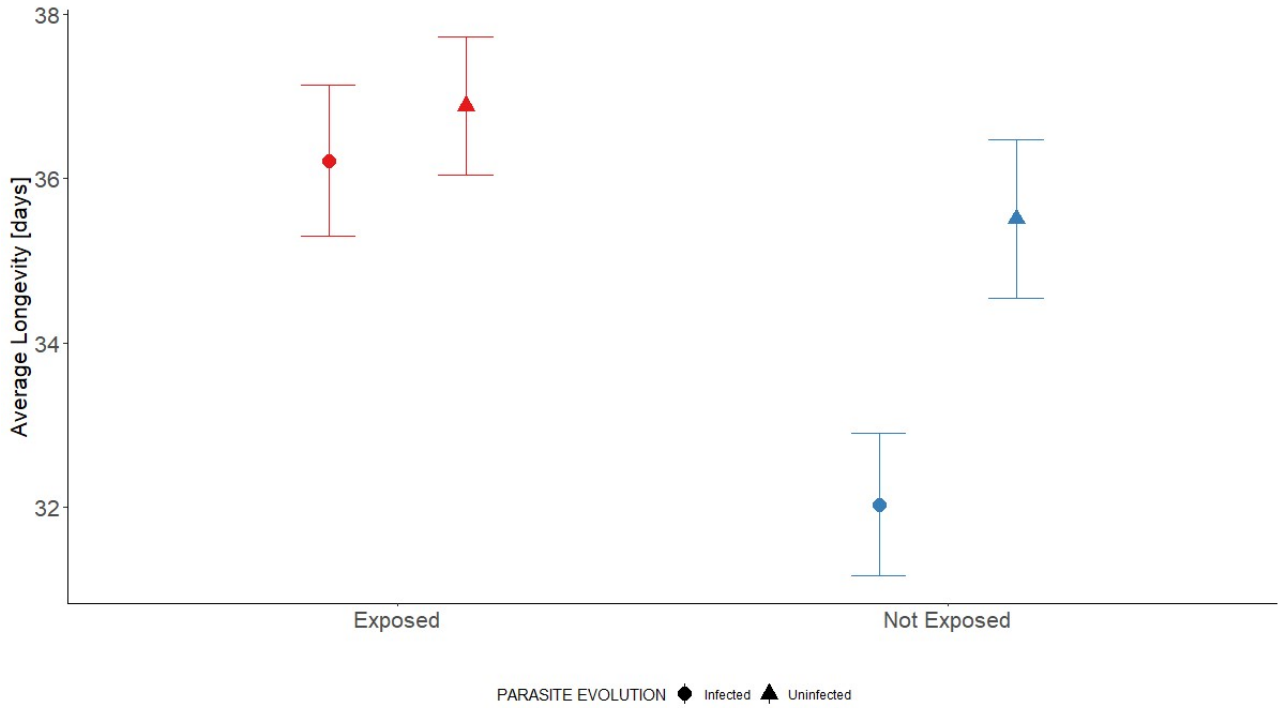

Figure 10: Average longevity (days after pupation) after being exposed to the parasite for ten generations during the selection (red) or not being exposed (blue). The triangles indicated the data for the individual infected with V. culicis during the final experiment. The circle *showed the data of the individual not being infected during the final experiment. The vertical lines indicate the 95% confidence interval.*

3.4.2. Oxidative stress analysis

The proportion of oxidised glutathione over the total amount: Lines selected for late-life reproduction (0.032, 0.030-0.034 ci) seem to have a slightly lower proportion of oxidised glutathione that the lines selected to reproduce early in life (0.037, 0.034-0.040) but not statistically significant ($\chi2$ = 3.11, df=1, p=0.077). The proportion was not influenced either by the age of the mosquito ($\chi2$ = 0.34, df=1, p=0.55) or by the exposition to *V. culicis* ($\chi2$ = 0.22, df=1, p=0.64) (Fig 11).

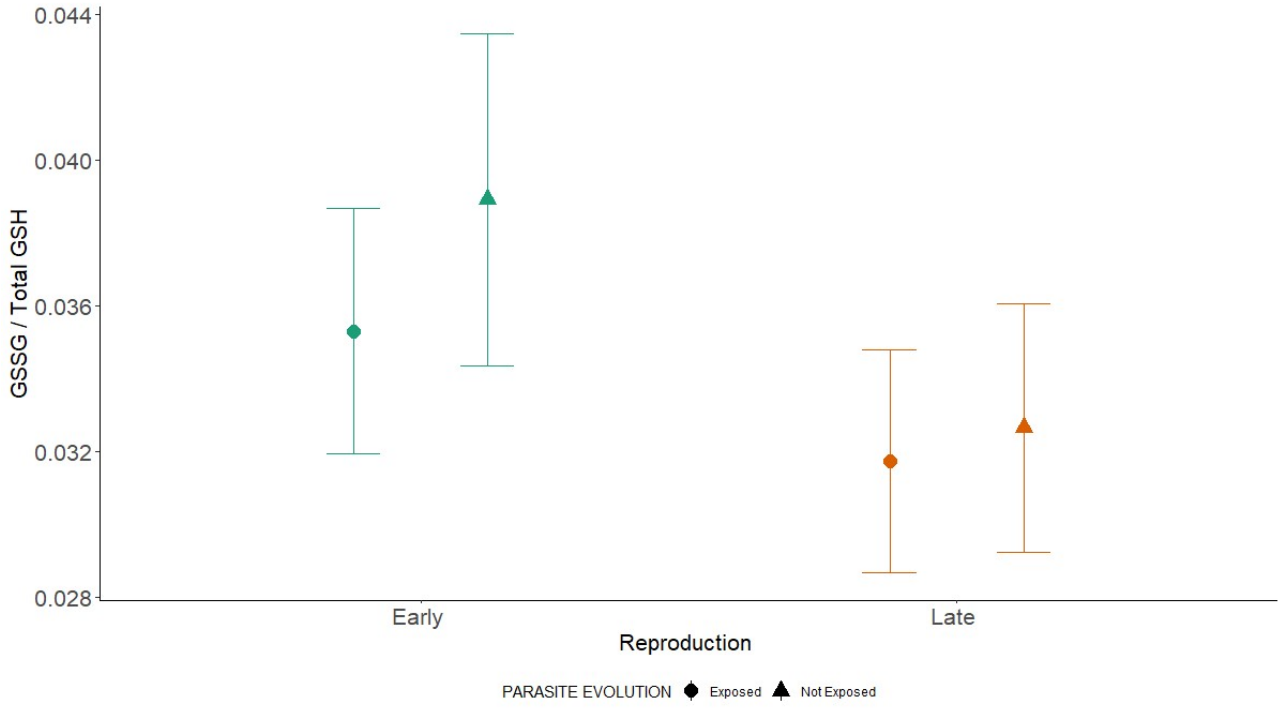

Figure 11: Proportion of oxidised glutathione (GSSG) over the total amount (Total GSH). Symbols represent the mean within treatments, and the vertical lines represent the 95% confidence interval. The green colour represents the selection for early reproduction; in contrast, orange represents the selection for late reproduction. Circles represent the treatment of being exposed to the parasite during selection. Conversely, the triangles represent the treatment of not being exposed during selection.

Total Glutathione: Older individuals (0.27 µg/mg, 0.26-0.28 ci) presented a lower physiological level of glutathione than younger individuals (0.32 µg/mg, 0.31-0.33 ci) ($\chi2$ = 33.91, df=1, p < 0.001). Unfortunately, neither the reproductive pattern selection ($\chi2$ = 1.74, df=1, p=0.19) (Early Life: 0.29 µg/mg, 0.28-0.30 ci vs Late Life: 0.30 µg/mg, 0.29-0.31 ci) nor the exposition to the microsporidian parasite ($\chi2$ = 0.24, df=1, p=0.62) (Exposed: 0.29 µg/mg, 0.28-0.30 ci vs Naive: 0.30 µg/mg, 0.29-0.31 ci) significantly influence the level of the glutathione (Fig 12).

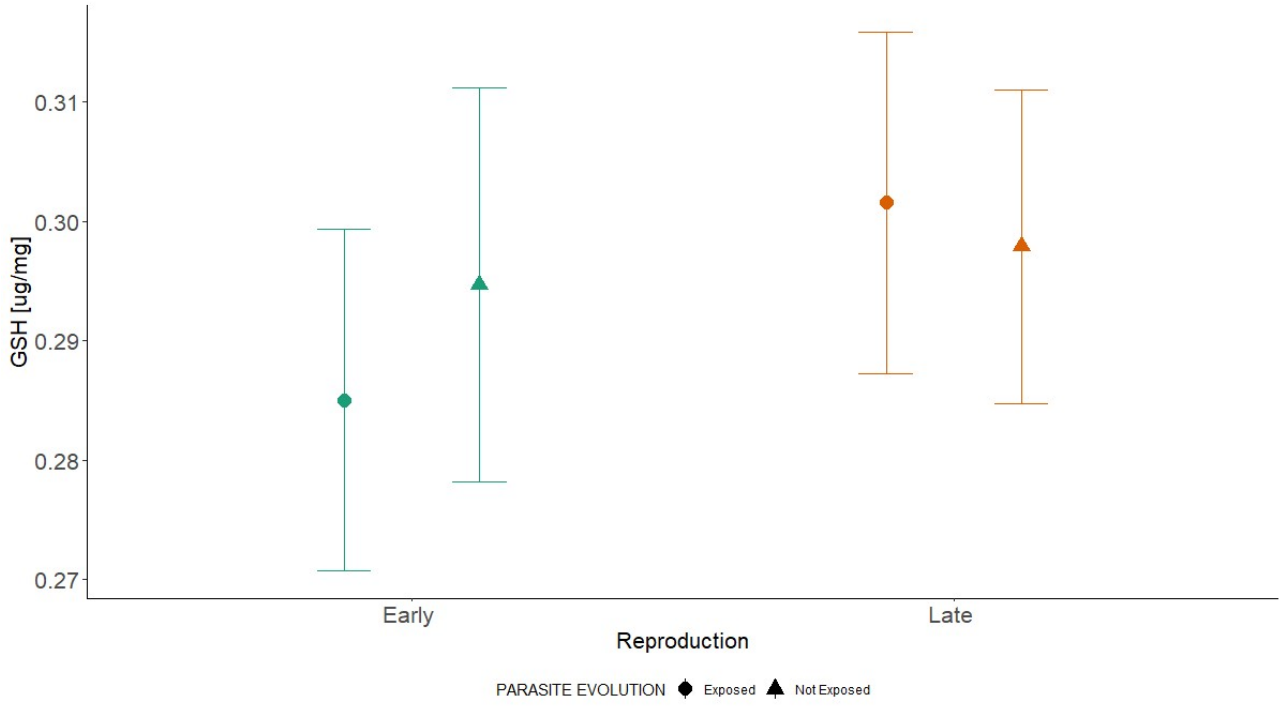

Figure 12: Physiological level of glutathione (GSH). Symbols represent the mean within treatments, and the vertical lines represent the 95% confidence interval. The green colour represents the selection for early reproduction; in contrast, orange represents the selection for late reproduction. Circles represent the treatment of being exposed to the parasite during selection. Conversely, the triangles represent the treatment of not being exposed during selection.

Oxidized glutathione: Older individuals (0.0093 µg/mg, 0.0086-0.0099 ci) presented a lower physiological level of oxidized glutathione than younger individuals (0.0099 µg/mg, 0.0094-0.105 ci) ($\chi2 = 4.18$, df=1, p = 0.041). Unfortunately, neither the exposition to the microsporidian parasite ($\chi2 = 0.35$, df=1, p=0.56) (Exposed: 0.0093 µg/mg, 0.0088-0.0099 ci vs Naive: 0.0098 µg/mg, 0.0092-0.0105 ci) nor the reproductive pattern selection ($\chi2 = 1.13$, df=1, p=0.29) (Early Life: 0.0100 µg/mg, 0.0093-0.0106 ci vs Late Life: 0.0092 µg/mg, 0.0086-0.0098 ci) nor significantly influence the level of the glutathione, even though the reproduction patterns seem to indicated a slight reduction of the oxidized form in the late reproducing lines (Fig 13).

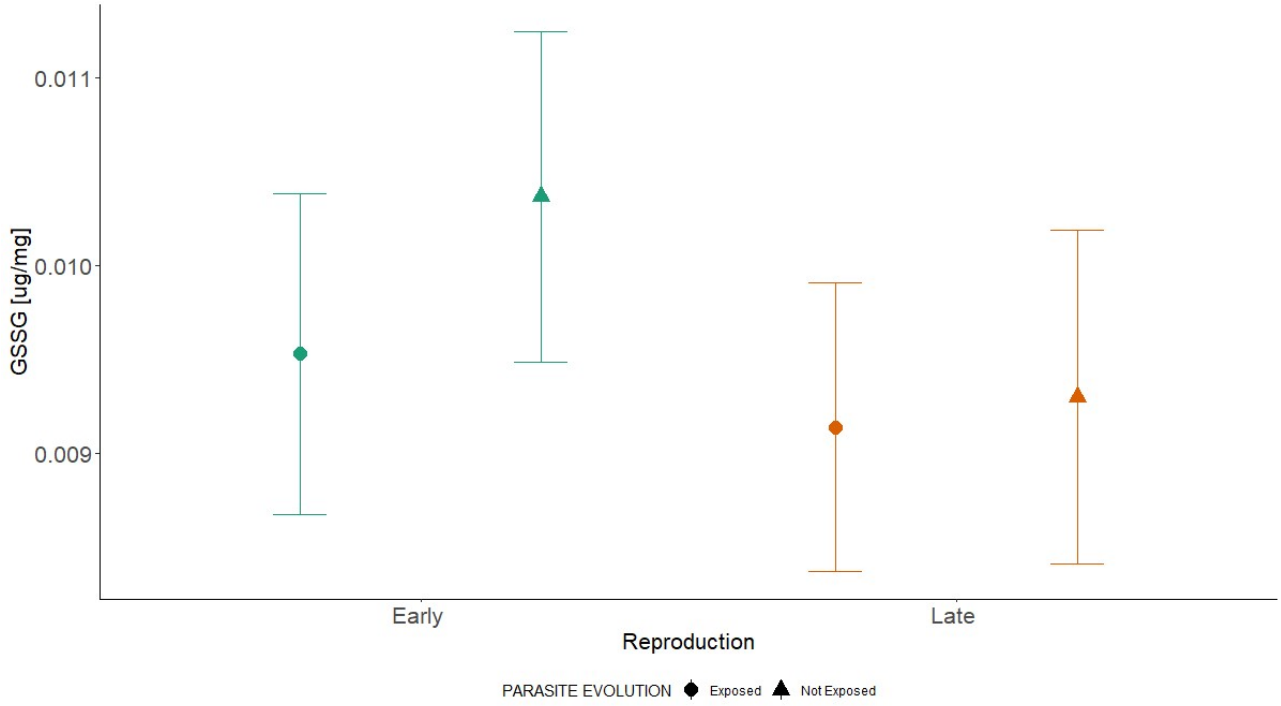

Figure 13: Physiological level of oxidized glutathione (GSSG). Symbols represent the mean within treatments, and the vertical lines represent the 95% confidence interval. The green colour represents the selection for early reproduction; in contrast, orange represents the selection for late reproduction. Circles represent the treatment of being exposed to the parasite during selection. Conversely, the triangles represent the treatment of not being exposed during selection.

Superoxide dismutase: also for the antioxidant, older individuals (0.0019 units/mg, 0.0018-0.0020 ci) presented a lower physiological level than younger individuals (0.0020 units/mg, 0.0020-0.0021 ci) ($\chi2 = 7.21$, df=1, $p = 0.007$) and, neither the reproductive pattern selection ($\chi2 = 0.14$, df=1, $p = 0.71$) (Early Life: 0.0020 units/mg, 0.0019-0.0020 ci vs Late Life: 0.0020 units/mg, 0.0019-0.0020 ci) nor the exposition to the microsporidian parasite ($\chi2 = 1.29$, df=1, $p = 0.26$) (Exposed: 0.0019 units/mg, 0.0019-0.0020ci vs Naive: 0.0020 units/mg, 0.0019-0.0021 ci) significantly influences the level of the superoxide dismutase (Fig 14).

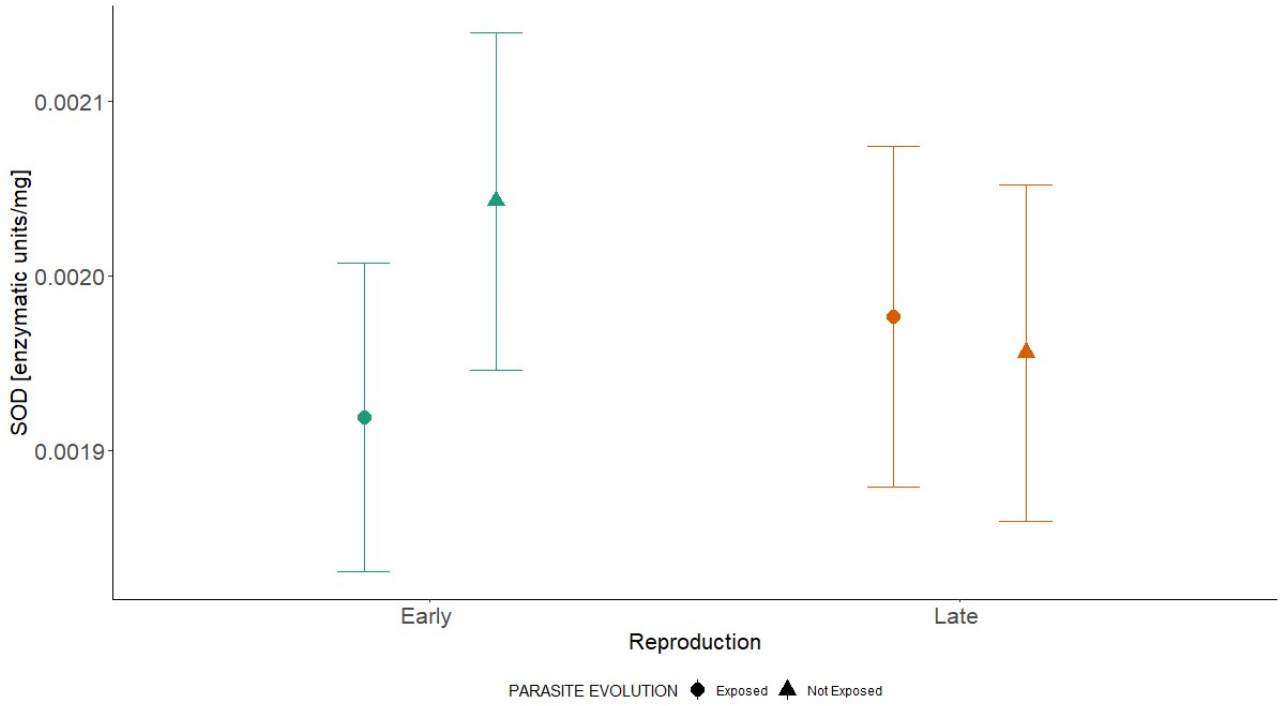

Figure 14: Physiological level of superoxide dismutase (SOD). Symbols represent the mean within treatments, and the vertical lines represent the 95% confidence interval. The green colour represents the selection for early reproduction; in contrast, orange represents the selection for late reproduction. Circles represent the treatment of being exposed to the parasite during selection. Conversely, the triangles represent the treatment of not being exposed during selection.

