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Sexual dimorphism in hemocyte responses to *Staphylococcus aureus* infection in *Drosophila melanogaster*

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AUTHORSHIP DECLARATION

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification except as specified.

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LAY SUMMARY

In the field of biology, individuals are classified either as a male or a female based on a combination of factors, including genomic differences (for example, in mammals, males possess one X and one Y chromosome, while females have two X chromosomes), reproductive organs and the action of specific hormones (chemical substances that regulate bodily functions). These factors influence how our bodies fight off infections. During the COVID-19 pandemic, men were experiencing more severe symptoms and higher mortality than females. This highlights the existence of sex-specific differences in disease susceptibility, the basis of which we still do not fully understand. *Staphylococcus aureus* is one of the common causes of bacterial bloodstream infections worldwide, and it disproportionately affects men. Although significant progress has been made in understanding how *S. aureus* infections develop, primarily signifying the role of macrophages (immune cells that engulf microbial invaders), it still needs to be fully understood why males are more susceptible to these infections than females. We used *Drosophila melanogaster*, commonly known as the fruit fly, which shares important similarities with humans, such as immune signalling pathways, many disease-causing genes and a macrophage-like immune cells.

Like in humans, male fruit flies were more vulnerable to *S. aureus* challenge, with higher mortality and bacterial counts, especially early in the infection. Additionally, female macrophage-like cells (hemocytes) engulfed the bacteria more actively. We used genetic tools to create flies without hemocytes, revealing that these cells play a crucial role in controlling *S. aureus* infection. Interestingly, differences in survival and

bacterial growth between male and female flies without the macrophage-like cells were eliminated in individuals without hemocytes, suggesting that females rely more on cellular immune responses. Unlike mammals, where sex determination is organism wide, in *Drosophila*, sex is determined cell by cell. By manipulating this phenomenon, we created male flies with female-like macrophage cells. These sex-switched individuals showed improved survival rates, nearly matching the survival rates of control females. This suggests that the female sexual identity of the macrophage-like cells offers a survival advantage during *S. aureus* infection. Furthermore, we studied a special *Drosophila* population that had evolved to resist parasitoid wasp attacks during their larval stages. In these flies, sex differences in *S. aureus* infection disappeared, suggesting that evolving a constitutive hemocyte response that allows individuals to better survive wasp attack early in life comes at the cost of resistance to *S. aureus* in adulthood, and this adaptation compresses sexual dimorphism in adult immune responses that rely on hemocytes.

ABSTRACT

Biological sex, determined by a combination of genetic, reproductive, and hormonal factors, significantly influences immune responses. This phenomenon is strikingly evident in the higher incidence of autoimmune diseases in females and the increased susceptibility to infectious diseases in males, as observed with COVID-19. Despite these disparities, less than 10% of immunological studies report their findings by sex, leaving the underlying mechanisms of this dimorphism unexplored.

Staphylococcus aureus is a gram-positive bacterium responsible for numerous nosocomial infections, disproportionately affecting men. *Drosophila melanogaster* is an excellent model organism for studying innate immune responses due to its available genetic resources, highly conserved immune pathways, and professional phagocytes similar to mammalian macrophages. Since *S. aureus* is primarily controlled through phagocytosis, in this thesis we employed *Drosophila melanogaster* to investigate sex-specific responses to the *S. aureus* challenge, mainly focusing on hemocytes (the insect equivalent of macrophages) as a foundation for the sexually dimorphic outcomes of this infection. We utilised various laboratory techniques, including the generation of transgenic lines, pathogen microinjection and survival assays, bacterial load quantification and a flow cytometry-based assay for the determination of phagocytic activity.

Our findings revealed that analogous to humans, males are more susceptible to *S. aureus* infection than females, accompanied by higher bacterial loads, especially in the early phase of infection. Additionally, females exhibited a higher proportion of phagocytic hemocytes compared to males. We implemented a genetic ablation

protocol to eliminate *Drosophila* hemocytes, resulting in significantly increased mortality rates in both sexes and higher *S. aureus* proliferation compared to controls. Interestingly, the depletion of hemocytes eliminated this dimorphism, indicating that both sexes rely on heavily on hemocytes to control the infection. Taking advantage of *Drosophila*'s unique cell-autonomous sex determination cascade, we generated male flies with genetically feminised hemocytes. Remarkably, these sex-switched hemocyte-carrying individuals demonstrated significantly improved survival rates to the *S. aureus* challenge, nearly matching those of control females. A similar trend was observed in bacterial proliferation, suggesting that female hemocytes are more efficient than their male counterparts, conferring a survival advantage in *S. aureus* infection. Lastly, we challenged *Drosophila* lines, which had been selected for survival against wasp parasitism during larval development. Intriguingly, the sex dimorphism in survival and bacterial load disappeared in these individuals, implying that survival to parasitism at the juvenile stages leads to the loss of dimorphism and a trade-off with resistance to *S. aureus* infection in adulthood. The research uncovers macrophages as an important source of sexually dimorphic susceptibility to *S. aureus* infection. It highlights the importance of considering "sex" as a variable in experimental design and its implications for personalised medicine.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

In biological terms, sex is determined by distinctions emerging from the genome, such as sex chromosome configuration, the development of reproductive organs, and the levels of gonadal hormones. These physiological differences between males and females extend beyond reproductive tissues and impact various bodily processes, including the immune system. Consequently, men are more susceptible to certain infections than women, whereas women are more prone to autoimmune diseases. Interestingly, these sex-specific immune differences are not unique to humans but are commonly observed in various species, including *Drosophila melanogaster*. Despite the clear disparities in immune responses between the sexes across the animal kingdom, our understanding of the underlying causes has been very limited. This is partly due to historical neglect of sex-based differences in research and the oversimplification of attributing them solely to the action of gonadal hormones, overlooking the influence of the chromosome complement within each cell. A deeper understanding of the interplay between hormonal and sex chromosome-driven differences in immune responses could facilitate more personalized treatment approaches, leading to improved outcomes for diseases with clear sex-biased susceptibility, such as *Staphylococcus aureus* infection, which disproportionately affects men. While the precise factors leading to this increased susceptibility in men remain elusive, professional phagocytes emerge as plausible candidates, given their importance in staphylococcal pathogenesis.

Drosophila melanogaster is an excellent model organism to study host-pathogen interactions, particularly due to its genetic similarities and conserved immune pathways, notably those related to phagocytosis, which parallel those in mammals. Additionally, the dimorphic physiology of *D. melanogaster*, coupled with the established role of its phagocytes in managing *S. aureus* challenge, positions it as a prime candidate for investigating how professional phagocytes contribute to sex-specific outcomes in *S. aureus* infection, a focal point of this thesis.

Herein, I present a review of important literature regarding the prevalent differences in immunity between the sexes, focusing on adaptive and immune responses, as well as their mediation through the combined action of hormonal and genetic factors. Additionally, I give an overview of the *D. melanogaster* immune system, particularly focusing on its involvement in the phagocytosis of bacteria and on the shared features between fruit flies and humans, ending with a brief revision of *S. aureus* pathogenesis and the accuracy of *Drosophila* as a model of staphylococcal infections.