Malondialdehyde: For the malondialdehyde, we detected a peculiar evolution. The older individual had a physiological level of MDA lower (1.03 ng/mg, 0.94-1.13 ci) than the younger individual (2.03 ng/mg, 1.88-2.18 ci) ($\chi2$ = 129.51, df=1, p <0.001) and we detected a significant effect of the interaction between the reproductive pattern and the exposition to parasite during selection ($\chi2$ = 4.67, df=1, p = 0.03) with early reproducing individual being exposed to the microsporidia having a higher level (1.66 ng/mg, 1.44-1.88 ci) than the individuals reproducing in their early life but not being exposed to *V. culicis* (1.34 ng/mg, 1.17-1.51 ci). This difference was not present in the individual reproducing late in their life (Late & Exposed 1.50 ng/mg, 1.28-1.72 ci); Late & Not Exposed 1.61 ng/mg, 1.41-1.81 ci) (Fig 15).

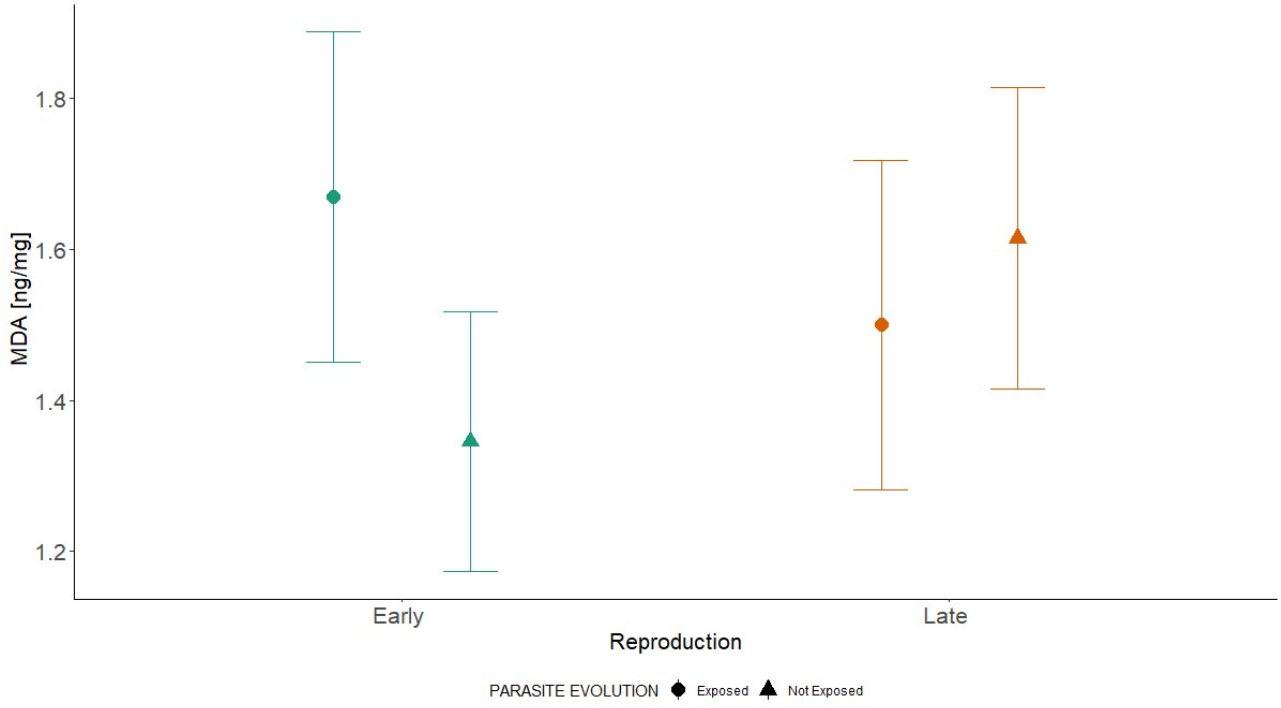

Figure 15: Physiological level of malondialdehyde (MDA). Symbols represent the mean within treatments, and the vertical lines represent the 95% confidence interval. The green colour represents the selection for early reproduction; in contrast, orange represents the selection for late reproduction. Circles represent the treatment of being exposed to the parasite during selection. Conversely, the triangles represent the treatment of not being exposed during selection.

3.4.3. Body mass

Older individuals were lighter (1.55 g, 1.52-1.58 ci) than younger individuals (1.67 g, 1.65-1.70 ci) ($\chi2 = 32.33$, df=1, $p < 0.001$) and mosquitoes from the exposed lines seemed to be tendentially heavier (1.64 g, 1.61-1.67 ci) than not exposed mosquitoes (1.59 g, 1.56-1.61 ci) ($\chi2 = 3.32$, df=1, $p = 0.06$). Neither the reproduction pattern selection ($\chi2 = 2.21$, df=1, $p = 0.14$) nor its interaction with the parasite exposition ($\chi2 = 0.1$, df=1, $p= 0.75$) was significant in determining the body weight of the mosquitoes (Fig. 16).

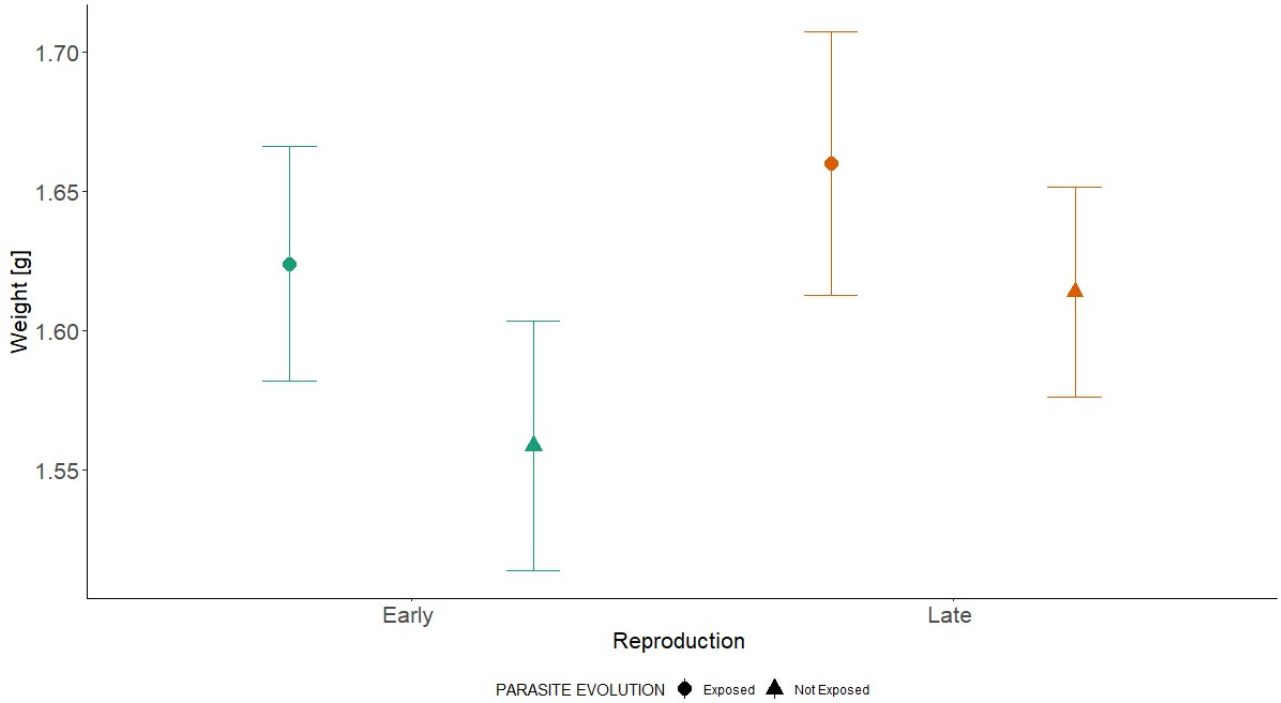

Figure 16 Average body mass. Symbols represent the mean within treatments, and the vertical lines represent the 95% confidence interval. The green colour represents the selection for early reproduction; in contrast, orange represents the selection for late reproduction. Circles represent the treatment of being exposed to the parasite during selection. Conversely, the triangles represent the treatment of not being exposed during selection.

3.5. Discussion

In this study, we aimed to experimentally evolve a longer lifespan and possibly a slower senescence phenotype in *Aedes aegypti* mosquitoes through selective breeding for late reproduction. Meanwhile, we also wanted to investigate the role of oxidative damage, as proposed by the free radical theory of ageing, in the observed changes in longevity and the possible interaction between immunity and longevity through the ROS-mediated damages produced during infection. After ten generations of selection, our late reproducing lines showed increased mean longevity. However, we did not find any directed evidence supporting the role of oxidative damage in this process, therefore supporting the theory. From our four oxidative stress markers, only the proportion of oxidised glutathione over the total amount tended to be reduced by our selection. We found a smaller proportion of oxidised glutathione in the late-reproducing mosquitoes, indicating a better redox potential than early reproducing. However, neither the amount of oxidate damage nor the level of antioxidant defences showed a difference between early or late reproduction.

Finally, we found that after ten generations of selection being exposed to parasitic infection, our lines evolve a resistance to and a tolerance of the parasite, but this does not affect their senescence directly. However, the interaction between early reproduction and exposition to the parasite was significant in determining the level of MDA, with the mosquitoes being exposed and early reproducers having more oxidative damage than the mosquito not being exposed and early reproducers or late reproducing mosquitoes, regardless of their exposition to the parasite.

The free radical theory of ageing suggests that oxidative damage resulting from reactive oxygen species (ROS) plays a pivotal role in the ageing process [10]. Based on this theory, we hypothesised that by selecting for late reproduction, we would indirectly select individuals with reduced oxidative damage and, thus, longer lifespan and slower senescence. While our results did not directly support the free radical theory of ageing, as we did not find a direct effect of the selection on oxidative damage, the potentially decreased proportion of oxidised glutathione over the total amount indicates an improvement in redox potential [142] and the visual tendency shown by the level of oxidized glutathione form to be reduced by the late reproduction seem to confirm this idea. Probably, our selection improved the antioxidative defence mechanisms of the selected lines, which were not measured in our experiments, such as catalase or glutathione reductase. Thus, we cannot confirm this suggestion from our results, as our measurements of antioxidants were not influenced. Globally, our finding implies that the evolved lines may possess an improved ability to counteract the detrimental effects of oxidative stress, potentially contributing to their slower senescence phenotype, which has already been suggested as a possible explanation for the different ageing rates among different species [134].

The lack of evidence of the direct involvement of oxidative damage, in our case MDA, in the increased longevity of our lines seems to invalidate the fundamental postulation of the free radical theory of ageing. However, there may be two reasons for our study's lack of such evidence. The first is that MDA has been

associated with ageing in several studies. However, the individuals showing such a relationship were in chronic inflammation, infection, or stress [143], [144]. Thus, we may think that our mosquitoes, which were free from parasites, relatively young and kept individually, may be living in a free-stress situation, which will prevent the induction of MDA during their ageing process. At least another study found that all causes of death in a cohort of more than 2'000 elderly were not associated with their physiological level of MDA but were correlated with their level of antioxidants [145].

Hence, their finding and our results seem to suggest that other markers of oxidative damage may need to be measured. This point is strictly related to the second reason why we might not have found direct evidence supporting the free radical theory of ageing, which is the change of the focal cause from oxidative damage caused by ROS to the disruption of other biological processes proposed by the updated theories. These new theories are less concentrated on oxidative damages as primary sources of ageing and more on the effect of ROS on other biological processes [36]. It has been proposed that ROS may reduce the fidelity of the polymerase y in mitochondria, which causes the somatic mutations of the mitochondrial DNA responsible for ageing [146]. Another paper proposed that ROS may first disrupt cell signalling, which causes ageing, and only if cells cannot cope with ROS, they undergo an oxidative stress status with cellular damage [147]. Thus, it seems that ROS causes ageing, which is the loss of the biological functions related to survival and reproduction, by interfering with other biological processes, and only if the cells cannot cope with free radicals do they suffer oxidative damage, but the ageing process is already ongoing. From these updated theories, the need to understand the role of ROS in the different biological processes seems necessary to choose multiple markers of oxidative stress to support their role in the ageing process.

Finally, our selection process included exposing the populations to a parasite, which led to the evolution of resistance to and improved tolerance of the parasite. However, these evolutionary responses did not directly impact longevity as we expected and found in other studies that long-living organisms had an altered immunity compared to short-living ones [148]. Interestingly, exposure to the parasite and early reproduction influenced the oxidative amount the mosquitoes suffered. Exposed early reproducing mosquitoes showed a significantly higher amount of oxidative damage than not exposed early reproducing mosquitoes. These results seemed inexplicable as the other oxidative stress markers were not different. Still, we detected that exposed mosquitoes had a bigger body mass than those not exposed. Hence, we hypothesised that the interaction between exposure to the parasite and early reproduction could induce a change in the metabolism of the mosquitoes, maybe to better compensate for the loss in fitness due to the parasite, when present, which resulted in a higher body mass but at the same time more oxidative damages when the parasite is absent. Moreover, we detected such interaction only in early reproduction because this selection theoretically selects individuals who invest more in reproduction to the detriment of other traits. Thus, parasite exposure may push this selection further and exacerbate the negative oxidative stress.

Interestingly, the fact that tolerance and resistance were increased as an evolutionary response to *V. culicis* infections suggests no negative genetic correlation between the two immune strategies, as suggested by other studies [76], [149]. Conversely, the positive correlation may result from a positive genetic correlation or an independent evolution of the two traits. Unfortunately, our experiment design could not indicate which possibility is correct. However, two other studies have already found a positive correlation [150], [151], and this outcome potentially impacts the study about the evolution of host-parasite interactions. Future research could further investigate the genetic and physiological mechanisms underlying the evolution of resistance and tolerance to the parasite and the potential links between these mechanisms and senescence. Elucidating the specific genetic variations and immune pathways involved in resistance and tolerance could provide valuable insights into disease control strategies and enhance our understanding of the complex interactions between senescence and immune function in *Aedes aegypti* mosquitoes.

In conclusion, our experimental evolution study aimed to evolve a slower senescence phenotype *in Aedes aegypti* mosquitoes through selective breeding for late reproduction. While the decrease in the proportion of oxidised glutathione over the total amount supported an improvement in antioxidative defence, our findings did not provide strong evidence supporting the role of oxidative damage in the ageing process, as initially suggested by the free radical theory of ageing. However, our results seem more aligned with the updated version of the theory, but it will be worth having a better understanding of the role of ROS in the different biological processes and from there, it might be possible to elucidate the cause of senescence. Additionally, the evolution of resistance and tolerance to the parasite observed during the selection process did not directly impact the senescence phenotype. These findings emphasise the intricate nature of the factors influencing senescence and immune responses and highlight the need for further research to unravel their underlying mechanisms fully.

Chapter 4

Effect of sex proportion on the physiology and immunity of male *Aedes aegypti*

Belli Alessandro[a] and Jacob C. Koella[a]

[a] Institute of Biology, University of Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland

4.1. Abstract

One of the most fundamental life-history trade-offs is the cost of reproduction, which is often reflected in survival, especially in males. However, other traits, such as resistance to stress or immunity, should be impacted. For that reason, we think that increasing the reproductive effort in *Aedes aegypti* males will increase stress, particularly oxidative stress, and as oxidative stress has been reported to impact survival and immunity negatively, we expect to see a decrease in such traits. We found that males in a female-biased group, thus having more mating opportunities, showed an increased malonaldehyde level correlated with oxidative damage. Additionally, we detected a decreased level of superoxide dismutase and glutathione, meaning an overall lover of antioxidant defence and, finally, a lower likelihood to survive a bacterial challenge, reflecting a lower tolerance to bacterial infection. However, we do not find an influence on melanization. These results were only observed in the males in the female-biased group but not in males in the male-biased group. Thus, we could suggest that the cost of reproduction in *Ae. Aegypti* males were not induced by male-male competition but probably by the cost of the mating itself and, more specifically, by producing sperm and seminal fluid. Finally, the findings indicate that susceptibility to oxidative stress might be an alternative mechanistic link to the energy allocation between reproduction and survival and maybe other life-history traits.

4.2. Introduction

Sexes present several differences in multiple aspects of their life. Indeed, differences have been reported for several species across the animal kingdom [152]–[158]. These can be explained by the different evolutionary pressures working on the sexes: females evolved traits to increase their longevity and reproductive lifespan, while males evolved traits to enhance their reproductive effort over longevity [159]. Reproduction may be the primary driver of such differences. In fact, among the evolutionary forces, sexual selection is among the most powerful. Hence, it is not impossible to think that it may be the driving force behind the sex difference and some trade-offs, such as between male reproduction and longevity. Sexual selection occurs when one sex competes for fertilization of the gametes of another sex, and it can be divided into two major forms: intrasexual selection and intersexual selection. In this study, we will focus only on the intrasexual selection that occurs when members of one sex compete for access to potential mates. Generally, males compete between them for access to females and endure significant costs. These costs might explain why increasing their reproductive effort decreases other traits, such as longevity. However, male costs may be due not only to competition but also to the mating itself. Even though the male sexual apparatus's development is genetically determined, and thus the cost is "paid" during the development stages. The individual modulates its activation according to the situation; consequently, the cost is also modulated. In addition, mating requires the implementation of behaviours that engender cost for the male. This selection pressure for male reproductive effort can decrease their longevity by at least two costs: the physiological cost of competition with other males for access to females and mating with the females. However, these costs should be reflected in other traits. The first possibility is oxidative stress resistance. Oxidative stress has been suggested as a cost of reproduction impacting the capacity of an individual to invest in other traits [160]. Oxidative stress is the physiological status in which antioxidant defences no longer balance reactive oxygen species (ROS) production. ROS are by-products of oxidative phosphorylation during energy metabolism [50], [161] and can damage biomolecules unless neutralized by antioxidant defences. Thus, if ROS production increases and antioxidants do not counterbalance them, the individual enters an oxidative stress status that can cause severe damage to the biomolecules and impair redox regulation and cellular signalling. In addition, oxidative stress has been suggested to participate in the onset of several diseases and the ageing process [129], [160], [162]. As said above, energy metabolism is the primary source of ROS; thus, investment in energetically demanding activities related to reproduction may increase ROS production and consequently cause oxidative stress [40], [163]. A second cost paid due to increased reproductive effort may be the decrease in the immune response. Innate immunity relies on several ROS to fight against pathogens. However, ROS are nonspecific reactive metabolites, and their use is a possible hazard source. They will damage the pathogen and the host if their surplus is not tightly controlled. So, the use of ROS during immunity and their effect strongly depends on the ability of the organism to manage the toxic side effects. Thus, if the organism needs to activate the immunity while already implicated in a stressful activity, such as reproduction, the ROS produced by the

immunity may have an autoimmune effect because of the overcoming of the detoxifying capacity of the organism. Stressful activities have been reported to increase ROS production and decrease antioxidant defences [163]. So, during periods of stress, such as reproductive season, the potential autoimmune effect of ROS may be amplified, and the immune responses may be less efficient [50]. Another way in which reproduction can negatively affect immunity is through the reallocation of resources. Several studies showed how individuals with high reproductive effort generally had decreased immune activity. In animals, this is often explained by the need for testosterone, which has an immunosuppressive effect, to produce the secondary sexual traits necessary to ensure mating [164], [165]. However, reproduction costs have also been reported in insects lacking sexual hormones [166], [167]. For this reason, it has been suggested that the existence of a model of a resource-based trade-off between immunocompetence and sexual traits can be applied independently to the presence of hormones [113]. Indeed, we can summarise this model in two steps. The first step is the food acquisition and processing that grants access to different nutrients, and the second step is the allocation of such nutrients to the different traits. Thus, one or more life-history traits may be limited by the availability of nutrients, which imposes on the organism to choose how to allocate the resources between the traits [168]. When the organism faces this situation, it faces a trade-off.

In this study, we focus on mosquitoes. Male mosquitoes, including the yellow fever mosquito *Aedes aegypti*, live considerably shorter than females [85]. But this difference appears to be a consequence of the cost of reproduction for the males, for males that are kept individually and thus do not compete or mate live as long as or even longer than females (Koella et al., unpublished results). For this reason, we designed an experiment to determine whether the cost of competing for access to mates or the cost of mating is more important for the mosquito *Ae. aegypti.* We thus kept mosquitoes in groups with sex ratios ranging from very male-biased, in which competition among males was intense, to very female-biased, where males had easy access to females. Since costs are often easier in undernourished individuals [168], we tested the mosquitoes maintained at the standard food regime of our lab and half of the standard food. Finally, we will measure the impact of experimental factors on the immunity and the physiology of the males and females. We predicted that females would show less evidence of costs than males. The costs of males should increase or decrease depending on the sex ratios of the group, according to whether the cost of mating or competing with other males is more important. We thought it was important to elucidate the cost source because this has dramatically different effects on population dynamics, for example, in the case of a mass release of sterile males for vector control. If the cause of the mortality is the competition between males, we will artificially increase the mortality of all the males in the population and by doing that, we can decrease the effect of our control measure. On the opposite hand, if the cause of male mortality is the mating opportunity, with an increase in the proportion of males in the population, we can expect a decrease in male mortality, and consequently, our released sterile male may survive longer than planned. This knowledge may be helpful when assessing the efficiency of the vector control strategy.

4.3. Material and methods

4.3.1. Ades aegypti

The mosquito *Aedes aegypti* is a vector of arboviruses like that causing yellow fever [81], dengue [85], Chikungunya and Zika [82]. It is ubiquitous in tropical and subtropical regions. We used the UGAL strain of the mosquito (obtained from Patrick Guérin, University of Neuchâtel), which we had maintained in our standard laboratory conditions for many years at 26.5 °C, 70% humidity, and 12:12 hours light-dark photoperiod with access to 6% sucrose solution.

4.3.2. General design

We reared mosquitoes at a high-food and a low-food diet and maintained adults at a sex ratio of 1:3, 1:1 or 3:1 and assayed the impact of the two factors on the mortality rate of the mosquitoes and their antibacterial and melanization immune responses and their oxidative stress at two ages.

Eggs were hatched synchronously under reduced air pressure. The larvae were reared individually in 12-well tissue-culture plates filled with 3 mL of deionized water. The larvae were reared at the standard diet of our laboratory (age 0: 0.06 mg of TetraMin™ fish food per larva, age 1 day: 0.08 mg, age 2: 0.16 mg, age 3: 0.32 mg, age 4: 0.64 mg, 0.32 mg from age 5 onwards) or at 50% of the diet. Each pupa was placed into a 50ml Falcon tube, and the emerging adults were provided with a cotton ball soaked with an 8% sugar solution. Two days after emergence, adults were moved in groups of 32 individuals to 0.8 L plastic jars in three sex ratios: 8 males and 24 females, 12 males and 12 females, or 24 males and 8 females. Jars per replicated three times per sex ratio for the 100% diet and 13 times per sex ratio for the 50% diet, giving 2592 mosquitoes. 48 hours after the transfer, three individuals of each sex were removed from each jar and assayed for their immune responses and oxidative stress. To maintain the sex ratio and density within the jars, we replaced these with mosquitoes of the same sex and age from our colony. These were previously marked with fluorescent powder so we could identify and ignore them in later analyses. Jars were checked daily, and dead mosquitoes were removed and replaced by marked mosquitoes of the same sex and age from our colony. Ten days after the transfer, the remaining mosquitoes were killed and assayed.

4.3.3. Oxidative stress assay

We estimated oxidative stress with three assays that quantify glutathione, malondialdehyde and superoxide dismutase. Two or ten days after emergence, the mosquitoes were killed at -80°C and left at -80°C until the first day of the assay. Then, the wings and legs of each mosquito were removed. The left wings were used to measure body size; they were mounted on a microscope slide and measured with the software ImageJ from the distal end of the alula to the tip of vein R3. The body was weighed to the nearest 0.01 mg with a microbalance. They were then placed into a 2 mL tube containing a 5mm steel bead and 120 µL of PBS and

homogenized with a tissue lyser for 4 minutes at 45 Hz and centrifuged for 10 minutes at 10'000 RPM at 4°C. Each homogenate was split into three aliquots and stored at -80°C.

Glutathione peroxidase quenches cellular ROS by oxidizing the glutathione in glutathione disulphide, so the proportion of oxidized glutathione over the total glutathione is a measure of the oxidative balance of the cells [105], [141]. To determine the glutathione levels in the reduced (GSH) and oxidized (GSSG) forms, we extracted glutathione on the day we homogenized the mosquitoes homogenization. We added 100 µL miliQ water, 5 µL formic acid 1.25% and 5 µL glutathione ethyl ester 1.25 µg/mL (GSHee; Sigma Aldrich, USA) to one of the aliquots (15 µL) of each homogenate, mixed with a vortex for 10s, and centrifuged the solution for 15 minutes at 15'000 RPM and 4°C. We then removed 115 µL of the extract and placed them in a syringe with a PTFE hydrophilic filter (pore size 0.22 µm), and gently blew it into a glass HPLC vial to ensure no particles were present in the samples. The two forms of glutathione were quantified with UHPLC-MS/MS following Rojas Mora et al. (2016) [107] and were added to obtain the total glutathione necessary to calculate the proportion of oxidized glutathione.

Superoxide dismutase is part of the antioxidant defence mechanisms of organisms. It was quantified with the Cayman Superoxide Dismutase Assay Kit (Cayman Chemical, USA) with minor modifications. In particular, the mosquito's homogenate was diluted 1 in 8 parts PBS before using it as described in the kit protocol. Samples were run in duplicates with an average within-plate coefficient of variation (CV) of 2.59%, and 35 samples were assayed in different plates, showing inter-plate repeatability of r = 0.92.

Malondialdehyde (MDA) is one of the final products of the peroxidation of polyunsaturated fatty acids in cells. Since its production releases free radicals, it is often used as an oxidative stress marker. We quantified MDA following the procedure described by Mendonça et al. (2017) [108] with minor modifications. 40 µL of NaOH 1.2 M and 10µL of mosquito homogenates were mixed and incubated for 30 minutes at 60°C for the protein hydrolysis. The samples were cooled at 4°C for 2 minutes, and 10 µL of the internal standard (d2-MDA 30 µM in 0.1 M HCl) was added. So that the proteins precipitated, we added 142 µL of trichloroacetic acid 20%, mixed, sonicated, and then centrifugated the samples for 5min at 9300 rcf. 180 µL of the supernatant were transferred to a 1.5 mL microcentrifuge tube. We added 18 µL of 2,4 dinitrophenylhydrazine 5mM and incubated the samples for 10 minutes at room temperature with gentle agitation to derive the MDA. 22 µL of NaOH 10 M were used to alkalize the sample. We added 250 µL of a solution of toluene and cyclohexane (1:1 v/v) and transferred the supernatant to a 1.5 mL microcentrifuge tube for the two phase-to-phase extractions. The recovered organic phase was evaporated in a SpeedVac ® at 35°C, and the pellet was reconstituted in methanol 50%. The extracts were finally filtered with a 22 µm PTFE filter (BGB, Germany) into an HPLC vial before being analyzed with an HPLC-MS. A standard curve, ranging from 0 to 40 µg/mL, was done with MDA tetrabutylammonium salt (Sigma-Aldrich Inc. St-Louis, Missouri), and all the standards went through the extraction protocol together with the samples.

4.3.4. Bacterial challenge assay

We assessed the efficacy of the mosquito's antimicrobial response by measuring the growth of green fluorescent E.coli strain BZB1011 [169] within the mosquito. Two or ten days after emergence, the mosquitoes were anaesthetized on ice for 2-5 min, and we injected 3'500 E.coli (0.2 µL of bacteria solution) into their thorax using glass micro capillaries. We kept the inoculated mosquitoes in individual cups for 48 hours and then assayed the proportion that survived and measured the bacterial load in the surviving mosquitoes. To do so, mosquitoes were briefly anaesthetized on ice, transferred in Eppendorf tubes and crushed with micro-pestles in 200µL of Luria-Bertani broth. The homogenate was diluted 20-fold in LA, and 100 µL was spread on LA agar plates. The agar plates were incubated at 37°C overnight, and bacteria colonies were counted under fluoresce light. The number of *E. coli* colonies was used to measure the bacterial load in the mosquitoes.

Injection doses were prepared by measuring the absorbance at a wavelength of 600 nm E.coli grown overnight in LA at 37°C and comparing this absorbance with a standard curve made before the experiment using E.coli solutions of known concentration. Serial dilutions were made until the desired absorbance was reached, corresponding to 17.5 x 106 *E. coli* per millilitre (3'500 bacteria per injection). The solution was kept on ice during the manipulation to avoid further bacterial growth, and a new solution was prepared every day of injection.

4.3.5. Melanization assay

Melanization ability was tested by inoculating mosquitoes with negatively charged carboxymethyl Sephadex® C-25 beads (Sigma-Aldrich Inc., St. Louis, Missouri), according to Barreaux et al. (2017) [170]. We anaesthetized mosquitoes in a Falcon™ tube placed on crushed ice for 5 to 10 minutes and then injected with a glass microcapillary one bead (30-100 µm diameter) into the mosquito's thorax. Injected adults were transferred to a plastic cup and were killed by freezing 48 hours after injection. The mosquitoes were dissected in a 0.1% methyl green saline solution, and a picture of each bead was taken under a microscope at 20x magnification. Melanization and the diameter of the bead were quantified with the software ImageJ. First, we manually selected the non-melanized parts of the beads and made them white to avoid counting them in the melanization index. Then, the mean grey value of each bead picture was extracted and used as a melanization index for statistical analyses. A grey value of 0 means that the bead is entirely white and not melanised, and a value of 256 indicates a black bead that is completely melanized.

4.3.6. Statistical analyses

All analyses were done using R software (R version 4.2.2 (2022-10-31 ucrt). Significance was assessed with the Anova function of the car library. If the interaction was significant, we used a type III SS and a type II SS

otherwise. All of the explanatory variables (age, sex, sex ratio and food regime) were considered categorical variables.

We analyzed survival during the experiment with a generalized mixed effect model (GLMer) with a family error binomial. Our response variable was binomial: dead (1) or alive (0). The explanatory variables were sex, sex ratio and the food regime, and the ID of the beaker was included as a random effect. To analyze the oxidative stress markers, we first transformed them with a BoxCox transformation to obtain the best possible normal distribution. Thus, GSH was square-transformed, the proportion of oxidized glutathione was log10 transformed, MDA was square-root-transformed, and the proportion of oxidized glutathione (GSSG) was SOD was used without transformation. The four markers were analyzed with a linearised mixed effect model (LMer).

The response variable was the marker, and the explanatory variables were the sex and age of the individual, the sex ratio and the food regime. The beaker ID was included as a random effect. We analyzed the melanization response (the grey value of each bead) with a linearised mixed effect model (LMer). The response variable was the grey value, and the explanatory variables were the sex and age of the individual, the sex ratio and the food regime. The beaker ID was included as a random effect, and the bead diameter was used as a covariable. The antibacterial response was assessed as the probability of death following the challenge and as the ability of the mosquito to clear the infection. We analyzed the probability of death and the probability of clearing the infection with generalized mixed-effect models with a binomial error distribution. In both analyses, the explanatory variables were the sex and age of the individual, the sex ratio and the food regime. The beaker ID was included as a random effect.

4.4. Results

4.4.1. Survival

Of the 2592 mosquitoes, 165 died. The mortality of males was about 15 times than that of females (165 males vs. 10 females died; χ^2 = 55.6, df=1, p <0.001), and the mortality was higher if the larvae had been fed at 50% of the standard food than if they had been well fed (100 deaths at low food vs 55 at high food; χ^2=5.44, df=1, p=0.020). The mortality of males was highest in female-dominated cages (23.6%, 18.3%-29.7% ci) and lowest in the male-dominated cages (5.1%, 3.3%-6.7% ci), but the mortality of females was affected only slightly by the sex ratio (interaction sex * sex ratio: χ^2=12.1, df=2, p=0.002) (Fig. 17).

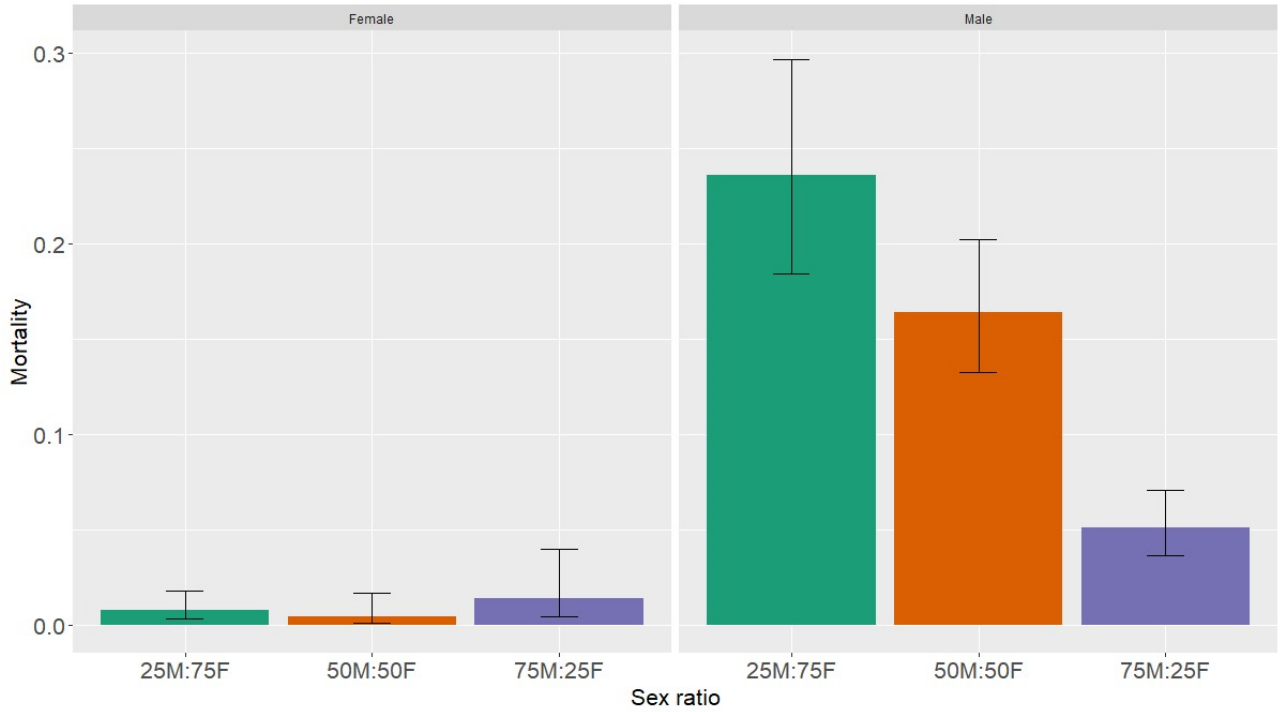

Figure 17: The bars indicate the proportion of the mosquitoes that die during the experiment (y-axis) for all the replicated of one specific ratio of sexes (x-axis). The left side of the figure shows the bars for the females. On the right side are the bars for the males. The vertical lines give a 95% confidence interval.

4.4.2. Bacterial challenge

The mortality within 48 hours of being inoculated with bacteria was higher for poorly fed mosquitoes (24.2%, 18.0%-31.6% cv) than for well-fed ones (13.8%, 9.4%-19.8% cv)(χ^2=4.16, df=1, p=0.041). While it was higher for males than for females (5.6%, 3.0%-10.2% cv) (χ^2=22.5, df=1, p<0.001), the difference between sexes was affected by the sex ratio (interaction sex * sex ratio: χ^2=7.42, df=2, p=0.024), with males in a female-dominated group having higher mortality (39.1%, 26.4%-53.5% cv) than those in a male-dominated group (27.8%. 17.6%-40.9% cv) and females in a female-dominated group having lower mortality (1.9%, 0.3%-9.8% cv) than those in a male-dominated group (13.0%, 6.4%-24.4% cv) (Fig. 18).