1.2 Sex-specific differences in immunity are apparent in epidemiological studies yet overlooked in research

In epidemiology studies, it is evident that men exhibit higher age-adjusted incidence of various infectious diseases than women (Walter et al., 2018, Westergaard et al., 2019). Additionally, men tend to experience more severe disease manifestations, leading to greater mortality. For instance, the occurrence of various bloodstream infections and subsequent mortality was higher for males across all age groups in Finland (Kontula et al., 2021). Similarly, in the majority of countries, tuberculosis

frequency was twice as high in men compared to women, with the exceeding number of deaths occurring among men (Horton et al., 2016). Such a tendency was also evident during the COVID-19 pandemic, when in almost all countries with available sex-disaggregated data, the risk of death was 1.7 times higher for men than women (Capuano et al., 2020).

In certain instances, the disease vulnerability is not solely attributed to males, as exemplified by the higher fatality of females infected with pathogenic influenza A viruses, despite men being more frequently exposed (Sabikunnahar et al., 2022). Also, women have about 1.6 times higher risk of developing HIV, as suggested by the sex-matched RNA loads (Meditz et al., 2011). These sex-specific differences are reflected by a combination of hormonal and genetic factors, leading to more robust innate and adaptive immune responses in females, which facilitate faster pathogen clearance compared to males. However, this delicate balance of generating a potent yet not overly aggressive immune response is often disturbed in females, making them more prone to autoimmune diseases, such as systemic lupus erythematosus or multiple sclerosis (Voskuhl, 2011). Despite clearly evident sex-specific differences in disease susceptibility in population-based data, mechanisms driving these disparities remain largely unexplained due to a skewed research perspective (Beery and Zucker, 2011).

The neglect of sex-related considerations is evident in preclinical research, including both *in vitro* and *in vivo* approaches, and clinical research. In the initial stages of preclinical screenings of novel therapeutics, cell lines are predominantly used. Due to shared structural characteristics, it is often assumed that cellular physiology remains

consistent between cells derived from males and females. Consequently, the sex of the cell line utilised in experiments is usually not accounted for (Shah et al., 2014). As a result, the cell model used in drug evaluations may be not representative of at least half of the population, negatively affecting the health of women (Correa-De-Araujo, 2006). The problem is becoming increasingly evident, as some of the first-line anti-inflammatory medications, such as aspirin (Ridker et al., 2005) and statins (Ridker et al., 2005), were found less effective in females. When it comes to the *in vivo* approach, female subjects in animal studies have been frequently excluded to eliminate confounding variables, such as hormonal fluctuations during reproductive cycles (Wald and Wu, 2010). Historically, women have also been omitted in clinical evaluations, in line with the US Food and Drug Administration guidelines, recommending the exclusion of females of childbearing age from drug trials (Liu and Mager, 2016). In addition to experimental design, the problem of not considering “sex” as a variable extends to data analysis, as immunology ranks the lowest among the ten biological disciplines for reporting the sex of subjects in publications, with fewer than 10% of articles analyzing data by sex (Beery and Zucker, 2011). As a result, sex-based differences have been overlooked at every possible stage, beginning with experimental design, *in vitro*, *in vivo* testing, human trials and finally, data analysis and reporting. However, new policies have been introduced (Klinge, 2008), providing some insight into sex differences in innate and adaptive immune responses.

1.3 Sex-specific differences in immunity are reported in innate and adaptive responses

1.3.1 Innate Immunity

The disparities in the innate immune defence mechanism can be seen in its cellular and humoral components. There are sex-specific differences in the number, relative distribution and activity of innate immune cells. For instance, men have more NK cells and monocytes (Abdullah et al., 2012, Piasecka et al., 2018) than women and they are more active than their female counterparts. Whereas females have more neutrophils in the peripheral circulation that are more phagocytic (Bain and England, 1975). Antigen-presenting cells derived from women are also more efficient (Weinstein et al., 1984), suggesting that they relay signals to adaptive immune components more efficiently than men. The ability to detect pathogens is also differentially regulated between sexes, as females have higher expression of pathogen-associated molecular pattern receptors. For instance, female leukocytes show higher expression levels of

TLR7, resulting in higher production of IFNα in women (Souyris et al., 2018). Additionally, females show a greater level of expression of genes associated with TLR pathways. However, male neutrophils express higher levels of TLR4, leading to increased production of TNF both at homeostasis and following the LPS activation (Aomatsu et al., 2013), which demonstrates that higher expression of inflammatory regulators is not exclusively skewed towards females.

As evident, the disparities in cell number and activity result in the differential production of cytokines and chemokines between the sexes, which further deepens

the sexually dimorphic nature of innate and adaptive responses. Although there are some exceptions, many cytokine and inflammatory mediators show higher expression in females compared to males, and such female-biased activation levels also extend to macrophages.

1.3.1.1 Macrophages

Sex has been demonstrated to influence macrophages, with sex hormones regulating the expression of genes (Ribas et al., 2011) and proliferation of these immune cells (Pepe et al., 2017). Herein, the focus will be on sex-specific differences in phenotype and function of macrophages, independent of the action of steroid hormones. Female rodent-derived peritoneal macrophages exhibit significantly upregulated expression of TLR2, TLR3 and TLR, indicating that the female phagocytic cells are more adept at pathogen detection than their male counterparts. Moreover, female macrophages exhibit enhanced phagocytosis and antimicrobial activity, as demonstrated by quicker zymosan particle engulfment and elevated antibacterial NADPH oxidase levels. Despite the higher macrophage count in women, their macrophage-derived cytokine production remained limited due to the increased presence of resident CD4+T lymphocytes (Scotland et al., 2011), showing that the differential macrophage regulation at the innate level may result in varying responses at the adaptive level. While another study did not report sex-specific expression of TLRs in peritoneal macrophages, a different type of pattern recognition receptor, C-type lectin receptor CD209b, was shown to be upregulated in female macrophages, enhancing their ability to control *S. pnuemoniae* peritonitis (Bain et al., 2020). Therefore, it appears that female macrophages heighten their sensitivity to infectious stimuli by upregulating

different classes of pattern recognition receptors. Numerous distinctions have been also reported in sex-specific differences in macrophage gene expression downstream of pathogen recognition. For instance, female macrophages derived from the bone marrow of recombinant chickens exhibited higher set points of IFN-responsive genes under basal conditions (Garcia-Morales et al., 2015). In murine macrophages, these genes are expressed through an autocrine induction of IFN-β in response to LPS dependent bacterial activation of TLR4 (Takeuchi and Akira, 2010). Therefore, female macrophages exhibit distinctive regulation not only at the level of pathogen recognition but also downstream of the immediate genes. Additionally, the elevated expression of IFN-responsive genes in female macrophages, even under homeostatic conditions, may potentially enhance their capacity to respond to a pathogen challenge.

In addition to differences in activity and gene expression, several studies have reported on sex-related disparities in the phenotypic states of murine macrophages. Under specific environmental stimuli, the lineage of monocyte-macrophage cells is distinguished by great plasticity and diversity (Sica and Mantovani, 2012, Mosser and Edwards, 2008), enabling them to transition through various activation stages. For simplification, we will focus on the two most extreme ones. In response to IFN-γ and LPS, macrophages can undergo classical activation (M1), facilitating the proinflammatory processes and microbial killing. M1 macrophages are characterized by increased production of pro-inflammatory cytokines and reactive oxygen species. Driven by IL-4 or IL-13, macrophages may become alternatively activated (M2), mediating processes of tissue repair and inflammation. M2 macrophages typically generate copious amounts of anti-inflammatory cytokines and arginase 1 (Murray et

al., 2014). For instance, rodent-derived bone marrow macrophages from males were more susceptible to LPS treatment and promoted M2 phenotype. Following *S. agalactiae* infection, the ratio of M1 to M2 macrophages in bronchoalveolar lavages was higher in male mice compared with female animals (Deny et al., 2022). Similarly, rodent-derived myocardial macrophages infected with Coxsackievirus B3, a causative agent of severe myocarditis in males but not females, expressed high levels of the M1 macrophage markers in males, whereas females showed elevated expression of markers associated with the M2 phenotype. As could be expected, the adoptive transfer of *ex-vivo* programmed M1 macrophages significantly increased myocarditis in both sexes and the transfer of M2 macrophages into infected males resulted in diminished inflammation of the myocardial tissue (Li et al., 2009). Therefore, the alternative activation phenotype commonly found in females may be one of the factors that confer them protection against infectious agents.

Hence, female macrophages exhibit better pathogen recognition ability, accompanied by greater bactericidal and engulfment properties compared to their male counterparts. Additionally, female macrophages seem to be better equipped to respond to infectious challenges. While macrophages are the primary mediators of the innate response, they also play a pivotal role in driving the effector phase of the adaptive immune response (Hirayama et al., 2017).

1.3.2 Adaptive Immunity

Although not relevant to the thesis, sexual differences have been also reported in the adaptive arm of the immune system, as exemplified by sex-specific activity and

distribution of lymphocyte subsets. At baseline, females have a higher frequency of CD4+ T cells, accompanied by a higher $CD4^+$ to $CD8^+$ ratio, whereas the count of circulating $CD8^+$ T cells is greater in males. Regardless, according to clinical studies, the ratios of $CD3^+$ to $CD4^+$ cells and $CD4^+$ to $CD8^+$ cells remain lower in males (Uppal et al., 2003). Additionally, adult females show more robust antibody responses, higher B cell numbers (Abdullah et al., 2012) and higher IgM and IgG titers (Obiandu et al., 2013, Ter Horst et al., 2016). Therefore, female adaptive immune cells typically demonstrate greater numbers, heightened activation and elevated levels of inflammation. Such more potent innate and adaptive immune responses in females stem from the mix of genetic and hormonal factors.

1.4 Biological sex is a combination of genetic and hormonal factors

1.4.1 Genetic Mediators

The sex of an embryo is determined by the presence of either two X chromosomes, driving the development of a homologous female, or the combination of one X and one Y chromosome, defining a heterologous male. Therefore, sexual differentiation commences at the moment of conception and becomes apparent early on in life. The human X chromosome contains about 1100 genes, such as IL-1 or IL-2, which are strongly implicated in the immune response (Libert et al., 2010). While the human Y chromosome harbours only 100 genes, including the *SRY* testis-determining protein, some of them have also been implicated in male immunity (Case et al., 2012, Case et al., 2013). In females, one of their two X chromosomes is randomly silenced in each cell to equalize gene product dosages between the sexes (Tukiainen et al., 2017). However, about 15% of genes on the X chromosome, particularly those governing

sex-specific immune responses, escape the inactivation, leading to their dual expression and higher copy number in females than males. For instance, the abovementioned TLR7, which acts as a critical intranuclear nucleic acid sensor critical to the induction of antiviral immunity, has been shown to escape chromosome inactivation in immune cells (Pisitkun et al., 2006). The X chromosome chosen for inactivation is determined randomly during embryonic development, resulting in cell mosaicism in females a phenomenon associated with survival advantage (Migeon, 2007). Additionally, polymorphisms from the Y chromosome and variations in autosomal genes encoding immunological proteins can also affect sex-dependent susceptibility (Case et al., 2012, Spach et al., 2009, Poland et al., 2008).

Aside from the differential gene expression between the sexes, the role of non-coding regions in the genome, especially microRNAs in mediating sex-specific variation is becoming increasingly evident (Cui et al., 2018). The X chromosome contains around 10% of all microRNAs found in the human genome (Sharma and Eghbali, 2014), while there are only two microRNAs located on the Y chromosome (Ghorai and Ghosh, 2014). In addition to quantitative differences, numerous microRNAs have been implicated in post-transcriptional mRNA expression of immunomodulatory genes. For example, the level of expression of the X chromosome-linked microRNA-223-3p, which has been previously shown to modulate inflammation, was upregulated in female rodent-derived macrophages compared to their male counterparts. Additionally, the overexpression of microRNA-223-3p in female lung macrophages correlated positively with the percentage of CD206+ macrophages (M2) and negatively with the percentage of CD80+ macrophages (M1), implying that the

differentially expressed microRNA-223-3p between the sexes may have a role in modulating the M1/M2 macrophage ratio.

Numerous sex-specific immunological differences are inherent in our genome. These distinctions between males and females emerge as early as conception, encompassing variations in chromosome configuration, gene content and regulation of their expression patterns. However, biological sex results from the interplay of both genetic and hormonal factors, which affect the dynamic, extent and changes of these sex-specific immunological distinctions embedded in our genome (Klein, 2012).

1.4.2 Hormonal Mediators

Sex steroid hormones, including testosterone, progesterone and estradiol, quantitatively and qualitatively influence immune responses by binding to their respective steroid receptors (Edwards, 2005) expressed in various cells, such as circulating lymphocytes or macrophages. As a result, they directly stimulate cell signalling pathways, leading to differential production of cytokines and chemokines (Klein and Roberts, 2010). Gonadal hormone level disparities originate from *in-utero* development, with testes leading to higher testosterone levels in males and ovaries inducing greater estrogen synthesis in females, establishing a life-long imbalance (Dias et al., 2022). Besides the immune system modulation, sex steroid hormones also influence bacterial metabolism and the expression of virulence determinants (GarcíaGómez et al., 2013), exerting effects both on the host and the pathogen. Several immune genes have androgen or estrogen response elements in their promoter regions (Hannah et al., 2008) and some microRNAs have been

demonstrated to be under hormonal regulation, clearly highlighting the connection between the genome and secreted hormones.