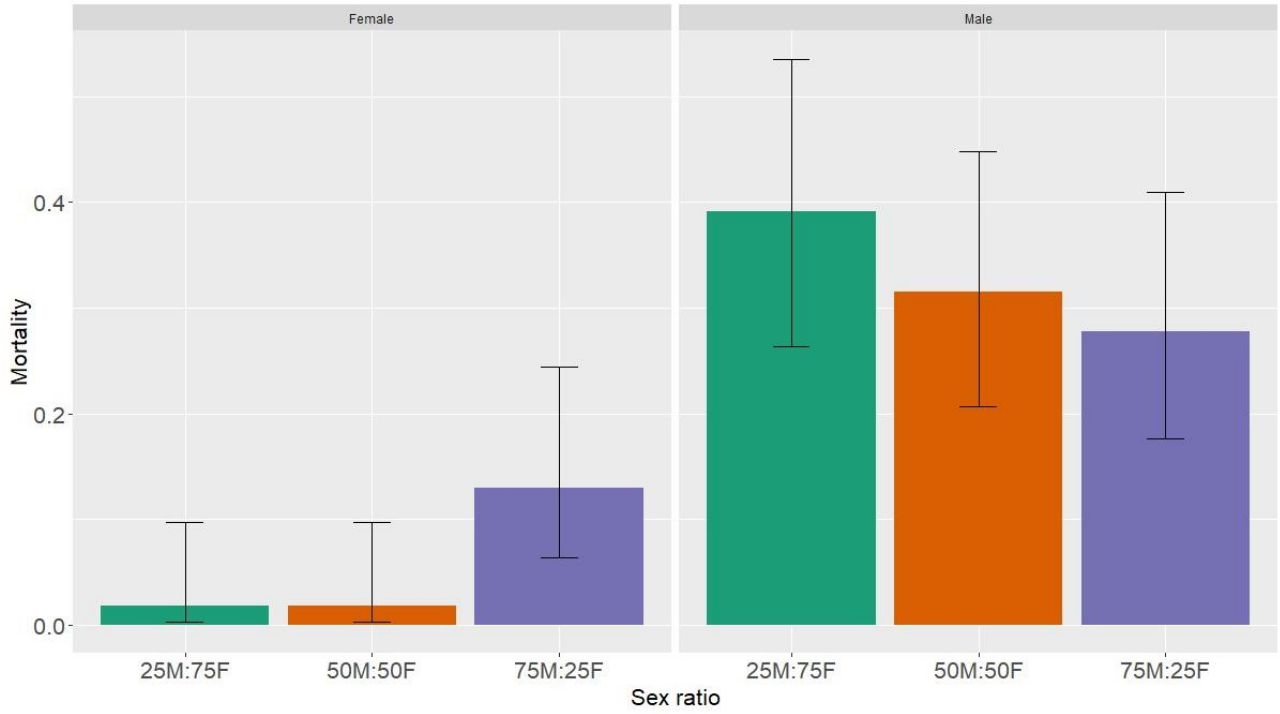

Figure 18: The bars indicate the proportion of the mosquitoes that die after the bacterial challenge (y-axis) for all the replicated of one specific ratio of sexes (x-axis). The left side of the figure shows the bars for the females. On the right side are the bars for the males. The vertical lines give a 95% confidence interval.

Within the mosquitoes that had survived 48 hours after being inoculated, fewer males than females cleared the infection (so no bacteria were detected) (χ^2=9.49, df=1, p=0.002), old mosquitoes were less likely to clear the infection than young ones (χ^2=12.9, df=1, p<0.001). On the capacity to clear an infection, neither the sex ratio (χ^2=0.39, df=1, p=0.82) nor the interaction sex ratio*sex had a significant effect (χ^2=0.86, df=2, p=0.65). Although it would appear that males living in a group dominated by females (14 clear individuals, 920 ci) are slightly better than the males in the other groups (25M:75F: 7 clear individuals, 4-12 ci and 50M:50F: 8 clear individuals, 4-14 ci). (Fig 19)

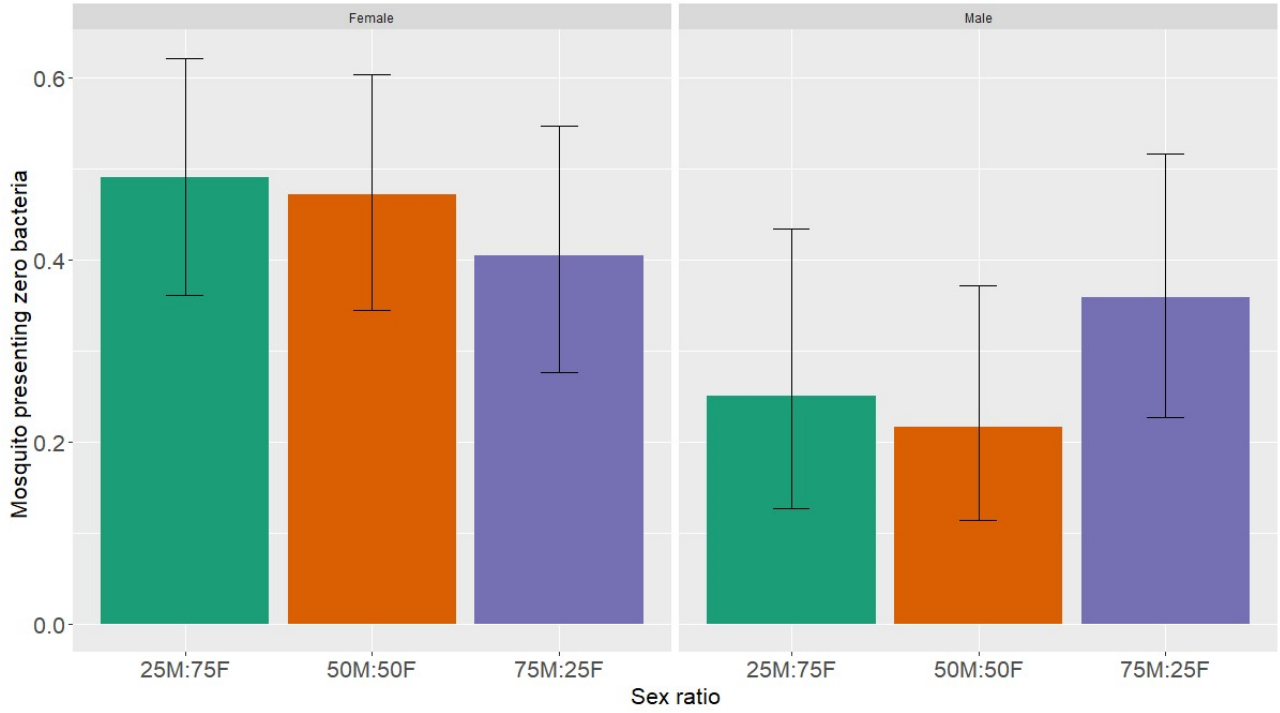

Figure 19: The bars indicate the proportion of the mosquitoes that presented zero bacteria after 48h from the inoculation (y-axis) for all the replicates of one specific ratio of sexes (x-axis). The left side of the figure shows the bars for the females. On the right side are the bars for the males. The vertical lines give a 95% confidence interval.

4.4.3. Melanization assay

The melanization score of males (18.03, 11.20-24.86 ci) was lower than that of females (72.78, 62.75-82.81 ci) (χ^2=51.33, df=1, p<0.001). No other factors had a significant effect. Neither the sex ratio (χ^2=1.99, df=2, p=0.37) nor the interaction between sex ratio and sex (χ^2=2.60, df=2, p=0.27) had a significant effect on the melanization index (Fig 20)

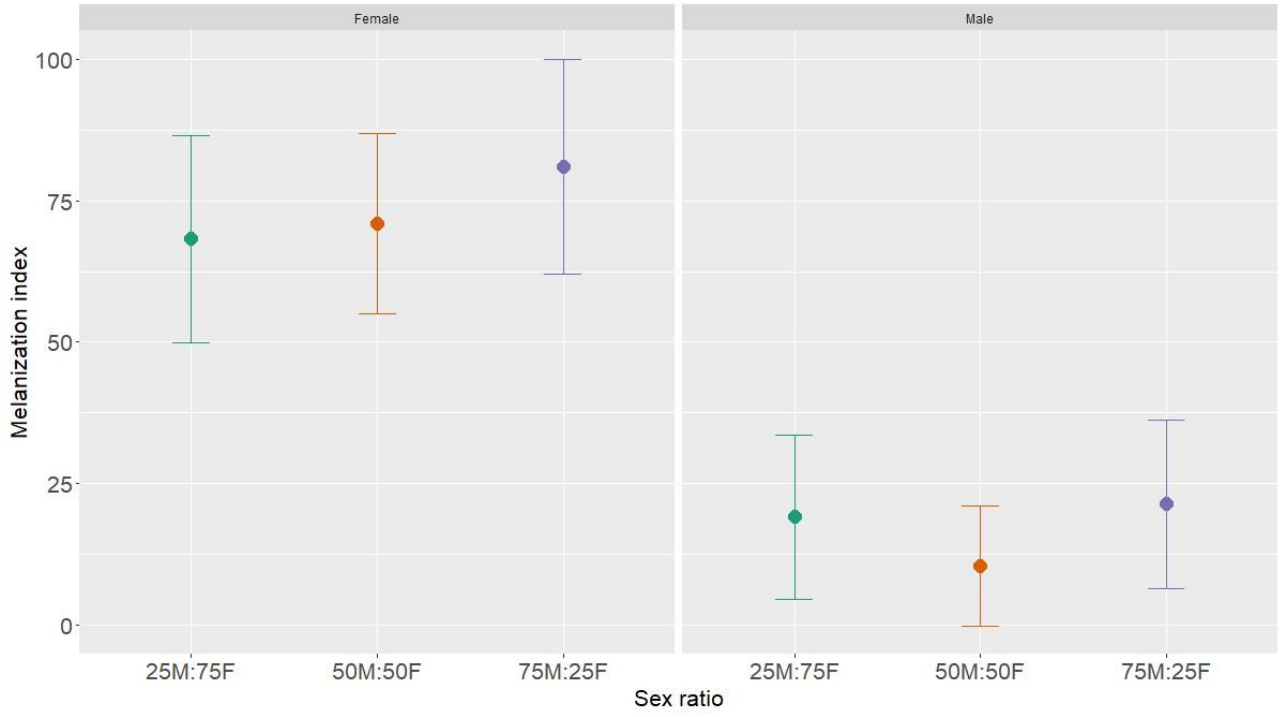

Figure 20: Plot of the melanization index in function of the ratio of sexes. The left side of the graph represents the values of the female mosquitoes, and the right side represents the values of male mosquitoes. The point represents the average value for the group. The vertical lines represent the 95% confidence interval.

4.4.4. Oxidative stress

Malondialdehyde: Old mosquitoes had more MDA than younger ones (χ^2=349, df=1, p<0.001), males had more MDA than females (χ^2=51.7, df=1, p<0.001), and the effect of age was stronger in males than in females (young males: 1.13 ng/mg, 0.90-1.36 ci; old males: 5.79 ng/mg, 5.17-6.41 ci; young females: 1.27 ng/mg, 1.09-1.45 ci; old females 2.93 ng/mg, 2.67-3.19 ci) (χ^2 = 85.17, df=1, p <0.001). Mosquitoes reared at low food (2.39 ng/mg, 2.04-2.74 ci) had less MDA than those reared at high food (3.11 ng/mg, 2.70-3.52 ci) (χ^2 = 14.29, df=1, p<0.001). Males living in a male-dominated group had less MDA (3.27 ng/mg, 2.36-4.18 ci) than males living in a female-dominated group (3.70 ng/mg, 2.77-4.63 ci), whereas females living in male-dominated group had more MDA than female living in female-dominated group (2.21 ng/mg, 1.83-2.59 ci vs. 1.86 ng/mg, 1.53-2.20 ci) (interaction sex * sex ratio: χ^2=6.49, df=2, p=0.039) (Fig. 21).

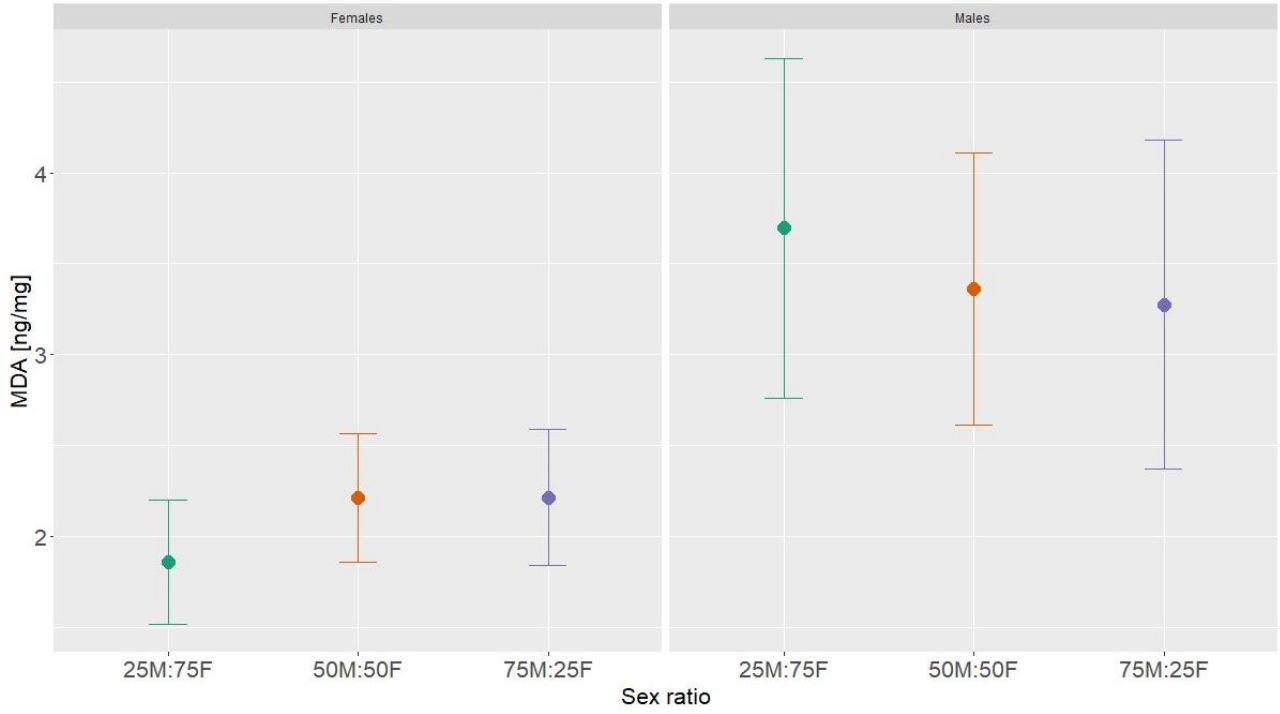

Figure 21: Plot of the MDA amount [ng] per mg of mosquito in the function of the ratio of sexes. The left side of the graph represents the values of the female mosquitoes, and the right side represents the values of male mosquitoes. The point represents the mean of the MDA amount of all mosquitoes of one ratio of sex and one sex. The vertical lines represent the 95% confidence interval.

Total glutathione: While young individuals (0.31 µg/mg, 0.30-0.32 ci) had a higher concentration of glutathione than old ones (0.28 µg/mg, 0.27-0.30 ci) (χ^2=9.59, df=1, p=0.002) and well-fed mosquitoes had more (0.31 µg/mg, 0.30-0.32 ci) than poorly fed ones (0.28 µg/mg, 0.26-0.29 ci) (χ^2=5.47, df=1, p=0.019), the effect of age was apparent only in the poorly fed ones (young: 0.32 ng/mg, 0.31-0.33 ci; old: 0.23 ng/mg, 0.21-0.26) but not in the well-fed individuals (young: 0.30 ng/mg, 0.29-0.31 ci]; old: 0.31 ng/mg, 0.30-0.33 ci) (interaction age * food: χ^2=22.94, df=1, p<0.001), and the effect of age was apparent only in females (young: 0.33 ng/mg, 0.32-0.34 ci; old: 0.25 ng/mg, 0.23-0.27 ci) but not in males (young: 0.29 ng/mg, 0.27-0.31; old: 0.30 ng/mg, 0.28-0.33 ci), (interaction age * sex: χ^2=57.4, df=1, p<0.001). The glutathione levels were affected by an interaction between the sex ratio and the mosquito's sex (χ^2=9.32, df=2, p=0.009) (Fig. 22), with males living in a male-dominated group having more glutathione (0.31 ng/mg, 0.30-0.33 ci) than those living in a female-dominated group (0.29 ng/mg, 0.26-0.31 ci) and females living in a male-dominated group having less glutathione (0.28 ng/mg, 0.26-0.30 ci) than females living in a female-dominated group (0.30 ng/mg, 0.28-0.32).

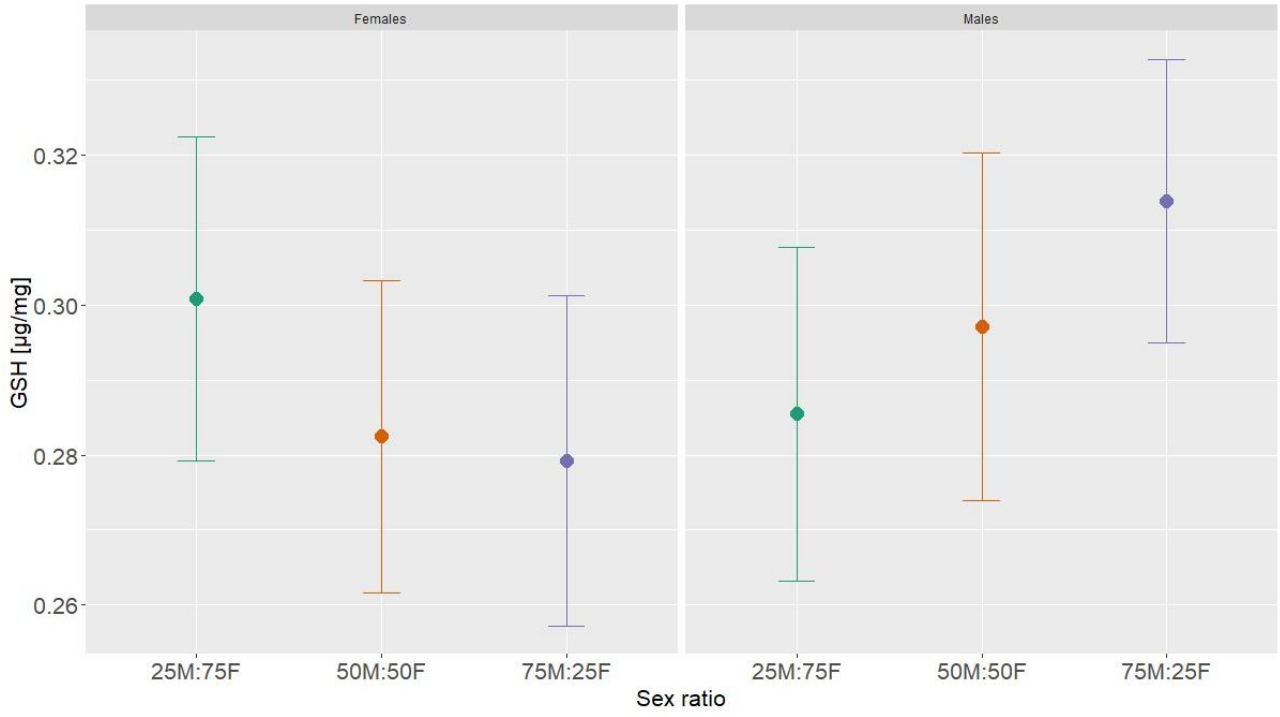

Figure 22: Plot of the total GSH amount [µg] per mg of mosquito in the function of the ratio of sexes. The left side of the graph represents the values of the female mosquitoes, and the right side represents the values of male mosquitoes. The point represents the mean of the GSH amount of all mosquitoes of one ratio of sex and one sex. The vertical lines represent the 95% confidence interval.