Generally, testosterone suppresses immune activity (Roberts et al., 2001), inhibiting natural killer (NK) cells (Hou and Zheng, 1988), neutrophils, macrophages and proinflammatory cytokine synthesis at early infection stages through the impairment of the transcriptional activity of NF-κB (D'Agostino et al., 1999). Whereas in the late stages of pathogen challenge, testosterone upregulates the production of anti-inflammatory cytokines via the androgen signalling (D'Agostino et al., 1999, Liva and Voskuhl, 2001). Additionally, it has been shown to decrease the expression of TLR4 on monocytes and macrophages, increasing disease susceptibility in male mice (Rettew et al., 2008). Progesterone also exhibits broad anti-inflammatory effects, suppressing the antibody-producing B cells (Lü et al., 2002), macrophages (Miller and Hunt, 1996, Savita and Rai, 1998) and NK cells (Furukawa et al., 1984), as well as the NF-κB signal transduction (Su et al., 2009). As a result, the synthesis of proinflammatory cytokines is reduced in favour of enhanced expression of anti-inflammatory mediators, thus preventing the development of bacterial infections and their subsequent spread to the bloodstream and sepsis (Vázquez-Martínez et al., 2018). Estrogens are mainly protective against bacterial infection and septic shock, with low concentrations enhancing T_H1-type responses and cell-mediated immunity, which stimulates the synthesis of pro-inflammatory cytokines and chemokines. Whereas high estradiol concentrations inhibit NF-κB activity, promoting T_H2-type responses and humoral immunity, which blocks pro-inflammatory pathways and promotes the production of anti-inflammatory cytokines (Bouman et al., 2005, Straub, 2007). Additionally, estrogen increases the expression of TLR4 on the surface of

peritoneal macrophages in rodents (Rettew et al., 2009). In general, estrogen has been demonstrated to exert a protective effect in many infections, including *S. aureus* (Castleman et al., 2018).

1.4.3 All cells have a sex

The theory of mammalian sex determination hinges on the two above-discussed components – the evolution of sex chromosomes, which results in different gene expression patterns on the X and Y chromosomes, and the sex chromosome dependent differentiation of either testes or ovaries, leading to the gonadal hormone regulation. Historically, the focus has centred on the role of sex hormones, specified by gonadal development, as the key drivers of sexual dimorphism between men and women (Arnold, 2019). As a result, the mechanisms based on the differences in X and Y gene dosage have been relatively understudied. However, research has shown that sex-related differences arise before the gonad differentiation (Bain et al., 2020) and in the context of immune responses, higher susceptibility to infections in males is observed from birth, before the action of pubertal hormones (Libert et al., 2010). Additionally, sex-specific distinctions in immune cell behaviour may not be exclusively governed by differing hormone levels, since the majority of cytokines in macrophages, which exhibited disparities between males and females, did not correlate with the concentrations of progesterone and testosterone. (Ter Horst et al., 2016, Jaillon et al., 2019). This suggests that while gonadal secretions play a crucial role in shaping sexual dimorphisms, the determination of sex and the manifestation of sex-specific traits extend beyond the influence of the gonads and their hormonal outputs. Consequently, the research perspective has shifted, focusing on the inherent imbalance of sex chromosomes within every single cell, beginning in the zygote,

functioning in the embryo and all through the lifespan. Therefore, sex differences in somatic tissues are an end product of complicated interactions between gonads, which define differences in sex hormones that, in turn, interact with the unequal effects of X and Y genes within cells. Although there are models, such as the FCG and XY* mice, that provide insights into the sex-biased and hormone independent effects of sex chromosome dosage on non-reproductive tissues, the differences observed are mostly phenotypic (Cox et al., 2014). The cell-autonomous influence of X and Y chromosome complement in non-gonadal tissues remains poorly investigated due to challenges in establishing a mammalian model that would allow for the manipulation of sex chromosome configuration at a cellular level (Straface et al., 2012). However, *Drosophila melanogaster*, whose sex determination is governed by a well-studied cascade of regulatory genes, enables the manipulation of sex at a cellular level by expressing sex-specific transcription factors. Such manipulation simulates the consequences of changing the chromosomal complement and provides clearer means to interpret chromosome-related differences between the sexes, all without the confounding effects of sex hormones.

1.5 *Drosophila* immunity

Drosophila relies entirely on an innate immune response which includes humoral and cellular factors, making it a powerful model to study innate immune response which otherwise could be masked by adaptive immune response. The humoral component depends on the production of antimicrobial peptides (AMPs), reactive oxygen species, cytokines and mediators of blood clotting and melanization (Vierstraete et al., 2004, De Gregorio et al., 2001, Agaisse et al., 2005). Whereas the cell-mediated component

involves phagocytosis of small microorganisms and encapsulation of larger parasites which are mediated by plasmatocytes and lamellocytes respectively (Lemaitre and Hoffmann, 2007). Herein, a brief overview of cellular recognition and signalling processes that *Drosophila* rely on to fight the infection will be given.

1.5.1 Humoral response

The humoral response in *Drosophila* encompasses various processes such as hemolymph coagulation and melanization. However, it is primarily driven by the secretion of AMPs, which are small, positively charged molecules that disrupt the negatively charged microbial membrane, resulting in cell death (Joo et al., 2016). While the synthesis of AMPs occurs mainly in the fat body, some may be produced in other tissues, such as epithelia (Tzou et al., 2000). To date, there are identified seven families of AMPs, including Drosomycins and Metchnikowins with antifungal properties (Fehlbaum et al., 1994, Levashina et al., 1995), Cecropins and Defensins with antibacterial and some antifungal characteristics (Ekengren and Hultmark, 1999, Cociancich et al., 1993, Tzou et al., 2002) and Drosocins, Attacins, and Diptericins, which primarily demonstrate antibacterial activity (Hedengren et al., 2000, Bulet et al., 1996). Depending on the type of pathogen, the expression of these AMPs is induced at the transcriptional level through the NF-κB-related Toll and IMD signalling pathways (Meister et al., 1997), activated by the recognition of pathogen-associated molecular patterns, such as lipopolysaccharides, β-glucans and peptidoglycans, which are recognized by specialized pattern recognition receptors. In the context of bacterial infections, there are thirteen peptidoglycan recognition proteins (PGRPs) within the

Drosophila genome (Werner et al., 2000), which distinguish the type of peptidoglycan, initiating signalling, facilitating phagocytosis, or doing both.

1.5.1.1 The Toll and Imd signalling pathways

The Toll pathway is analogous to the MYD88-dependent TLR pathway in mammals (Leulier et al., 2003, Kaneko et al., 2004). Contrary to vertebrate Toll-like receptors, Toll is not involved in pathogen recognition directly but is rather activated upon binding with a cleaved form of a cytokine called Spätzle (Weber et al., 2003). In response to LYS-type peptidoglycan and glucan, mostly found in gram-positive bacteria and fungi, PGRP-SA, PGRP-SD and GNBP3 become activated, initiating the processing of Spätzle pro-form. As a result, the Toll molecule undergoes dimerization, recruiting three death domain-containing proteins to the plasma membrane, which induces phosphorylation and subsequent degradation of the Cactus. Then, transcription factors Dorsal and Dif are translocated to the nucleus, inducing the expression of genes encoding AMPs, such as Drosomycin and Attacin. Additionally, a separate group of peptides, known as the Bomanins, which are regulated by the Toll pathway, contribute to the killing of gram-positive bacteria and fungi in the fly hemolymph (Clemmons et al., 2015).

The Imd pathway, often compared to the TNF and TRIF-dependent TLR pathways in mammals (Buchon et al., 2014), is triggered by DAP-type peptidoglycan found in gram-negative bacteria and *Bacillus* species (Boulet et al., 2021b, Georgel et al., 2001). Direct binding of DAP-type peptidoglycan to the surface-bound PGRP-LC or cytosolic PGRP-LE results in the recruitment of a signalling complex, followed by

phosphorylation and cleavage of the transcription regulator Relish. As a result, rel-68 translocates to the nucleus, leaving the inhibitory domain in the cytoplasm, and induces the expression of AMPs, such as Diptericin and Drosocin.

1.5.1.2 The JAK/STAT signalling pathway

Similarly to mammals, the *Drosophila* JAK/STAT pathway mediates multiple immune processes, including responses to mechanical stress, e.g. due to wounding or parasitation by wasps. In response to infection, hemocytes release three cytokines from the Unpaired (Upd) family, Upd1, Upd2 and Upd3 (Myllymäki and Rämet, 2014). These cytokines bind to the Domeless receptor (Brown et al., 2001), which is inherently associated with Hopscotch (Perrimon and Mahowald, 1986). As a result, Domeless dimerizes, activating Hopscotch which in turn phosphorylates transcriptional regulator STAT92E (Brown et al., 2003). This leads to STAT92E dimerization and nuclear translocation, activating genes from the turandot (Tot) and thioester-containing protein (TEP) families (Myllymäki and Rämet, 2014). TEPs, which phylogenetically share similarities with mammalian complement factors and macroglobulins, are mostly expressed in hemocytes, the fat body and specific regions of the epithelia. While some studies have demonstrated that a concurrent deficiency of TEP1, TEP2 and TEP4 does not impact survival to bacterial and fungal infections (Bou Aoun et al., 2011), others have reported that flies lacking all the TEPs are more susceptible to some fungi, gram-positive bacteria and parasitoid wasp infections, yielding contradictory results. Additionally, the TEP-deficient flies exhibited a compromised phagocytic activity and decreased Toll activation (Dostálová et al., 2017), implying their role in particle engulfment and signalling crosstalk. Genes from

the Tot family, although relatively understudied, have been implicated in response stress induced by bacterial and viral infections (Kemp et al., 2013, Ekengren and Hultmark, 2001).

1.5.2 *D. melanogaster* cellular immune response is primarily mediated by hemocytes

The cellular response in *D. melanogaster* involves phagocytosis of small microorganisms and encapsulation of larger parasites and is primarily mediated by circulating blood cells, collectively known as hemocytes. The *D. melanogaster* blood cell system shares highly conserved features with the vertebrate hematopoietic system at the functional and developmental levels. Much like in vertebrates, there are two blood lineages, the embryonic lineage, which resembles self-renewing tissue macrophages in vertebrates, and the lymph gland lineage, which is analogous to progenitor-based hematopoiesis in vertebrates (Mase et al., 2021). These lineages give rise to three types of hemocytes, plasmatocytes, lamellocytes and crystal cells, each with a distinct function and structure. In general, hemocytes are scattered around the body, mostly in sessile clusters, and accumulate along the heart in the abdomen (Lanot et al., 2001).

Both lineages are induced in the pupal stages and continue into adulthood, with the embryonic lineage primarily supplying immune cells under normal conditions, while the lymph gland lineage aids in immune challenges, providing support. In adult animals, the production of new blood cells halts, and the number of hemocytes decreases. It was widely believed that at every developmental stage beyond the embryo, 90% of all cells were plasmatocytes and small numbers of crystal cells.

However, recent data has challenged this notion, suggesting that the adult hematopoietic system is essentially composed of plasmatocytes (85%), crystal cells (8 to 10%) and a small population of undifferentiated cells (~4%) (Boulet et al., 2021).

1.5.2.1 Plasmatocytes

Plasmatocytes, which are characterized by a round and small appearance, account for the majority of circulating hemocytes in larvae and adults. They perform a diverse array of functions, such as the production of AMPs, cytokines, extracellular matrix components, uptake of lipids, secretion of metabolic mediators, and, more importantly, phagocytosis of apoptotic cells and pathogens. Although various types of *Drosophila* cells have been implicated in the engulfment of particles (Shklover et al., 2015, Serizier and McCall, 2017), plasmatocytes demonstrate the greatest phagocytic ability. Usually, the process of phagocytosis starts with the binding of molecules exposed on the surface of microorganisms or apoptotic cells to dedicated phagocytic receptors. Upon ligand recognition, phagocytic receptors, either directly or indirectly, activate downstream signalling pathways, triggering particle uptake (Stuart and Ezekowitz, 2005). The process has been demonstrated to involve various phagocytic machinery, including the above-discussed PGRPs, scavenger receptors (SRs), integrins, opsonins, and the Down syndrome cell adhesion molecule (Dscam). Most phagocytic receptors overlap with molecular markers of plasmatocytes, aiding in their analysis.

Scavenger receptors bind to a wide range of polyanionic ligands and are grouped into the class B and class C families. One of the four members of the class C family, dSrCI,

is expressed exclusively on the surface of plasmatocytes, mediating the binding of both gram-positive and gram-negative bacteria, such as *S. aureus* and *E. coli*, but not yeast (Pearson et al., 1995, Rämet et al., 2001). Croquemort, the class B family member, is also specifically expressed in plasmatocytes, aiding in the clearance of apoptotic cells and gram-positive microbes (Stuart et al., 2005, Franc et al., 1996). More specifically, it primarily mediates phagosome maturation rather than the initial recognition. Another class B member, Peste, has been implicated in the engulfment of selected gram-positive bacteria (Philips et al., 2005, Agaisse et al., 2005). The Nimrod family of phagocytic receptors, which is characterized by the presence of epidermal growth factor-containing repeats, comprises twelve members, including NimrodC1, Eater and Draper. These receptors are associated with the clearance of apoptotic cells and the elimination of both gram-positive and gram-negative bacteria, and fungi (Kurucz et al., 2007, Melcarne et al., 2019, Kocks et al., 2005, Bretscher et al., 2015, Freeman et al., 2003, Cuttell et al., 2008). Integrins, which are heterodimeric proteins formed by α and β subunits, have been demonstrated to facilitate the uptake of both apoptotic cells and gram-positive bacteria. Although with contrasting results, PGRP-LC, has been suggested to have a minor in the phagocytosis of gram-negative, but not gram-positive bacteria (Rämet et al., 2002). Whereas another pattern recognition receptor, PGRP-SC1 *Drosophila* Down Syndrome Cell Adhesion Molecule 1, whose genetic composition allows for the generation of alternatively-spliced isoforms, has been shown to act as both a phagocytic receptor of *E. coli* and an opsonin (Watson et al., 2005). Additionally, for the actin-dependent engulfment to happen, the target molecule must be tethered to the phagocyte, which is achieved either by direct recognition of microbial determinants or previously mentioned TEPs. In *Drosophila*, certain TEPs, specifically TEP2 and TEP3, have been shown to

opsonize certain pathogens, such as *E. coli* and *S. aureus*, before phagocytosis (Stroschein-Stevenson et al., 2005, Dostálová et al., 2017).

Several morphological similarities between mammalian macrophage-mediated and *Drosophila* plasmatocyte-facilitated phagocytosis have been observed, with approximately 70% of the mammalian phagosome proteins present in *Drosophila* S2 cell phagosomes. This highlights a high degree of conservation in the phagocytic machinery shared between fruit flies and humans (Stuart et al., 2007). In *Drosophila*, once the phagocyte binds to the target particle, a phagosome is formed around it through the remodeling of the plasma membrane, which is facilitated by the actin cytoskeleton (Pearson et al., 2003). Then, the newly formed phagosome fuses with cellular endosomes and lysosomes, which release hydrolases. Small GTPases of the Rab family, especially Rab5 and Rab7, mediate the initial and late fusion events respectively (Horn et al., 2014, Yousefian et al., 2013, Cheng et al., 2005). For the proper activation of the hydrolases, the phagosome lumen undergoes acidification mediated by the proton-pumping vacuolar ATPase, resulting in microbe destruction (Cheng et al., 2005, Philips et al., 2005).