Oxidized glutathione: Well-fed mosquitoes had more oxidized glutathione (0.0073 µg/mg, 0.0078-0.0068 ci) than poorly fed ones (0.0060 µg/mg, 0.0066-0.0054 ci) (χ^2=14.26, df=1, p < 0.001), the effect of age was different in the poorly fed ones with a decrease of the oxidized form with age (young: 0.0065 µg/mg, 0.0060-0.0070 ci; old: 0.0056 µg/mg, 0.0044-0.0065), at the opposite in the well-fed individuals we detected an increase of the oxidized from with age (young: 0.0070 µg/mg, 0.0064-0.0076 ci]; old: 0.077 µg/mg, 0.0066-0.0086 ci) (interaction age * food: χ^2=5.51, df=1, p=0.19). Similar age decreased the oxidized form in females (young: 0.0068 µg/mg, 0.0063-0.0073 ci; old: 0.0058 µg/mg, 0.0052-0.0064 ci) but increased in males (young: 0.0067 µg/mg, 0.0061-0.0073; old: 0.0076 µg/mg, 0.0065-0.0087 ci), (interaction age * sex: χ^2=5.96, df=1, p=0.015). The levels of oxidized glutathione were affected by an interaction between the sex ratio and the mosquito's sex (χ^2=9.04, df=2, p=0.011) (Fig. 23), with males living in a male-dominated group having more glutathione (0.0083 µg/mg, 0.0080-0.0097 ci) than those living in a female-dominated group (0.0063 µg/mg, 0.0053-0.0073 ci) and females living in a male-dominated group having less glutathione (0.0063 µg/mg, 0.0057-0.0069 ci) than females living in a female-dominated group (0.0068 µg/mg, 0.0060-0.0074).

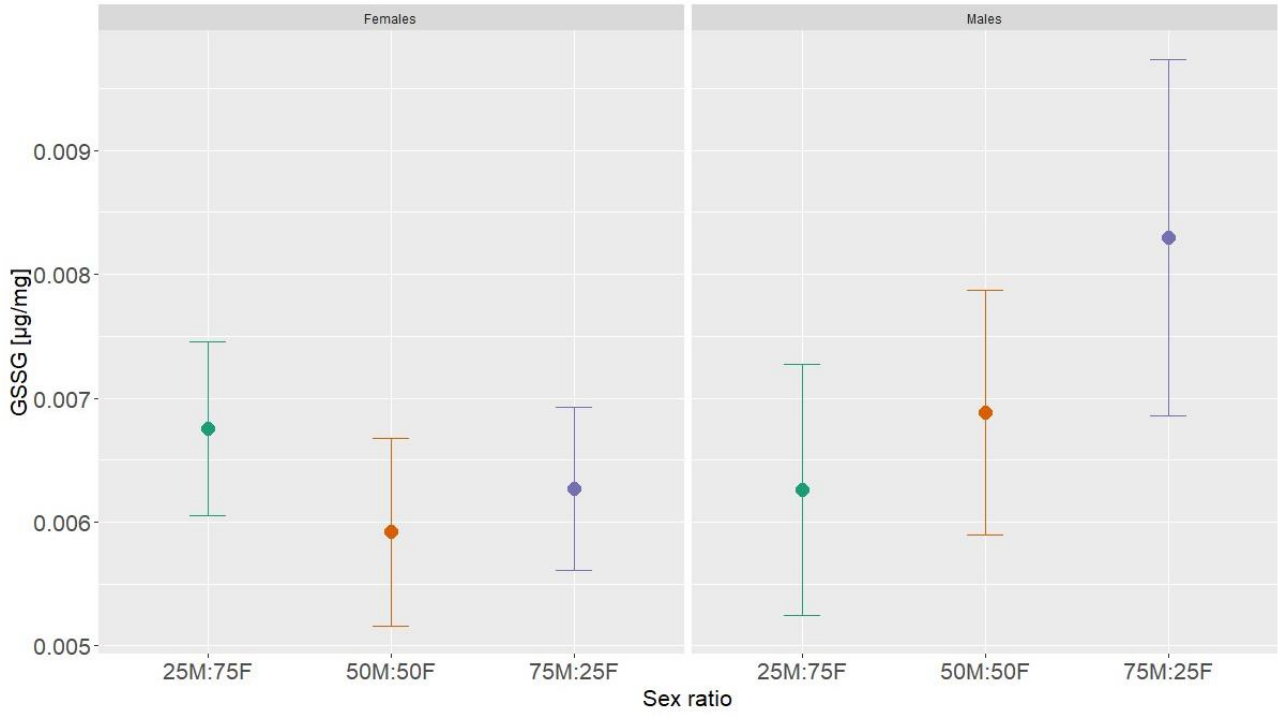

Figure 23: Plot of the oxidized glutathione (GSSG) amount [µg] per mg of mosquito in the function of the ratio of sexes. The left side of the graph represents the values of the female mosquitoes, and the right side represents the values of male mosquitoes. The point represents the mean of the GSH amount of all mosquitoes of one ratio of sex and one sex. The vertical lines represent the 95% confidence interval.

The proportion of oxidized glutathione over total amount: Well-fed mosquitoes (0.024, 0.022-0.026 ci) had a higher proportion of oxidized glutathione than poorly fed mosquitoes (0.022, 0.020-0.023 ci) (χ^2=5.57, df=1, p=0.018). The interaction between the age and the diet of the mosquitoes had a significant role in determining the proportion of oxidized glutathione (χ^2=5.30, df=1, p=0.021), the effect of age was stronger in poorly fed mosquitoes (young: 0.020, 0.019-0.021 ci; old: 0.023, 0.022-0.024 ci) than in well-fed ones (young: 0.024, 0.023-0.025 ci; old: 0.025, 0.023-0.026 ci). Neither the sex ratio (χ^2=0.64, df=2, p=0.73) nor the interaction between the sex ratio and mosquito's sex (χ^2=0.35, df=2, p=0.84) was significant in influencing the proportion of oxidized glutathione, although it would seem that male in a male dominated-group (0.026, 0.022.0.030 ci) had a slightly higher proportion than the males in the others groups (50M:50F: 0.023 &, 0.020-0.026 ci and 25M:75F: 0.023, 0.020-0.026 ci) (Fig 24).

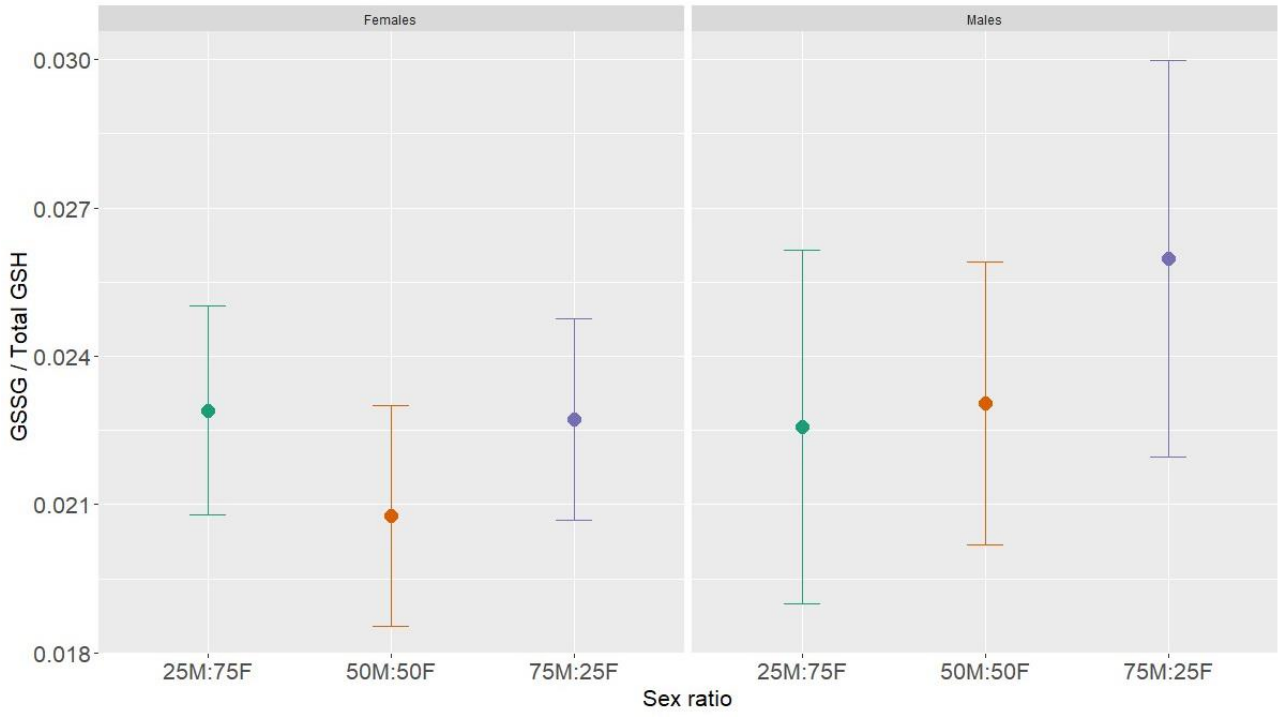

Figure 24: Plot of the proportion of oxidized glutathione over the total amount [%] in the function of the ratio of sexes. The left side of the graph represents the values of the female mosquitoes, and the right side represents the values of male mosquitoes. The point represents the average proportion of all mosquitoes of one ratio of sex and one sex. The vertical lines represent the 95% confidence interval.

Superoxide dismutase: Males (0.0024 units/mg, 0.0023-0.0025 ci) had more enzymes than females (0.0021 units/mg, 0.0020-0.0021 ci) (χ^2=50.5, df=1, p<0.001). While well-fed mosquitoes had more SOD than poorly-fed ones (χ^2=64.5, df=1, p<0.001), the effect of food was stronger in young mosquitoes (well-fed: 0.0026 units/mg, 0.0025-0.0027 ci; poorly fed: 0.0018 units/mg, 0.0017-0.0019 ci) than in old ones (well fed: 0.0024 units/mg, 0.0023-0.0025 ci; poorly fed: 0.0021 units/mg, 0.0020-0.0022 ci) (interaction food * age: χ^2=14.0, df=1, p<0.001). The amount of SOD was affected by an interaction between the sex ratio and the sex of the individual (χ^2=8.89, df=2, p=0.011), with males in a male-dominated group having more SOD (0.0025 units/mg, 0.0023-0.0028 ci) than males a female-dominated group (0.0023 ng/mg, 0.0021-0.0025 ci) and females in a female-dominated group having more SOD level than those in other groups (Fig. 25).

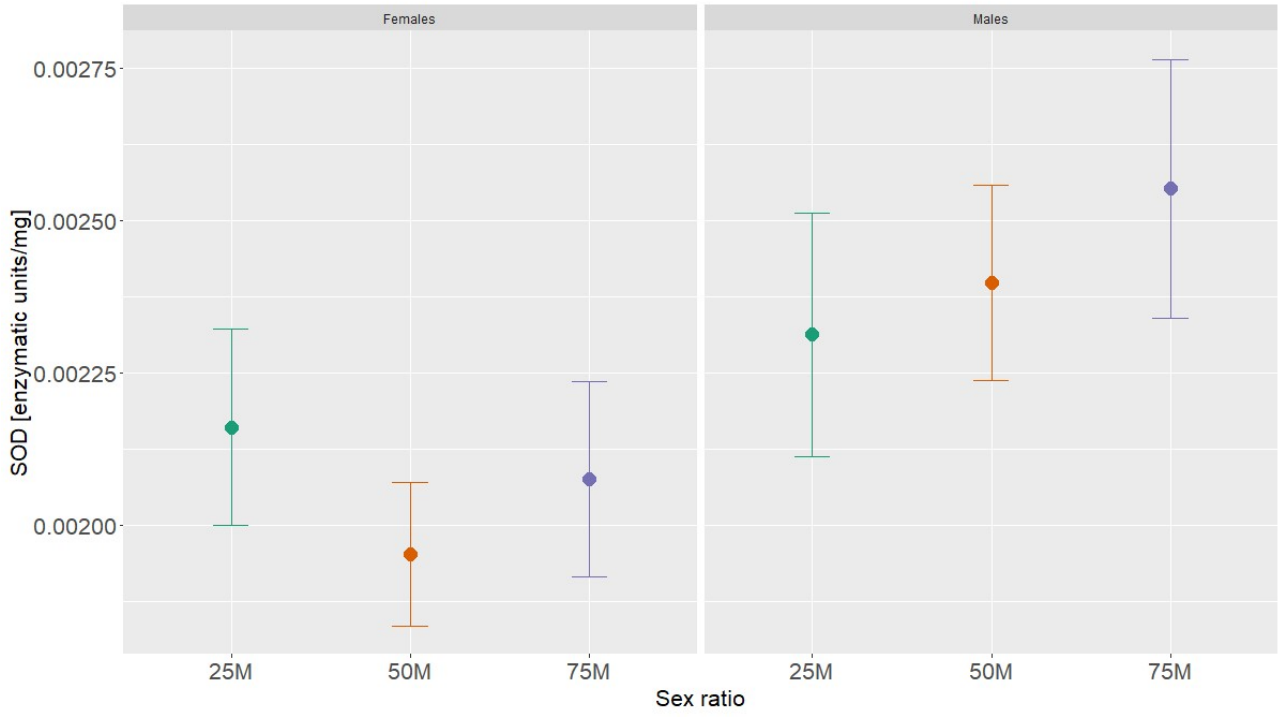

Figure 25: Plot of the SOD amount [units of enzyme] per mg of mosquito in the function of the ratio of sexes. The left side of the graph represents the values of the female mosquitoes, and the right side represents the values of male mosquitoes. The point represents the mean of the SOD amount of all mosquitoes of one ratio of sex and one sex. The vertical lines represent the 95% confidence interval.

4.5. Discussion

Our experiment manipulated the sex ratio of groups of the mosquito *Aedes aegypti* to be highly female-biased (giving males ready access to females), 50:50, or highly male-biased (forcing males to compete for access to females). As the number of females increased, the mortality rate and the cellular oxidative damage of the males increased, while their antioxidant defences and the likelihood that they survived a bacterial challenge decreased. The results suggest that competing with other males for access to females is less costly than the act of mating. Furthermore, although the pattern for females is less clear than for males, it suggests that being in a group with many males is more damaging than being with few males, and thus, for females, being harassed by many males is costly.

The cost of reproduction is one of the central concepts to understand the evolution of life history traits [1], [171], and several energy-based models have been used to describe the link between reproduction and other life-history traits, such as longevity [163]. However, the precise mechanistic bases of this trade-off have rarely been studied [55], [172], and commonly, energy is the proposed currency for reproduction costs. Nevertheless, Alonso-Alvarez has proposed that susceptibility to oxidative stress may be another mechanism linking reproduction and longevity [163] instead of energy reallocation. He found that manipulating the reproductive effort of the male bird showed a decrease in the total antioxidant defences.

Interestingly, our results showed that males exposed to more females had decreased antioxidant defences while suffering more cellular damage and a higher mortality rate. This data supported the idea that oxidative stress may be the currency of reproduction cost and that the link between reproduction and longevity may not directly depend on energy allocation. There is no doubt that reproduction demands energy to fulfil all reproductive behaviours. For that reason, the organisms increase their basal and field metabolic rates [173], and this requires the reallocation of energy from other traits, which was suggested to be the cause of the trade-off between reproduction and longevity. However, it might be that the organisms faced such a trade-off because to increase their metabolic rate, they must inevitably increase oxygen consumption, which can lead to an augmentation of the reactive oxygen species production. ROS are highly reactive molecules with great potential to damage cells and macromolecules [35], [174], [175], leading to premature tissue senesce and organism death. So, given the cytotoxic effects of ROS and their role in the ageing process, reproduction might negatively impact longevity but induce ROS production.

Additionally, we found that tolerance to bacterial infection was also negatively impacted by reproduction. Tolerance is defined as all processes that reduce the decline of fitness during infection [75], [176], and even though the exact general mechanisms of tolerance are not entirely understood, functional studies using RNA knock-down have shown that in Drosophila melanogaster genes involved in metabolic processes, immune regulation and tissue protection can mechanistically determine the tolerance of infection. Thus, since reproduction increases the susceptibility of males to oxidative stress, which is a form of tissue protection,

the males' tolerance to infection is reduced. Hence, results indicate that oxidative stress may also be the mechanistic link between reproduction and tolerance.

In summary, we found that the possible mechanism by which reproduction costs are reflected in longevity and tolerance to bacteria is the increase of the organism's susceptibility to oxidative stress, but for the moment, we do not know the proximal cause of the costs. We supposed that direct competition between males might be the cause. However, we detected that males in a male-biased group showed less cost than males in a female-biased group. These results align with the observations about the ecology of *Aedes aegypti* males in the swarm. Swarms are essential in the *Aedes aegypti* mating behaviour and consist of males flying in an aggregate around the host, waiting for females [177], [178]. So even though swarms are small groups, they are male-biased [179], so it is not feasible for a male to become the dominant and block access to females to all other males. For this reason, males seem to have evolved through a scramble competition, in which all males are ready to try to be the first to mate with the coming females instead of competing to have access to the mate [180], [181]. Thus, our results and the field observation seem to agree that males did not compete with females, and consequently, no or little costs are imposed on their fitness.

After excluding the intrasexual competition, our results indicated that mating itself, specifically the sperm and the seminal fluid, might be the source of the reproductive cost. During copulation, males transfer a wide variety of seminal fluid proteins to females together with sperm. These proteins are the effectors of a wide range of female post-mating responses, including refractoriness to re-mating, induction of ovogenesis and egg-laying behaviour, changing female flight and feeding behaviour, inducing antimicrobial activities, and modulating sperm storage parameters [182], [183], in the specific case of *Aedes aegypti*, a male transfers up to 870 sperm proteins and 280 seminal fluid proteins (SFPs) to the female [184]. The absence of such SFPs has been reported to affect the reproduction success of both sexes negatively, and nutrient-deprived males gradually reduced their fecundation success with an increasing number of mating, probably due to the reduced production of sperm and SFPs because of the limiting resources [185]. Accordingly, the evidence may indicate that SFPs are essential for males' fitness but energetically costly. However, *Ae. aegypti* males may gain a significant fitness advantage by increasing the resources invested in SFPs. *Ae. aegypti* is considered a monogamous species, so if the first insemination is good enough, it is sufficient to fertilize the eggs produced during a female's lifespan. It has been proved that multiple mating has no critical effect on progeny paternity [178]. So, evolutionarily speaking, natural selection should evolve males to reduce the cost of mating to increase the number of acts they can perform during their lives. However, it has been reported that an *Ae. aegypti* male can inseminate a maximum of 5 to 7 females, and its ability to reproduce sperm is limited [186]. Thus, males may have increased their fitness by investing in the SFPs to guarantee that each ejaculate induces the post-mating female responses, among which is the refractoriness to mating. Even more importantly, SFPs must ensure the viability of the sperm for the entire female's life. Doubtless, this explanation only holds if males mate less frequently when the sex ratio is male-biased, that is, if females do

not let males mate whenever they want. In nature, it has been known for a long time and for various species that females may react against copulation with unwanted males [187].