1.5.2.2 Lamellocytes

Lamellocytes, known for their large and flat appearance, play a key role in the encapsulation of large immune targets. While they originate in larvae, lamellocytes are not typically present in healthy juveniles or adolescents unless under immune challenges, such as parasitoid wasp egg infestation, when their differentiation is massively induced (Lanot et al., 2001). *Drosophila* may be infected by more than 50 species of hymenopteran parasites, where female wasps lay their eggs in the larval

hemocoel (Carton et al., 1986). The parasitic eggs are too large to be engulfed by *Drosophila* phagocytes, so circulating plasmatocytes only attach to the eggs, the number of crystal cells increases (Sorrentino et al., 2002), promoting the significant differentiation of lamellocytes (Lanot et al., 2001), which subsequently encapsulate the invader, resulting in its elimination.

1.5.2.3 Crystal cells

Crystal cells, which are typically found in small numbers in adult animals, play a pivotal role in clotting and melanin-mediated wound healing. They are distinguished by the presence of an enzyme called prophenoloxidase that is stored in the form of crystalline inclusions. Upon cell degranulation, prophenoloxidase is solubilized in the hemolymph, leading to enzyme activation. Subsequently, phenols and quinones are oxidized, resulting in the polymerization of these molecules into melanin (Vlisidou and Wood, 2015). Because of the cytotoxic nature of certain intermediate products in melanin synthesis, a Toll pathway-dependent protease inhibitor Serpin 27-A strictly controls the localization of the reaction (De Gregorio et al., 2002). Melanin may be synthesized either at the site of infection or injury, aiding in wound closure, or sequestered onto the pathogen itself, promoting lamellocyte adherence and subsequent encapsulation of the particle too big to be phagocytosed.

1.5.2.4 Hemocytes are a heterogenous population with various functional subtypes

Recent advancements in single-cell RNA sequencing have unveiled the heterogeneity of *Drosophila* blood cells, revealing distinct hemocyte subpopulations, and

challenging the notion of adult hemocytes as a uniform and terminally differentiated cell population (Ghosh et al., 2015). Circumstances, such as immune challenges, developmental transitions, or generation-long selective pressures, may change the transcriptional profiles of plasmatocytes. As previously mentioned, plasmatocytes can differentiate into lamellocytes in response to parasitism by wasps (Leitão et al., 2020). There are also dome*Meso* cells expressing progenitor markers that have been reported to proliferate and differentiate in response to infection (Boulet et al., 2021a). In response to bacterial stimuli, plasmatocytes undergo metabolic reprogramming, a process regulated by HIF1α. Remarkably, this metabolic transition resembles the M1 macrophage activation observed in mammals, which enhances phagocytic activity and promotes a pro-inflammatory phenotype. (Cattenoz et al., 2020). Lastly, changes in hemocyte populations are not constrained to the individual level but can also accumulate over generations, as evident in larvae that have evolved under high levels of wasp parasitism, resulting in the constitutive presence of lamellocytes regardless of immune stimuli (Leitão et al., 2020).

1.5.2.5 The crosstalk of *Drosophila* hemocytes with other immunocompetent tissues

Hemocytes send signals to other immunocompetent tissues, such as the fat body. While signalling pathways responsible for regulating the expression of AMPs are indeed present and activated within hemocytes (Vlisidou and Wood, 2015), their contribution to AMP production is yet to be fully elucidated. Nevertheless, several pieces of evidence suggest a connection between hemocytes and the systemic activation of AMPs. For example, mutants for *psidin* lysosomal protein were unable to both engulf bacteria and activate the expression of Defensin in the fat body (Brennan

et al., 2007). Individuals with mutations in the *domino* gene, which dramatically reduces the pool of both circulating and sessile hemocytes, displayed increased lethality but still mounted a humoral response upon septic injury (Braun et al., 1998). Similarly, genetic ablation of larval hemocytes also underscored the importance of hemocytes in systemic immune response upon gut and systemic challenge. Moreover, hemocyte-specific knockdown of the cytokine-like Toll ligand Spätzle impeded the expression of Drosomycin, a known target of the Toll pathway (Shia et al., 2009), suggesting that in larvae, there is an integration of the cellular response with humoral response through a cytokine-based regulatory signal. Additionally activation of *totA* gene, which has been demonstrated to regulate JAK-STAT pathway in the fat body during response to septic injury, is based on the hemocyte-specific expression of gene encoding Unpaired-like cytokine (Agaisse et al., 2003), suggesting that the cellular response influence humoral response through cytokine-based regulatory signals. However, the understanding becomes more complicated when looking at fully developed individuals. In adult *Drosophila*, even when hemocytes are depleted, there is still a robust expression of AMPs (Defaye et al., 2009, Charroux and Royet, 2009), indicating that the induction of AMP production in adulthood occurs independently of a hemocyte-specific signal.

1.5.3 Sexual dimorphism in immune responses across taxa: insights from *Drosophila*

Sexual dimorphism of innate immunity is not restricted to humans, but disparities are evolutionarily conserved across taxa, including insects. Different theories seek to explain the factors contributing to the widely observed susceptibility of males, some proposing that the diminished immune response, which can be energetically costly,

represents a trade-off linked to the positive selection of traits enhancing reproductive fitness (Zuk, 2009). Regarding genetic parallels with mammals, *D. melanogaster* possesses X and Y chromosomes, which, unlike humans, regulate sex determination cell-autonomously. Additionally, *Drosophila* exhibits X-linked variation in genes encoding innate signalling proteins (Hill-Burns and Clark, 2009).

While sexually dimorphic immune responses in *Drosophila* are evident even without the action of steroid hormones (Belmonte et al., 2019), some involvement of hormonal mediators has been demonstrated. Compared to mammals, the endocrine regulation in fruit flies is relatively uncomplicated, involving three major hormones: 20hydroxyecdysone, Juvenile Hormone, insulin/insulin-like growth factor signalling and adipokinetic hormone (Keith, 2023). Research in this area has been predominantly focused on developmental aspects, and the understanding of immune regulation, especially in adult *Drosophila*, is still emerging (Nunes et al., 2021). For instance, 20hydroxyecdysone has been implicated in the activation of hemocytes and their immune functions, such as response to a pathogenic challenge in pupae (Regan et al., 2013). In cell culture experiments, Juvenile Hormone, which exerts an immunosuppressive effect, has been shown to counteract the immunostimulatory functions of 20-hydroxyecdysone, suggesting potentially more complex interactions (Flatt et al., 2008). Therefore, while fruit flies may share some similarities in endocrine immune regulation with mammals, the extent of the parallels is largely unknown, with genetic differences considered as the main mediators of sexually dimorphic outcomes in *D. melanogaster*.

In addition to chromosomal and hormonal disparities, the sex-specific responses to infections are most likely distinctly mediated by different immune components (Regan et al., 2013). For instance, the number and activity of key cellular immunity representatives, hemocytes, have been reported to differ between males and females (Leech et al., 2019a, Duneau et al., 2017b). Several studies have also reported sex disparities in *Drosophila* humoral immunity. Various transmembrane Toll receptors are differentially regulated between male and female flies, with Toll-5 and Toll-7 demonstrating higher basal expressions in males (Duneau et al., 2017b, Chowdhury et al., 2019). Certain Toll receptors, like Toll-1 and Toll-3, show elevated expression in females (Chowdhury et al., 2019) and males (Duneau et al., 2017b) respectively. However, they regulate both immunity and gonad-specific processes, easily explaining their sex-specific expression (Duneau et al., 2017b, Levin and Malik, 2017). Differences have also been observed downstream of these receptors, e.g. at the level of other signalling components and in AMP induction. For instance, sexually dimorphic induction of Drosomycin following *P. rettgeri* challenge was lost in flies devoid of cytokine Spätzle (Duneau et al., 2017b), implicating its role in mediating the sexually distinct expression of AMPs. In the IMD pathway, unchallenged males have been shown to express Diptericin at higher levels, particularly upon infection with *P. carotovorum* (Regan et al., 2016), although another study reported higher expression of Diptericin in females (Meister et al., 1994). Finally, the JAK-STAT also exhibits dimorphic regulation, as exemplified by the above-mentioned totA genes, whose expression were significantly upregulated in males (Duneau et al., 2017b). Therefore, even though males exhibit a higher expression of many Toll-regulated genes and to a lesser extent IMD-regulated genes, the Toll pathway appears to be

crucial for the sexually dimorphic outcome of both gram-positive and gram-negative bacterial infections in both sexes (Duneau et al., 2017b).

Despite the differential outcomes of bacterial challenges between the sexes, it is not possible to attribute the direction of sex-biased survival to infection to one signalling pathway or sex. Whenever immune responses and survival of males and females are directly compared, survival appears to be pathogen-dependent (Regan et al., 2013). For instance, systemic infections with the *Providencia* species, the extracellular gram-negative bacteria, render males more resistant than females (Buchanan et al., 2018, Duneau et al., 2017a), whereas males challenged with *P. fluorescens*, another gram-negative representative, die quicker compared to females (Leech et al., 2019b). Although there is a paucity of studies assessing the response of both sexes to gram-positive bacteria, female-biased survival to *S. aureus* is well-established (Leech et al., 2019b), providing the foundation for further research. Various *Drosophila* laboratory models demonstrate both female- and male-specific survival to gram-positive and gram-negative species (Regan et al., 2013). Additionally, the nature of the immune performance in *D. melanogaster*, and consequently its sexually dimorphic outcome, is heavily dependent on a variety of factors. For instance, mating status has been shown to have an immunosuppressive impact on females. More specifically, mated females were more susceptible to infection and had a higher pathogen load compared to virgin females (Duneau et al., 2017a, Short and Lazzaro, 2010, Schwenke and Lazzaro, 2017). In a recent study, male-biased susceptibility to *S. marcescens*, a gram-negative bacteria, was only observed in two out of four lines (Kutch and Fedorka, 2017), suggesting that the direction of sexual dimorphism is also dictated by genetic background. Therefore, *Drosophila* is a powerful model to

investigate the sexually dimorphic nature of bacterial infections, such as *S. aureus*, provided that there are appropriate controls put in place.

1.5.4 *Drosophila* sex determination is cell-autonomous

Whereas in mammals, the Y chromosome is involved in both genetic and gonadal sex determination (Gilbert, 2000), the *Drosophila* Y chromosome does not influence genetic sex determination, and its sole role is limited to the male germline differentiation (Manolakou et al., 2006). Although there is some evidence of endocrine regulation of sexual dimorphism in the *Drosophila* (Bilen et al., 2013), none has been implicated in the sex determination cascade, making cellular sex independent of hormonal mediation. Therefore, except for germ cells, whose sexual identity is dictated by both independent and soma-associated inductive signalling (DeFalco et al., 2008), somatic sex determination in *Drosophila* is cell-autonomous. However, there are a few inconsistencies, such as the muscle of Lawrence (Lawrence and Johnston, 1986), whose sexually dimorphic appearance is also influenced by cell-to-cell interactions. Regardlessly, such exceptions prove the rule that the sexual identity of somatic cells is mainly determined in a cell-autonomous manner by a ratio of female determinants on the X chromosome, called the numerator proteins (Cline, 1988), and male determinants on the autosomes, known as the denominator proteins (Younger Shepherd et al., 1992), which determine which sex-specific pattern of transcription will be initiated. Normally, flies have either one or two X chromosomes (X) and two sets of autosomes (A). In individuals with two X chromosomes and two sets of autosomes, the numerator proteins activate the early expression of the *Sex-lethal (Sxl)* gene, which results in female-specific processing of its own transcript. Once expressed, it is

maintained through the positive autoregulatory circuit (Bell et al., 1991). *Sxl* is required for female-specific processing of the *transformer (tra)* pre-mRNA (McKeown et al., 1987), which together with constitutively synthesised Transformer-2 protein, enhances alternative splicing of the doublesex (*dsx*) pre-mRNA (Inoue et al., 1992). As a result, a female-specific DSX-F protein isoform is produced (Burtis and Baker, 1989). Whereas in individuals with one X chromosome and two sets of autosomes, the denominator proteins inhibit numerator proteins, blocking the early *Sxl* expression (Younger-Shepherd et al., 1992). As a result, the *Sxl* transcription is initiated from the late promoter, resulting in a default processing of *Sxl* and *tra* transcripts, which contain an early codon stop, producing a nonfunctional protein (Boggs et al., 1987). As a result, a male-specific DSX-M isoform is expressed. Both DSX-F and DSX-M are key transcription factors that predominantly control sexual differentiation outside of the central nervous system (Burtis and Baker, 1989), whereas an additional transcription factor in the cascade, *fruitless*, regulates sex-specific behaviour (Ryner et al., 1996).

1.6 *Staphylococcus aureus*

Staphylococcus aureus, a gram-positive bacterium, primarily resides in the upper respiratory tract (Wertheim et al., 2005b) but may be also found in other locations within the human host (Albrecht et al., 2015). It is a common member of the human microbiota, colonizing approximately 40% of newborns and up to 50% of adults, either periodically or permanently, but not causing any harm (Wertheim et al., 2005b, Peacock et al., 2003). However, when host defences are breached, *S. aureus* can become pathogenic and cause a spectrum of diseases, ranging from minor skin and soft tissue infections to potentially deadly conditions like bacteremia and meningitis

(Naber, 2009). With the rise of health-seeking behaviour, *S. aureus* has become now a major pathogen causing both community and hospital-acquired infections (Prestinaci et al., 2015). Although the overall rates of *S. aureus* infections have remained stable over the last two decades (Tong et al., 2015), it was still associated with over one million deaths in 2019 (Ikuta et al., 2022). The rise of antibiotic resistance, particularly the methicillin-resistant form of S. aureus (MRSA), substantially contributes to high mortality rates of *S. aureus*-induced community and nosocomial infections (Lee et al., 2018a). Apart from the antibiotic resistance affecting outcomes of the staphylococcal challenge, sex is also a notable risk factor. Generally, men are more susceptible to various forms of *S. aureus* infection than females. *S. aureus* colonizes men more often than females (Humphreys et al., 2015), predisposing them to infection, dissemination of the bacterium into the bloodstream (Allard et al., 2008), and subsequent death (Mohus et al., 2022). This aforementioned tendency is also evident in the United Kingdom, where the incidence of methicillin-sensitive *S. aureus* bloodstream infections has been increasing since 2014 and regardless of age, the rate was always markedly higher in men compared to women (PHE, 2019). Together with the clinical observations, the aforementioned tendency has also been observed in *ex vivo* and *in vivo* laboratory studies. For instance, neutrophils from female rodents showed an increased bactericidal capacity of *S. aureus* compared to their male counterparts and enhanced protection against tissue damage accompanied by faster bacterial clearance (Castleman et al., 2018).