Several studies reported aggressive female behaviours (i.e. kicks, writhing, biting, etc.) to avoid copulating with the wrong male [187]–[189]. And this appears to be the case also in *Ae. aegypti*, where we already observed the appearance of refractory behaviour, an increase of the kicks with the posterior legs of the females within a short time from the first mating (~2 hours) [180]. Additionally, it has been reported that polyandry is very limited (~6%) in natural populations of *Ae. aegypti* [190]. Finally, a study proves that wild-type *Ae. aegypti* kept in close captivity with refractory females do not show any reduced lifespan, while males kept with virgin females do [191]. This evidence corroborates the hypothesis that the essential role of SFPs for male fitness is the cause of the reproduction cost we have observed on longevity and tolerance to infections in *Ae. aegypti* males when the number of females increases and, consequently, access to reproduction.

Ultimately, we detected a pattern in females, indicating that being harassed by males is costly. This cost might be explained by the fact that even though mated females showed refractory behaviour that prevented them from being inseminated by multiple males, this does not mean that females cannot couple when they are refractory. By couple, we mean the act of the male seizing the female and trying to have genitalia contact [186]. Consequently, being in a group with more males can result in contestant harassment that finally costs energy and time for females, as already shown by the study of Helinski et al. (2012) [192].

In conclusion, our results suggested that in males, susceptibility to oxidative stress might be the mechanism by which the reproduction cost is reflected in survival and that seminal fluid protein production and transfer to females might be the proximal cause of the reproduction cost.

Chapter 5

General Discussion

5.1. Discussion

The Free Radical Theory of Aging (FRTA) proposed by Denham Harman in the 1950s posits that the accumulation of free radicals, or more in particular reactive oxygen species (ROS), might be a significant contributor to the ageing process [10]. ROS are highly reactive molecules produced naturally during cellular metabolism, particularly in mitochondria. According to the FRTA, over time, the unchecked accumulation of ROS leads to cellular damage, oxidative stress, and functional decline, ultimately causing ageing-related diseases. Since its postulation, the theory has been very successful, and numerous studies have attempted to prove its veracity. Several lines of evidence supported the theory.

First, the level of oxidative damage to various cellular components (DNA, lipids and proteins) increased with age among various species. Second, long-living animals showed reduced oxidative damage and higher oxidative resistance compared to short-living animals of the same species or among species [193]. However, almost all the evidence was correlative, so some researchers aimed to have direct proof that manipulating oxidative damages by increased reactive oxygen species production or decreasing antioxidant defences resulted in a shorter lifespan. To do so, they use genetic manipulations targeting the expression of antioxidant genes on *C. elegans* or mice. In both cases, some mutants' lines showed higher oxidative stress and an increased life span. To test that the higher oxidant level was the real cause of the increased longevity, they treated the mutants with antioxidants and found a reduced lifespan. These results proved the theory wrong and started requesting the role of ROS [147], [194], which allowed recent research to reveal exciting and diverse roles of ROS beyond their classical association with cellular damage.

ROS have been found to play a critical role in cell signalling involved in various cellular processes. They can activate specific signalling pathways and transcription factors, modulating gene expression and influencing cell proliferation, differentiation, and survival [25], [195]. Additionally, they have been suggested to play an essential role in the immune response by participating in phagocytosis, the process by which immune cells engulf and eliminate foreign invaders. ROS influence immune cell functions, such as antimicrobial defence and inflammatory responses [27], [196], [197]. With our study on the correlation between antioxidant defences and immunity, we were able to show that not only ROS are essential for resistance, but also the antioxidant capacity of an organism might influence its ability to mount an efficient immune response. ROS have been suggested to modulate autophagy, a cellular process responsible for recycling damaged organelles and proteins, and has been reported to be regulated by ROS [198]. Proper autophagy plays a crucial role in cellular quality control and longevity.

Regarding longevity, ROS are implicated in both the induction and regulation of cellular senescence, a state in which cells lose their ability to divide and function properly. ROS can drive senescence at high levels, while moderate levels can promote tissue repair and regeneration. Finally, ROS can trigger mitohormesis, a cellular

response in which low levels of ROS induce adaptive responses that improve cellular health and resilience. This phenomenon challenges the notion that all ROS are solely harmful [198], [199].

These newfound roles of ROS and their multifaceted functions had the potential to reshape the Free Radical Theory of Aging in several ways. First, ROS is now considered to have a dual nature. At first, the traditional FRTA considered ROS as exclusively harmful molecules driving ageing and age-related diseases. However, the current understanding of ROS as signalling molecules and regulators of essential cellular processes reveals their dual nature. ROS can have detrimental and beneficial effects on cells, depending on their levels and context. Second, in the beginning, the theory focused on oxidative damage and the ways to reduce ROS. The emphasis shifts to redox homeostasis, thus maintaining the delicate balance between ROS and antioxidants, which is crucial for cellular health and longevity. Finally, the theory focuses more on mitochondria and their pivotal role in ROS regulation and ageing. Dysfunctional mitochondria can contribute to increased ROS production, while functional mitochondria can implement mitohormesis and adaptive responses. We were able to see such a reshaping of the FRTA in our chapter about the evolution of senescence, where we manipulated the reproductive pattern of our mosquitoes, and we were able to select mosquitoes that have a longer lifespan and a potentially better redox potential, without detecting any change in the amount of oxidative damage they suffer. These results supported the idea that ROS are implicated in more processes than causing oxidative damage.

The reshaping of the FRTA also impacted the medical approach and the possibility of developing health treatment. Since the postulation of the theory, the thinking was antioxidant-based therapies as a one-size-fits-all approach. However, with the recent insights into ROS function, it might be necessary to re-evaluate this approach and focus on targeting more specific ROS pathways and promoting redox balance to reduce ageing-related diseases [200]–[202].

On the side of evolutionary biology, the discovery of new roles for ROS introduces the concept that ROS could be a mechanism of trade-offs in life history decisions. Life history theory explores the allocation of energy and resources to various life functions throughout an organism's lifespan, and the trade-off between immunity and longevity has already been suggested to be mediated by ROS. High ROS levels may accelerate ageing but could also play a role in promoting immunity. Organisms might face a trade-off between investing resources in immunity, where ROS may be beneficial and allocating resources to survival, where minimizing ROS-induced damage is advantageous. In general, ROS can influence various life history traits, such as growth rate, age at maturity, and reproductive output [40], [203], [204]. We detected that the cost of reproduction was reflected in survival by an increased susceptibility to oxidative stress and oxidative damages, suggesting a possible mechanistic role of ROS in the reproduction-survival trade-off. Organisms might employ ROS-mediated signalling pathways to adjust their life history strategies based on environmental conditions and available resources. Incorporating ROS as a mechanism of trade-offs in life history theory adds a new

dimension to our understanding of how organisms allocate resources and energy to maximize fitness and adapt to their environments.

In conclusion, the work in this thesis supports the idea that the Free Radical Theory of Aging has evolved significantly with the discovery of new roles for ROS in cellular function. The updated understanding of ROS as signalling molecules and regulators challenges the traditional view of ROS as solely damaging entities. This new knowledge emphasizes the importance of redox balance and mitochondrial function in ageing. Furthermore, the possibility that ROS may act as a mechanism of trade-offs in life history decisions opens up exciting avenues for further research in ageing and evolutionary biology.

5.2. Synthesis and further perspective

In the first chapter, we found a genetic correlation between resistance to parasites and the total amount of glutathione. This correlation was found between the basal physiological level of uninfected family individuals and the proportion of infection in the same family.

The next step could be to look at ROS evolution during infection in individuals with different genetic resistance. And see if an experimental manipulation of ROS and antioxidants, for example, through dietary supplements, impacts resistance. Additionally, most of the genetic differences in oxidative traits were influenced by maternal effects, meaning a maternal effect may exist. A further step would be to monitor the oxidative stress status of the mother and see whether offspring are negatively influenced by it, meaning the mother does not have the resources to protect them, or whether the oxidative stress induces a priming response that increases the mother investment in eggs to protect offspring against oxidative stress. Understanding more of the mechanisms by which oxidative stress operates transgenerational would be interesting.

The second chapter describes how different reproductive patterns select for different longevity. We increased the lifespan of our mosquitoes by selecting for late reproduction. In addition, we exposed some of the mosquitoes to the microsporidian parasite *V. culicis*, and these lines evolved an increased resistance and a greater tolerance to the parasite by reducing the proportion of infected individuals and, at the same time, increasing the longevity of the infected one. Finally, we detected that our selection for late reproduction, hence potentially slower senesce, decreased the proportion of oxidized glutathione over the total amount by possibly reducing the amount of oxidized glutathione. This data could indicate the redox potential improvement in long-living mosquitoes.

The next step could be quantifying other physiological parameters (carbohydrates, lipids, proteins) between early reproducers and late reproducers and makers of ROS-mediated damages, such as a mutation in mitochondrial DNA or protein damages, allowing us to understand if the evolution of other physiological parameters determines the senescence trajectory of our lines. In addition, it would be interesting to investigate whether our selection for late reproduction influences other life-history traits, which could influence our mosquitoes' oxidative stress physiology. For example, we detected a higher level of oxidative damage in young individuals, which could be associated with the development and metamorphosis from pupae to adults. Thus, it would be interesting to investigate whether late reproducing lines could evolve at a slower growth rate to reduce oxidative damage in this development stage and increase their adult life span. In addition, it would be interesting to know the mechanism of the acquired resistance against *V. culicis* by looking at immune gene expression and other immunity-related parameters. On the side of the parasite, it would be good to explore whether parts of the parasite's virulence are caused by a parasite-induced increase in the total metabolic rate, which increases the production of free radicals cellular damage and accelerates

ageing processes [205]. Finally, testing the evolution of ROS in the different lines when infected could underline some hidden interaction between ROS, immunity, and senescence that we fail to detect in our uninfected individuals.

In the third chapter, we found that ROS might be the mechanistic link between the cost of reproduction and survival in *Aedes aegypti* males. Our results indicated that males in a female-biased group had higher oxidative damage, higher mortality rate, lower antioxidant defences and lower probability of surviving a bacterial infection. In contrast, females did not show a clear pattern. However, living with more males seemed to increase oxidative damage, probably because of the constant harassment.

In our study, females were mated but not allowed to blood feed. Hence, a further step could be to allow females to have access to a blood meal and lay eggs multiple times. This approach could allow us to assess their stress marker after each reproductive episode and understand whether the investment in the actual reproduction affects future reproduction and survival through oxidative stress in females. Additionally, if oxidative stress is an actual cost of life, defending themselves against ROS may not be limited by energy but rather by the availability of antioxidants or their precursors. For both sexes, a further step could be to experimentally manipulate the level of ROS and antioxidants to confirm the role of oxidative stress as a mechanistic link between life-history traits. One possibility is to supplement antioxidants in the diet, and this approach will have the advantage of allowing us to test the effect of such manipulation at different ages. Finally, ROS might be a link between traits that are temporarily separated. Thus, a third possible perspective is to look at the life-history traits of the offspring of parents, from whom we know their oxidative stress level. Because ROS can damage sperm DNA that can be vertically transmitted, or eggs from a "stressed" mother can be less protected against ROS in early life, which can be translated into degraded health in adulthood. In conclusion, focusing on more measurements, such as DNA damage, is more important than the only balance between ROS and antioxidants in adults because measurement in adults could not reflect the oxidative stress during development.

References

[1] K. Winemiller, *The Evolution of Life Histories*, vol. 123, no. 5. Oxford university press Oxford, 1994.

[2] D. Roff, *Evolution of life histories: theory and analysis*. Springer Science & Business Media, 1993.

[3] S. C. Stearns, "Life history evolution: Successes, limitations, and prospects," *Naturwissenschaften*, vol. 87, no. 11, pp. 476–486, 2000.

[4] S. C. Stearns, M. Ackermann, M. Doebeli, and M. Kaiser, "Experimental evolution of aging, growth, and reproduction in fruitflies," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 97, no. 7, pp. 3309–3313, 2000.

[5] S. C. Stearns, "Trade-offs in life-history evolution," *Funct. Ecol.*, vol. 3, no. 3, pp. 259–268, 1989.

[6] M. K. Skinner, *Encyclopedia of reproduction*. Academic Press, 2018.

[7] T. B. L. Kirkwood and R. Holliday, "The evolution of ageing and longevity," *Proc. R. Soc. London - Biol. Sci.*, vol. 205, no. 1161, pp. 531–546, 1979.

[8] T. B. L. Kirkwood and S. N. Austad, "Why do we age?," *Nature*, vol. 408, no. 6809, pp. 233–238, 2000.

[9] C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, and G. Kroemer, "The hallmarks of aging," *Cell*, vol. 153, no. 6, p. 1194, 2013.

[10] D. Harman, "Free radical theory of aging," *Mutat. Res. DNAging*, vol. 275, no. 3–6, pp. 257–266, Sep. 1992.

[11] A. Phaniendra, D. B. Jestadi, and L. Periyasamy, "Free radicals: properties, sources, targets, and their implication in various diseases," *Indian J. Clin. Biochem.*, vol. 30, pp. 11–26, 2015.

[12] A. Ayala, M. F. Muñoz, and S. Argüelles, "Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal," *Oxid. Med. Cell. Longev.*, vol. 2014, 2014.

[13] M. Khoubnasabjafari, K. Ansarin, and A. Jouyban, "Reliability of malondialdehyde as a biomarker of oxidative stress in psychological disorders," *BioImpacts*, vol. 5, no. 3, pp. 123–127, 2015.

[14] P. V. Vignais, "The superoxide-generating NADPH oxidase: Structural aspects and activation mechanism," *Cell. Mol. Life Sci.*, vol. 59, no. 9, pp. 1428–1459, 2002.

[15] T. Finkel, "Redox-dependent signal transduction," *FEBS Lett.*, vol. 476, no. 1–2, pp. 52–54, 2000.

[16] H. P. Souza, F. R. M. Laurindo, R. C. Ziegelstein, C. O. Berlowitz, and J. L. Zweier, "Vascular NAD(P)H oxidase is distinct from the phagocytic enzyme and modulates vascular reactivity control," *Am. J. Physiol. - Hear. Circ. Physiol.*, vol. 280, no. 2 49-2, pp. 658–667, 2001.

[17] H. M. Schmidt, E. E. Kelley, and A. C. Straub, "The impact of xanthine oxidase (XO) on hemolytic diseases," *Redox Biol.*, vol. 21, p. 101072, 2019.

[18] E. T. Land, *Free radicals in biology and medicine*, vol. 58, no. 4. Oxford university press, USA, 1990.

[19] R. Harrison, "Structure and function of xanthine oxidoreductase: where are we now?," *Free Radic. Biol. Med.*, vol. 33, no. 6, pp. 774–797, 2002.

[20] F. Collin, "Chemical basis of reactive oxygen species reactivity and involvement in neurodegenerative diseases," *Int. J. Mol. Sci.*, vol. 20, no. 10, 2019.

[21] J. Zielonka and B. Kalyanaraman, "'ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis'-a critical commentary," *Free Radic. Biol. Med.*, vol. 45, no. 9, pp. 1217–1219, 2008.

[22] M. S. Weng, J. H. Chang, W. Y. Hung, Y. C. Yang, and M. H. Chien, “The interplay of reactive oxygen species and the epidermal growth factor receptor in tumor progression and drug resistance,” *J. Exp. Clin. Cancer Res.*, vol. 37, no. 1, pp. 1–11, 2018.

[23] S. Yang and G. Lian, “ROS and diseases: role in metabolism and energy supply,” *Mol. Cell. Biochem.*, vol. 467, no. 1–2, pp. 1–12, 2020.

[24] S. Salim, “Oxidative stress and the central nervous system,” *J. Pharmacol. Exp. Ther.*, vol. 360, no. 1, pp. 201–205, 2017.

[25] S. G. Rhee, “Redox signaling: Hydrogen peroxide as intracellular messenger,” *Exp. Mol. Med.*, vol. 31, no. 2, pp. 53–59, 1999.

[26] X. Zhou *et al.*, “OGG1 is essential in oxidative stress induced DNA demethylation,” *Cell. Signal.*, vol. 28, no. 9, pp. 1163–1171, 2016.

[27] Y. Yang, A. V. Bazhin, J. Werner, and S. Karakhanova, “Reactive oxygen species in the immune system,” *Int. Rev. Immunol.*, vol. 32, no. 3, pp. 249–270, 2013.

[28] R. Zug and P. Hammerstein, “Wolbachia and the insect immune system: What reactive oxygen species can tell us about the mechanisms of Wolbachia-host interactions,” *Front. Microbiol.*, vol. 6, no. OCT, pp. 1–16, 2015.

[29] A. Meister, “On the discovery of glutathione,” *Trends Biochem. Sci.*, vol. 13, no. 5, pp. 185–188, 1988.

[30] G. Noctor, A. C. M. Arisi, L. Jouanin, K. J. Kunert, H. Rennenberg, and C. H. Foyer, “Glutathione: Biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants,” *J. Exp. Bot.*, vol. 49, no. 321, pp. 623–647, 1998.

[31] I. F. F. Benzie, “Evolution of antioxidant defence mechanisms,” *Eur. J. Nutr.*, vol. 39, no. 2, pp. 53–61, 2000.

[32] M. Lawson, K. Jomova, P. Poprac, K. Kuca, K. Musílek, and M. Valko, “Free radicals and antioxidants in human disease,” *Nutr. Antioxid. Ther. Treat. Perspect.*, vol. 39, no. 1, pp. 283–305, 2018.

[33] B. A. Stanley *et al.*, “Thioredoxin reductase-2 is essential for keeping low levels of H 2O 2 emission from isolated heart mitochondria,” *J. Biol. Chem.*, vol. 286, no. 38, pp. 33669–33677, 2011.

[34] H. K. Biesalski, “Free radical theory of aging.,” *Curr. Opin. Clin. Nutr. Metab. Care*, vol. 5, no. 1, pp. 5–10, Sep. 2002.

[35] A. P. Wickens, “Ageing and the free radical theory,” *Respir. Physiol.*, vol. 128, no. 3, pp. 379–391, Nov. 2001.

[36] G. Barja, “Updating the mitochondrial free radical theory of aging: An integrated view, key aspects, and confounding concepts,” *Antioxidants Redox Signal.*, vol. 19, no. 12, pp. 1420–1445, Oct. 2013.

[37] J. L. Hsu, Y. Hsieh, C. Tu, D. O’Connor, H. S. Nick, and D. N. Silverman, “Catalytic properties of human manganese superoxide dismutase,” *J. Biol. Chem.*, vol. 271, no. 30, pp. 17687–17691, 1996.

[38] T. D. Oury, J. D. Crapo, Z. Valnickova, and J. J. Enghild, “Human extracellular superoxide dismutase is a tetramer composed of two disulphide-linked dimers: A simplified, high-yield purification of extracellular superoxide dismutase,” *Biochem. J.*, vol. 317, no. 1, pp. 51–57, 1996.

[39] J. Uchmanski and A. Kaliszewicz, “Darwinian Demons.,” no. January 2016, 2006.

[40] C. Selman, J. D. Blount, D. H. Nussey, and J. R. Speakman, “Oxidative damage, ageing, and life-history evolution: Where now?,” *Trends Ecol. Evol.*, vol. 27, no. 10, pp. 570–577, Oct. 2012.

[41] E. P. Chen *et al.*, “Myeloid Cell COX-2 deletion reduces mammary tumor growth through enhanced cytotoxic T-lymphocyte function,” *Carcinogenesis*, vol. 35, no. 8, pp. 1788–1797, 2014.

[42] T. B. L. Kirkwood, “The disposable soma theory: Origins and evolution,” *Evol. Senescence Tree Life*, pp. 23–39, 2017.

[43] S. Bouwhuis, A. Charmantier, S. Verhulst, and B. C. Sheldon, “Individual variation in rates of senescence: Natal origin effects and disposable soma in a wild bird population,” *J. Anim. Ecol.*, vol. 79, no. 6, pp. 1251–1261, Nov. 2010.

[44] J. R. Downie, “Developmental biology,” *Nature*, vol. 272, no. 5648, p. 97, 1978.

[45] R. L. Walford, “the Immunologic Theory of Aging,” *Immunol. Rev.*, vol. 2, no. 1, pp. 171–171, 1969.

[46] L. Hayflick, “Origins of longevity,” *Mod. Biol. Theor. aging*, pp. 21–34, 1987.

[47] A. Sanz and R. K. A. Stefanatos, “The Mitochondrial Free Radical Theory of Aging: A Critical View,” *Curr. Aging Sci.*, vol. 1, no. 1, pp. 10–21, 2010.

[48] G. Barja, “The mitochondrial free radical theory of aging,” *Prog. Mol. Biol. Transl. Sci.*, vol. 127, pp. 1–27, 2014.

[49] R. Ramzan, S. Vogt, and B. Kadenbach, “Stress-mediated generation of deleterious ROS in healthy individuals - role of cytochrome c oxidase,” *J. Mol. Med.*, vol. 98, no. 5, pp. 651–657, 2020.

[50] D. K. Dowling and L. W. Simmons, “Reactive oxygen species as universal constraints in life-history evolution,” *Proc. R. Soc. B Biol. Sci.*, vol. 276, no. 1663, pp. 1737–1745, 2009.

[51] N. Nur, “The consequences of brood size for breeding blue tits. III. Measuring the cost of reproduction: survival, future fecundity, and differential dispersal,” *Evolution (N. Y).*, vol. 42, no. 2, pp. 351–362, 1988.

[52] T. W. Arnold, E. A. Roche, J. H. Devries, and D. W. Howerter, “Costs of reproduction in breeding female mallards: Predation risk during incubation drives annual mortality,” *Avian Conserv. Ecol.*, vol. 7, no. 1, p. 2, 2012.

[53] B. Charlesworth, *Evolution in age-structured populations*, vol. 2. Cambridge University Press Cambridge, 1994.

[54] L. G. Harshman and A. J. Zera, “The cost of reproduction: the devil in the details,” *Trends Ecol. Evol.*, vol. 22, no. 2, pp. 80–86, 2007.

[55] A. J. Zera and L. G. Harshman, “The physiology of life history trade-offs in animals,” *Annu. Rev. Ecol. Syst.*, vol. 32, no. 1, pp. 95–126, 2001.

[56] J. R. Speakman *et al.*, “Oxidative stress and life histories: Unresolved issues and current needs,” *Ecol. Evol.*, vol. 5, no. 24, pp. 5745–5757, 2015.

[57] A. Kumar *et al.*, “Mosquito innate immunity,” *Insects*, vol. 9, no. 3, 2018.

[58] J. F. Hillyer and M. R. Strand, “Mosquito hemocyte-mediated immune responses,” *Curr. Opin. Insect Sci.*, vol. 3, pp. 14–21, 2014.

[59] M. T. Aliota *et al.*, “Mosquito transcriptome profiles and filarial worm susceptibility in Armigeres subalbatus,” *PLoS Negl. Trop. Dis.*, vol. 4, no. 4, pp. 1–15, 2010.

[60] L. C. Bartholomay *et al.*, “Description of the transcriptomes of immune response-activated hemocytes from the mosquito vectors *Aedes aegypti* and Armigeres subalbatus,” *Infect. Immun.*, vol. 72, no. 7, pp. 4114–4126, 2004.

[61] L. C. Bartholomay *et al.*, “Profiling infection responses in the haemocytes of the mosquito, *Aedes aegypti*,” *Insect Mol. Biol.*, vol. 16, no. 6, pp. 761–776, 2007.

[62] S. B. Pinto *et al.*, “Discovery of Plasmodium modulators by genome-wide analysis of circulating hemocytes in Anopheles gambiae,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 50, pp. 21270–21275, 2009.

[63] Y. J. Choi, J. F. Fuchs, G. F. Mayhew, H. E. Yu, and B. M. Christensen, “Tissue-enriched expression profiles in *Aedes aegypti* identify hemocyte-specific transcriptome responses to infection,” *Insect Biochem. Mol. Biol.*, vol. 42, no. 10, pp. 729–738, 2012.

[64] L. A. Baton, A. Robertson, E. Warr, M. R. Strand, and G. Dimopoulos, “Genome-wide transcriptomic profiling of Anopheles gambiae hemocytes reveals pathogen-specific signatures upon bacterial challenge and Plasmodium berghei infection,” *BMC Genomics*, vol. 10, no. 1, pp. 1–13, 2009.

[65] J. F. J. F. Hillyer, “Chapter 12: Mosquito Immunity,” in *Invertebrate Immunity*, vol. 708, Springer, Boston, MA, 2010, pp. 218–238.

[66] J. Moretti and J. M. Blander, “Insights into phagocytosis-coupled activation of pattern recognition receptors and inflammasomes,” *Curr. Opin. Immunol.*, vol. 26, pp. 100–110, 2014.

[67] L. Zheng, L. Zhang, H. Lin, M. T. McIntosh, and A. R. Malacrida, “Toll-like receptors in invertebrate innate immunity,” *Invertebr. Surviv. J.*, vol. 2, no. 2, pp. 105–113, 2005.

[68] J. L. Ramirez *et al.*, “Reciprocal tripartite interactions between the Aedes aegypti midgut microbiota, innate immune system and dengue virus influences vector competence,” *PLoS Negl. Trop. Dis.*, vol. 6, no. 3, pp. 1–11, 2012.

[69] G. K. Christophides *et al.*, “Immunity-related genes and gene families in Anopheles gambiae,” *Science (80-.).*, vol. 298, no. 5591, pp. 159–165, 2002.

[70] C. Lowenberger *et al.*, “Insect immunity: Isolation of three novel inducible antibacterial defensins from the vector mosquito, Aedes aegypti,” *Insect Biochem. Mol. Biol.*, vol. 25, no. 7, pp. 867–873, 1995.

[71] S. Kumar *et al.*, “The role of reactive oxygen species on Plasmodium melanotic encapsulation in Anopheles gambiae,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 100, no. SUPPL. 2, pp. 14139–14144, 2003.

[72] A. Molina-Cruz *et al.*, “Reactive oxygen species modulate Anopheles gambiae immunity against bacteria and Plasmodium,” *J. Biol. Chem.*, vol. 283, no. 6, pp. 3217–3223, Feb. 2008.

[73] D. R. Ardia, J. E. Gantz, B. C. Schneider, and S. Strebel, “Costs of immunity in insects: An induced immune response increases metabolic rate and decreases antimicrobial activity,” *Funct. Ecol.*, vol. 26,

no. 3, pp. 732–739, 2012.

[74] L. Råberg, “How to Live with the Enemy: Understanding Tolerance to Parasites,” *PLoS Biol.*, vol. 12, no. 11, p. e1001989, 2014.

[75] L. Råberg, A. L. Graham, and A. F. Read, “Decomposing health: Tolerance and resistance to parasites in animals,” *Philos. Trans. R. Soc. B Biol. Sci.*, vol. 364, no. 1513, pp. 37–49, Jan. 2009.

[76] L. Råberg, D. Sim, and A. F. Read, “Disentangling genetic variation for resistance and tolerance to infectious diseases in animals,” *Science (80-.).*, vol. 318, no. 5851, pp. 812–814, Nov. 2007.

[77] E. L. Simms, “Defining tolerance as a norm of reaction,” *Evol. Ecol.*, vol. 14, no. 4–6, pp. 563–570, 2000.

[78] A. M. P. Schieber and J. S. Ayres, “Thermoregulation as a disease tolerance defense strategy,” *Pathog. Dis.*, vol. 74, no. 9, pp. 936–941, 2016.

[79] J. S. Adelman and D. M. Hawley, “Tolerance of infection: A role for animal behavior, potential immune mechanisms, and consequences for parasite transmission,” *Horm. Behav.*, vol. 88, pp. 79–86, 2017.

[80] G. Kuno, “Early history of laboratory breeding of Aedes aegypti (Diptera: Culicidae) focusing on the origins and use of selected strains,” *J. Med. Entomol.*, vol. 47, no. 6, pp. 957–971, 2010.

[81] O. Tomori, “Yellow fever: The recurring plague,” *Critical Reviews in Clinical Laboratory Sciences*, vol. 41, no. 4. Taylor & Francis, pp. 391–427, 19-Jan-2004.

[82] J. A. Phillips and A. Neyland, “Zika Virus,” *Work. Heal. Saf.*, vol. 64, no. 8, p. 396, Apr. 2016.

[83] G. Kuno, “Zika virus,” *Mol. Detect. Hum. Viral Pathog.*, vol. 374, no. 16, pp. 313–320, 2016.

[84] T. R. Southwood, G. Murdie, M. Yasuno, R. J. Tonn, and P. M. Reader, “Studies on the life budget of Aedes aegypti in Wat Samphaya, Bangkok, Thailand.,” *Bull. World Health Organ.*, vol. 46, no. 2, pp. 211–226, 1972.

[85] R. H. Foote, “Aedes Aegypti (L.), the Yellow Fever Mosquito. Its life history, bionomics, and structure. Sir S. Rickard Christophers. Cambridge University Press, New York, 1960. xii + 739 pp. Illus. $14.50,” *Science (80-.).*, vol. 133, no. 3463, pp. 1473–1474, 1961.

[86] V. Nene *et al.*, “Genome sequence of Aedes aegypti, a major arbovirus vector,” *Science (80-.).*, vol. 316, no. 5832, pp. 1718–1723, Jun. 2007.

[87] L. E. Munstermann, “Care and maintenance of Aedes mosquito colonies,” *Mol. Biol. Insect Dis. Vectors*, pp. 13–20, 1997.

[88] C. F. Oliva, D. Damiens, and M. Q. Benedict, “Male reproductive biology of Aedes mosquitoes,” *Acta Trop.*, vol. 132, no. 1, pp. S12–S19, 2014.

[89] nature, “Aedes aegypti life cycle,” *Nature*, 2016. [Online]. Available: http://www.nature.com/scitable/content/aedes-aegypti-life-cycle-22400575.

[90] M. Wittner and L. M. Weiss, *The Microsporidia and Microsporidiosis*, vol. 2. ASM press Washington, DC, 1999.

[91] J. Weiser and M. Coluzzi, “The microsporidian Plistophora culicis Weiser, 1946 in different mosquito hosts.,” *Folia Parasitol. (Praha).*, vol. 19, no. 3, pp. 197–202, 1972.

[92] J. J. Becnel, S. E. White, and A. M. Shapiro, “Review of microsporidia-mosquito relationships: From the simple to the complex,” *Folia Parasitol. (Praha).*, vol. 52, no. 1–2, pp. 41–50, 2005.

[93] T. G. Andreadis, “Microsporidian parasites of mosquitoes,” *J. Am. Mosq. Control Assoc.*, vol. 23, no. 2 SUPPL., pp. 3–29, Jan. 2007.

[94] C. A. Desjardins *et al.*, “Contrasting host-pathogen interactions and genome evolution in two generalist and specialist microsporidian pathogens of mosquitoes,” *Nat. Commun.*, vol. 6, no. May, 2015.

[95] P. Agnew and J. C. Koella, “Life history interactions with environmental conditions in a host-parasite relationship and the parasite’s mode of transmission,” *Evol. Ecol.*, vol. 13, no. 1, pp. 67–91, 1999.

[96] S. Bedhomme, P. Agnew, C. Sidobre, and Y. Michalakis, “Virulence reaction norms across a food gradient,” *Proc. R. Soc. B Biol. Sci.*, vol. 271, no. 1540, pp. 739–744, 2004.

[97] D. J. Betteridge, “What is oxidative stress?,” *Metabolism.*, vol. 49, no. 2 SUPPL. 1, pp. 3–8, 2000.

[98] R. K. Chaitanya, K. Shashank, and P. Sridevi, “Oxidative Stress in Invertebrate Systems,” in *Free Radicals and Diseases*, R. Ahmad, Ed. Rijeka: IntechOpen, 2016.

[99] M. S. Dionne, L. N. Pham, M. Shirasu-Hiza, and D. S. Schneider, “Akt and foxo Dysregulation Contribute to Infection-Induced Wasting in Drosophila,” *Curr. Biol.*, vol. 16, no. 20, pp. 1977–1985, 2006.

[100] J. S. Ayres and D. S. Schneider, “The role of anorexia in resistance and tolerance to infections in Drosophila,” *PLoS Biol.*, vol. 7, no. 7, p. e1000150, 2009.

[101] M. C. Chambers, K. H. Song, and D. S. Schneider, “Listeria monocytogenes Infection Causes Metabolic Shifts in Drosophila melanogaster,” *PLoS One*, vol. 7, no. 12, p. e50679, 2012.

[102] S. A. Adamo, “Parasitic suppression of feeding in the tobacco hornworm, Manduca sexta: Parallels with feeding depression after an immune challenge,” *Arch. Insect Biochem. Physiol.*, vol. 60, no. 4, pp. 185–197, 2005.

[103] M. M. Lissner and D. S. Schneider, “The physiological basis of disease tolerance in insects,” *Curr. Opin. Insect Sci.*, vol. 29, pp. 133–136, 2018.

[104] J.-M. G. and D. S. Falconer, *Introduction to Quantitative Genetics*, vol. 17, no. 1. 1962.

[105] N. H. P. Cnubben, I. M. C. M. Rietjens, H. Wortelboer, J. Van Zanden, and P. J. Van Bladeren, “The interplay of glutathione-related processes in antioxidant defense,” *Environ. Toxicol. Pharmacol.*, vol. 10, no. 4, pp. 141–152, Sep. 2001.

[106] E. T. Land, *Free radicals in biology and medicine*, vol. 58, no. 4. 1990.

[107] A. R. Mora, M. Meniri, G. Glauser, A. Vallat, and F. Helfenstein, “Badge size reflects sperm oxidative status within social groups in the house sparrow Passer domesticus,” *Front. Ecol. Evol.*, vol. 4, no. JUN, p. 67, Jun. 2016.

[108] R. Mendonça *et al.*, “Sensitive and selective quantifcation of free and total malondialdehyde in plasma using UHPLC-HRMS,” *J. Lipid Res.*, vol. 58, no. 9, pp. 1924–1931, 2017.

[109] A. J. Nappi, E. Vass, F. Frey, and Y. Carton, “Superoxide anion generation in Drosophila during melanotic encapsulation of parasites,” *Eur. J. Cell Biol.*, vol. 68, no. 4, pp. 450–456, 1995.

[110] A. J. Nappi and E. Vass, “Hydrogen peroxide production in immune-reactive Drosophila melanogaster,” *J. Parasitol.*, vol. 84, no. 6, pp. 1150–1157, 1998.

[111] H. Y. Yi, M. Chowdhury, Y. D. Huang, and X. Q. Yu, “Insect antimicrobial peptides and their applications,” *Appl. Microbiol. Biotechnol.*, vol. 98, no. 13, pp. 5807–5822, 2014.

[112] C. J. Bayne, “Phagocytosis and Non-Self Recognition in Invertebrates,” *Bioscience*, vol. 40, no. 10, pp. 723–731, 1990.

[113] J. Kurtz, A. Wiesner, P. Götz, and K. P. Sauer, “Gender differences and individual variation in the immune system of the scorpionfly Panorpa vulgaris (Insecta: Mecoptera),” *Dev. Comp. Immunol.*, vol. 24, no. 1, pp. 1–12, Jan. 2000.

[114] G. Pan *et al.*, “Invertebrate host responses to microsporidia infections,” *Dev. Comp. Immunol.*, vol. 83, pp. 104–113, 2018.

[115] R. S. Khush, F. Leulier, and B. Lemaitre, “Drosophila immunity: two paths to NF-κB,” *Trends Immunol.*, vol. 22, no. 5, pp. 260–264, 2001.

[116] C. A. Brennan and K. V. Anderson, “Drosophila: The genetics of innate immune recognition and response,” *Annu. Rev. Immunol.*, vol. 22, pp. 457–483, 2004.

[117] D. Ferrandon, J. L. Imler, C. Hetru, and J. A. Hoffmann, “The Drosophila systemic immune response: Sensing and signalling during bacterial and fungal infections,” *Nat. Rev. Immunol.*, vol. 7, no. 11, pp. 862–874, 2007.

[118] B. Lemaitre and J. Hoffmann, “The host defense of Drosophila melanogaster,” *Annu. Rev. Immunol.*, vol. 25, pp. 697–743, 2007.

[119] C. Hetru and J. A. Hoffmann, “NF-kappaB in the immune response of Drosophila.,” *Cold Spring Harb. Perspect. Biol.*, vol. 1, no. 6, p. a000232, 2009.

[120] M. S. Hayden and S. Ghosh, “Shared Principles in NF-κB Signaling,” *Cell*, vol. 132, no. 3, pp. 344–362, 2008.

[121] S. Vallabhapurapu and M. Karin, “Regulation and function of NF-κB transcription factors in the immune system,” *Annu. Rev. Immunol.*, vol. 27, pp. 693–733, 2009.

[122] M. J. Morgan and Z. G. Liu, “Crosstalk of reactive oxygen species and NF-κB signaling,” *Cell Res.*, vol. 21, no. 1, pp. 103–115, 2011.

[123] T. O. Tollefsbol, “Transgenerational Epigenetics,” in *Transgenerational Epigenetics*, Elsevier, 2014, pp. 1–396.

[124] R. Q. Moraes-Souza *et al.*, “Oxidative Stress Profile of Mothers and Their Offspring after Maternal Consumption of High-Fat Diet in Rodents: A Systematic Review and Meta-Analysis,” *Oxid. Med. Cell. Longev.*, vol. 2021, 2021.

[125] E. I. K. Vitikainen *et al.*, “Evidence of oxidative shielding of offspring in a wild mammal,” *Front. Ecol. Evol.*, vol. 4, no. MAY, pp. 1–10, 2016.

[126] D. Costantini, M. Rowe, M. W. Butler, and K. J. McGraw, “From molecules to living systems: Historical and contemporary issues in oxidative stress and antioxidant ecology,” *Funct. Ecol.*, vol. 24, no. 5, pp. 950–959, 2010.

[127] S. Deepashree, T. Shivanandappa, and S. R. Ramesh, “Life History Traits of an Extended Longevity Phenotype of Drosophila melanogaster,” *Curr. Aging Sci.*, vol. 10, no. 3, Jul. 2017.

[128] K. W. Kelley, “NIH public access policy,” *Brain. Behav. Immun.*, vol. 22, no. 5, p. 629, Oct. 2008.

[129] K. B. Beckman and B. N. Ames, “The free radical theory of aging matures,” *Physiol. Rev.*, vol. 78, no. 2, pp. 547–581, Apr. 1998.

[130] R. S. Sohal, R. J. Mockett, and W. C. Orr, “Mechanisms of aging: An appraisal of the oxidative stress hypothesis,” *Free Radic. Biol. Med.*, vol. 33, no. 5, pp. 575–586, 2002.

[131] A. Jacobson *et al.*, “Aging enhances pressure-induced arterial superoxide formation,” *Am. J. Physiol. - Hear. Circ. Physiol.*, vol. 293, no. 3, pp. H1344–H1350, Sep. 2007.

[132] A. J. Donato *et al.*, “Direct evidence of endothelial oxidative stress with aging in humans: Relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-κB,” *Circ. Res.*, vol. 100, no. 11, pp. 1659–1666, 2007.

[133] B. Lener *et al.*, “The NADPH oxidase Nox4 restricts the replicative lifespan of human endothelial cells,” *Biochem. J.*, vol. 423, no. 3, pp. 363–374, Oct. 2009.

[134] H. H. Ku, U. T. Brunk, and R. S. Sohal, “Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species,” *Free Radic. Biol. Med.*, vol. 15, no. 6, pp. 621–627, Dec. 1993.

[135] R. B. Price and E. J. Sutow, “Micrographic and profilometric evaluation of the finish produced by diamond and tungsten carbide finishing burs on enamel and dentin,” *J. Prosthet. Dent.*, vol. 60, no. 3, pp. 311–316, 1988.

[136] L. A. Sena and N. S. Chandel, “Physiological roles of mitochondrial reactive oxygen species,” *Mol. Cell*, vol. 48, no. 2, pp. 158–167, 2012.

[137] A. Panday, M. K. Sahoo, D. Osorio, and S. Batra, “NADPH oxidases: An overview from structure to innate immunity-associated pathologies,” *Cell. Mol. Immunol.*, vol. 12, no. 1, pp. 5–23, 2015.

[138] B. Zwaan, R. Bijlsma, and R. F. Hoekstra, “Direct selection on life span in Drosophila melanogaster,” *Evolution (N. Y).*, vol. 49, no. 4, pp. 649–659, 1995.

[139] K. Garschall and T. Flatt, “The interplay between immunity and aging in Drosophila,” *F1000Research*, vol. 7, 2018.

[140] H. B. Rubins *et al.*, *Gemfibrozil for the Secondary Prevention of Coronary Heart Disease in Men with Low Levels of High-Density Lipoprotein Cholesterol*, vol. 341, no. 6. World Health Organization, 1999.

[141] E. T. Land, “Free radicals in biology and medicine,” *Int. J. Radiat. Biol.*, vol. 58, no. 4, pp. 725–725, 1990.

[142] G. W. Felton and C. B. Summers, “Antioxidant systems in insects,” *Arch. Insect Biochem. Physiol.*, vol. 29, no. 2, pp. 187–197, 1995.

[143] Z. Singh, I. P. Karthigesu, P. Singh, and R. Kaur, “Use of malondialdehyde as a biomarker for assessing oxidative stress in different disease pathologies: A review,” *Iran. J. Public Health*, vol. 43, no. 3, pp. 7–16, 2014.

[144] A. Sönnerborg, G. Carlin, B. Åkerlund, and C. Jarstrand, “Increased production of malondialdehyde in patients with HIV infection,” *Scand. J. Infect. Dis.*, vol. 20, no. 3, pp. 287–290, 1988.

[145] C. Mao *et al.*, “Associations between superoxide dismutase, malondialdehyde and all-cause mortality in older adults: A community-based cohort study,” *BMC Geriatr.*, vol. 19, no. 1, pp. 1–9, 2019.

[146] A. S. Ziada, M. S. R. Smith, and H. C. F. Côté, “Updating the Free Radical Theory of Aging,” *Front. Cell Dev. Biol.*, vol. 8, no. September, pp. 1–5, 2020.

[147] J. Viña, C. Borras, K. M. Abdelaziz, R. Garcia-Valles, and M. C. Gomez-Cabrera, “The free radical theory of aging revisited: The cell signaling disruption theory of aging,” *Antioxidants Redox Signal.*, vol. 19, no. 8, pp. 779–787, 2013.

[148] D. K. Fabian *et al.*, “Evolution of longevity improves immunity in Drosophila,” *Evol. Lett.*, vol. 2, no. 6, pp. 567–579, 2018.

[149] C. M. Vincent and N. P. Sharp, “Sexual antagonism for resistance and tolerance to infection in Drosophila melanogaster,” *Proc. R. Soc. B Biol. Sci.*, vol. 281, no. 1788, p. 20140987, 2014.

[150] M. Zeller and J. C. Koella, “The role of the environment in the evolution of tolerance and resistance to a pathogen,” *Am. Nat.*, vol. 190, no. 3, pp. 389–397, 2017.

[151] V. M. Howick and B. P. Lazzaro, “Genotype and diet shape resistance and tolerance across distinct phases of bacterial infection,” *BMC Evol. Biol.*, vol. 14, no. 1, pp. 1–13, 2014.

[152] M. Tarka, A. Guenther, P. T. Niemelä, S. Nakagawa, and D. W. A. Noble, “Sex differences in life history, behavior, and physiology along a slow-fast continuum: a meta-analysis,” *Behav. Ecol. Sociobiol.*, vol. 72, no. 8, 2018.

[153] K. A. Lee, “Linking immune defenses and life history at the levels of the individual and the species,” *Integr. Comp. Biol.*, vol. 46, no. 6, pp. 1000–1015, 2006.

[154] O. Restif and W. Amos, “The evolution of sex-specific immune defences,” *Proc. R. Soc. B Biol. Sci.*, vol. 277, no. 1691, pp. 2247–2255, 2010.

[155] P. R. Murgatroyd, *Energy metabolism in animals and man*, vol. 1. CUP Archive, 1990.

[156] L. S. Katz, *An introduction to behavioral endocrinology*, vol. 52, no. 1–2. Sinauer Associates, 1997.

[157] W. Schuett, T. Tregenza, and S. R. X. Dall, “Sexual selection and animal personality,” *Biol. Rev.*, vol. 85, no. 2, pp. 217–246, 2010.

[158] B. R. Smith and D. T. Blumstein, “Fitness consequences of personality: A meta-analysis,” *Behav. Ecol.*, vol. 19, no. 2, pp. 448–455, 2008.

[159] T. Antao, *Evolutionary parasitology applied to control and elimination policies*, vol. 27, no. 6. Oxford University Press, 2011.

[160] M. Garratt *et al.*, “Tissue-dependent changes in oxidative damage with male reproductive effort in house mice,” *Funct. Ecol.*, vol. 26, no. 2, pp. 423–433, 2012.

[161] L. Gustafsson, D. Nordling, M. S. Andersson, B. C. Sheldon, and A. Qvarnstrom, “Infectious diseases, reproductive effort and the cost of reproduction in birds,” *Philos. Trans. - R. Soc. London, B*, vol. 346, no. 1317, pp. 323–331, 1994.

[162] D. Harman, “Free radical theory of aging: An update - Increasing the functional life span,” *Ann. N. Y. Acad. Sci.*, vol. 1067, no. 1, pp. 10–21, May 2006.

[163] C. Alonso-Alvarez, S. Bertrand, G. Devevey, J. Prost, B. Faivre, and G. Sorci, “Increased susceptibility to oxidative stress as a proximate cost of reproduction,” *Ecol. Lett.*, vol. 7, no. 5, pp. 363–368, 2004.

[164] D. Nordling, M. Andersson, S. Zohari, and L. Gustafsson, “Reproductive effort reduces specific immune response and parasite resistance,” *Proc. R. Soc. B Biol. Sci.*, vol. 265, no. 1403, pp. 1291–1298, 1998.

[165] C. Deerenberg, V. Arpanius, S. Daan, and N. Bos, “Reproductive effort decreases antibody responsiveness,” *Proc. R. Soc. B Biol. Sci.*, vol. 264, no. 1384, pp. 1021–1029, 1997.

[166] S. N. Gershman, C. A. Barnett, A. M. Pettinger, C. B. Weddle, J. Hunt, and S. K. Sakaluk, “Give ’til it hurts: Trade-offs between immunity and male reproductive effort in the decorated cricket, Gryllodes sigillatus,” *J. Evol. Biol.*, vol. 23, no. 4, pp. 829–839, 2010.

[167] J. Contreras-Garduño, M. C. Rodríguez, M. H. Rodríguez, A. Alvarado-Delgado, and H. Lanz-Mendoza, “Cost of immune priming within generations: Trade-off between infection and reproduction,” *Microbes Infect.*, vol. 16, no. 3, pp. 261–267, 2014.

[168] C. L. Boggs, “Understanding insect life histories and senescence through a resource allocation lens,” *Funct. Ecol.*, vol. 23, no. 1, pp. 27–37, 2009.

[169] D. A. I. Mavridou, D. Gonzalez, W. Kim, S. A. West, and K. R. Foster, “Bacteria Use Collective Behavior to Generate Diverse Combat Strategies,” *Curr. Biol.*, vol. 28, no. 3, pp. 345-355.e4, 2018.

[170] A. M. G. Barreaux, P. Barreaux, M. B. Thomas, and J. C. Koella, “Inoculating Anopheles gambiae mosquitoes with beads to induce and measure the melanization immune response,” *J. Vis. Exp.*, vol. 2017, no. 119, pp. 1–6, 2017.

[171] D. C. Culver, “Life history evolution,” *Encycl. Caves*, no. May 2012, pp. 465–468, 2012.

[172] A. I. Barnes and L. Partridge, “Costing reproduction,” *Anim. Behav.*, vol. 66, no. 2, pp. 199–204, 2003.

[173] J. Å. Nilsson, “Metabolic consequences of hard work,” *Proc. R. Soc. B Biol. Sci.*, vol. 269, no. 1501, pp. 1735–1739, 2002.

[174] A. Görlach *et al.*, “Reactive oxygen species, nutrition, hypoxia and diseases: Problems solved?,” *Redox Biol.*, vol. 6, no. 8, pp. 372–385, Dec. 2015.

[175] J. Vina, J. Gambini, R. Lopez-Grueso, K. M. Abdelaziz, M. Jove, and C. Borras, “Females Live Longer than Males: Role of Oxidative Stress,” *Curr. Pharm. Des.*, vol. 17, no. 36, pp. 3959–3965, Dec. 2012.

[176] A. A. Agrawal, S. Y. Strauss, and M. J. Stout, “Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish,” *Evolution (N. Y).*, vol. 53, no. 4, pp. 1093–1104, 1999.

[177] D. E. Haskell, C. R. Webster, D. J. Flaspohler, and M. W. Meyer, “The American Midland Naturalist,” *Am. Midl. Nat.*, vol. 169, no. 1, pp. 1–16, 2013.

[178] H. F. Schoof, “Mating, resting habits and dispersal of Aedes aegypti.,” *Bull. World Health Organ.*, vol. 36, no. 4, pp. 600–601, 1967.

[179] W. K. Hartberg, “Observations on the mating behaviour of Aedes aegypti in nature.,” *Bull. World*

Health Organ., vol. 45, no. 6, pp. 847–850, 1971.

[180] L. J. Cator, C. A. S. Wyer, and L. C. Harrington, “Mosquito Sexual Selection and Reproductive Control Programs,” *Trends Parasitol.*, vol. 37, no. 4, pp. 330–339, 2021.

[181] W. K. Dodds and M. R. Whiles, “Nonpredatory Interspecific Interactions Among Plants and Animals in Freshwater Communities,” in *Freshwater Ecology*, W. K. Dodds and M. R. B. T.-F. E. (Third E. Whiles, Eds. Academic Press, 2020, pp. 653–670.

[182] F. W. Avila, L. K. Sirot, B. A. LaFlamme, C. D. Rubinstein, and M. F. Wolfner, “Insect seminal fluid proteins: identification and function,” *Annu. Rev. Entomol.*, vol. 56, pp. 21–40, 2011.

[183] S. Wigby *et al.*, “Seminal fluid protein allocation and male reproductive success,” *Curr. Biol.*, vol. 19, no. 9, pp. 751–757, 2009.

[184] E. C. Degner, Y. H. Ahmed-Braimah, K. Borziak, M. F. Wolfner, L. C. Harrington, and S. Dorus, “Proteins, transcripts, and genetic architecture of seminal fluid and sperm in the mosquito aedes aegypti,” *Mol. Cell. Proteomics*, vol. 18, no. March, pp. S6–S22, 2019.

[185] M. E. H. Helinski and L. C. Harrington, “Male mating history and body size influence female fecundity and longevity of the dengue vector Aedes aegypti,” *J. Med. Entomol.*, vol. 48, no. 2, pp. 202–211, 2011.

[186] R. W. Gwadz, G. B. Craig, and W. A. Hickey, “Female sexual behavior as the mechanism rendering Aedes aegypti refractory to insemination.,” *Biol. Bull.*, vol. 140, no. 2, pp. 201–214, Apr. 1971.

[187] A. Pomiankowski, “The costs of choice in sexual selection,” *J. Theor. Biol.*, vol. 128, no. 2, pp. 195–218, 1987.

[188] B. Hölldobler, “The behavioral ecology of mating in harvester ants (Hymenoptera: Formicidae: Pogonomyrmex),” *Behav. Ecol. Sociobiol.*, vol. 1, no. 4, pp. 405–423, 1976.

[189] G. Sacca, “ Comparative Bionomics in the Genus Musca ,” *Annu. Rev. Entomol.*, vol. 9, no. 1, pp. 341–358, 1964.

[190] J. B. Richardson, S. B. Jameson, A. Gloria-Soria, D. M. Wesson, and J. Powell, “Evidence of limited polyandry in a natural population of Aedes aegypti,” *Am. J. Trop. Med. Hyg.*, vol. 93, no. 1, pp. 189–193, 2015.

[191] I. Bargielowski, L. Alphey, and J. C. Koella, “Cost of mating and insemination capacity of a genetically modified mosquito Aedes aegypti OX513A compared to its wild type counterpart,” *PLoS One*, vol. 6, no. 10, pp. 2–6, 2011.

[192] M. E. H. Helinski and L. C. Harrington, “The role of male harassment on female fitness for the dengue vector mosquito Aedes aegypti,” *Behav. Ecol. Sociobiol.*, vol. 66, no. 8, pp. 1131–1140, Aug. 2012.

[193] A. Bokov, A. Chaudhuri, and A. Richardson, “The role of oxidative damage and stress in aging,” *Mech. Ageing Dev.*, vol. 125, no. 10-11 SPEC. ISS., pp. 811–826, 2004.

[194] V. I. Pérez *et al.*, “Is the oxidative stress theory of aging dead?,” *Biochim. Biophys. Acta - Gen. Subj.*, vol. 1790, no. 10, pp. 1005–1014, Oct. 2009.

[195] V. J. Thannickal and B. L. Fanburg, “Reactive oxygen species in cell signaling,” *Am. J. Physiol. - Lung Cell. Mol. Physiol.*, vol. 279, no. 6 23-6, pp. L1005-28, Dec. 2000.

[196] Z. M. Moghadam, P. Henneke, and J. Kolter, “From Flies to Men: ROS and the NADPH Oxidase in Phagocytes,” *Front. Cell Dev. Biol.*, vol. 9, no. March, 2021.

[197] C. Kohchi, H. Inagawa, T. Nishizawa, and G. I. Soma, “ROS and innate immunity,” *Anticancer Res.*, vol. 29, no. 3, pp. 817–822, 2009.

[198] L. Li, J. Tan, Y. Miao, P. Lei, and Q. Zhang, “ROS and Autophagy: Interactions and Molecular Regulatory Mechanisms,” *Cell. Mol. Neurobiol.*, vol. 35, no. 5, pp. 615–621, 2015.

[199] C. Bárcena, P. Mayoral, and P. M. Quirós, “Mitohormesis, an Antiaging Paradigm,” *Int. Rev. Cell Mol. Biol.*, vol. 340, pp. 35–77, 2018.

[200] F. E. Harrison, “A critical review of vitamin C for the prevention of age-related cognitive decline and alzheimer’s disease,” *J. Alzheimer’s Dis.*, vol. 29, no. 4, pp. 711–726, 2012.

[201] M. E. Rusu, I. Fizeșan, L. Vlase, and D. S. Popa, “Antioxidants in Age-Related Diseases and Anti-Aging Strategies,” *Antioxidants*, vol. 11, no. 10, pp. 1–5, 2022.

[202] L. S. Wieland *et al.*, “Risks and benefits of antioxidant dietary supplement use during cancer treatment: Protocol for a scoping review,” *BMJ Open*, vol. 11, no. 4, pp. 1–7, 2021.

[203] N. B. Metcalfe and C. Alonso-Alvarez, “Oxidative stress as a life-history constraint: The role of reactive oxygen species in shaping phenotypes from conception to death,” *Funct. Ecol.*, vol. 24, no. 5, pp. 984–996, 2010.

[204] P. Monaghan, N. B. Metcalfe, and R. Torres, “Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation,” *Ecol. Lett.*, vol. 12, no. 1, pp. 75–92, 2009.

[205] I. M. M. van Leeuwen, J. Vera, and O. Wolkenhauer, “Dynamic energy budget approaches for modelling organismal ageing,” *Philos. Trans. R. Soc. B Biol. Sci.*, vol. 365, no. 1557, pp. 3443–3454, 2010.