The primary mechanism through which the immune system controls *S. aureus* infection is via phagocytosis by specialized cells, such as macrophages. The importance of macrophages in controlling the staphylococcal challenge has been

underscored in different model organisms such as mice (Surewaard et al., 2016, Yajjala et al., 2016, Martin et al., 2011, Kitur et al., 2015) or zebrafish (Prajsnar et al., 2008, Pollitt et al., 2018, Prajsnar et al., 2021). Usually, *S. aureus* enters the human body cavity through breaches in the host's physical defences, e.g. a wound, where it encounters macrophages and neutrophils, comprising the first line of the host defence (Pidwill et al., 2020). Although macrophages are generally effective in phagocytosing and breaking down most of the bacteria, a small fraction manages to escape the destruction, facilitating dissemination within the host (Pollitt et al., 2018). Additionally, *S. aureus* can establish intracellular persistence within macrophages, creating a reservoir for recurrent and chronic infections (Kubica et al., 2008). The bacterium can manipulate and evade macrophage responses, hampering their recruitment and phagocytic functions (Flannagan et al., 2015). Furthermore, differences in macrophage polarization states can also influence host resistance. Whilst most microbes typically induce the M1 polarization state (Galli and Saleh, 2021, Benoit et al., 2008), *S. aureus* has been reported to trigger both the M1 and M2 responses, depending on the infection context (Werz et al., 2018). For example, abscess formation in dermal *S. aureus* has been linked to M1-activated macrophages, while uncontrolled bacterial dissemination has been associated with M2-activated macrophages (Asai et al., 2010). Additionally, the formation of small colony variants, which are atypical *S. aureus* subpopulations often present in chronic infections, in the *in vitro* liver-on-chip model has been associated with M2-activated macrophages, promoting increased cell death and impaired monocyte recruitment (Siwczak et al., 2022).

Given the protective effect of estrogen against gram-positive bacteria (Saia et al., 2015) and the contribution of alpha-hemolysin (Hla), a toxin causing pore formation and inflammation, to the staphylococcal pathogenesis (Kennedy et al., 2010), the apparent sex bias has been thus far predominantly considered in an estrogen-dependent manner (Castleman et al., 2018), with very few finding reported at the molecular level, especially in respect to macrophages (Pokhrel et al., 2020).

1.6.1 *Drosophila* as a model for *Staphylococcus aureus* infection

Invertebrates, such as *D. melanogaster*, are validated as suitable models of human staphylococcal infections if the considered bacterium is pathogenic in a given model and if the previously characterized virulence regulators are required for pathogenicity in that model (García-Lara et al., 2005). The infection dynamics following *S. aureus* injection have been well-characterized, beginning with bacterial proliferation at the injury site, subsequent systemic infection and mortality. Notably, the increase in bacterial load is exponential and proportional to the number of bacteria inoculated, resulting in fly death, irrespective of the bacterial strain. None of the well-characterized virulence regulators essential for full pathogenicity in several mammalian systems play an important role in the *D. melanogaster* model (Needham et al., 2004). However, individuals infected with the *perR* and *pheP* mutants died significantly slower than those injected with wild-type *S. aureus*. Both *perR* and *pheP* mutants were also attenuated in the murine skin abscess model (Horsburgh et al., 2001, Horsburgh et al., 2004), showing that there are virulence components essential for both *D. melanogaster* and mice.

D. melanogaster serves as an excellent model organism for studying staphylococcal phagocytosis because plasmatocytes, independent of humoral responses, are critical to the survival of *S. aureus* infection (Nehme et al., 2011, Defaye et al., 2009). As a result, phagocytic machinery involved in the uptake and elimination of the bacterium, especially at the stage of particle recognition, is relatively well-researched. Several phagocytic receptors, such as dSr-CI (Pearson et al., 1995), Croquemort (Stuart et al., 2005), NimrodC1 (Kurucz et al., 2007), Eater (Kocks et al., 2005), GRP-SC1a (Garver et al., 2006), Draper and integrin βv have been implicated in the engulfment of *S. aureus* by *Drosophila*. Interactions between some of the receptors have also been reasonably well-defined. For instance, both integrin βv and Draper have been shown to act together to mediate the engulfment of *S. aureus* by recognizing the lipoteichoic acid and peptidoglycan (Shiratsuchi et al., 2012, Hashimoto et al., 2009). Additionally, another integrin subunit, αPS3, has been shown to act as a heterodimer with the βv subunit in mediating the phagocytosis of *S. aureus* (Nonaka et al., 2013). However, not all bacterial recognition happens directly, as TEP4 has been implicated in *S. aureus* opsonization, improving the specificity and efficiency of its engulfment (Stroschein-Stevenson et al., 2005). Once activated by the bacterium, nucleation-promoting factors D-SCAR and D-WASP regulate the actin network at the engulfment site, resulting in plasma membrane zippering and engulfment of *S. aureus* (Pearson et al., 2003). Substantial progress has also been made in deciphering processes related to phagosome formation and maturation in the staphylococcal challenge. For instance, another RabGTPase, particularly Rab14, has been shown to facilitate phagosome maturation (Garg and Wu, 2014). Despite *S. aureus* primarily triggering the cellular response, the bacterium has been demonstrated to induce the production

of AMPs and reactive oxygen species through the Toll (Ramond et al., 2021) and unexpectedly IMD pathways (Hori et al., 2018).

Therefore, the well-characterized infection dynamics, virulence regulators, and phagocytic machinery involved in *S. aureus* control, combined with the relatively straightforward and well-defined cellular component of *Drosophila* immunity, establishes a good host-pathogen model for further research. Within this thesis, we will utilise this model to study the sexually dimorphic outcome of the staphylococcal challenge, focusing on phagocytosis as a source of the sex-specific intricacies.

CHAPTER 2: METHODS

2.1 Fly strains

2.1.1 Fly Strains (CHAPTER 3)

All transgenic lines were backcrossed for at least six generations into the wild-type, outbred strain *white*^{Dahomey} (gifted by Partridge Laboratory at the Institute of Healthy Aging). The reporter line, *hmlΔ-GAL4, UAS-GFP* (BDSC: 30140) (Sinenko and Mathey-Prevot, 2004) was used for the determination of basal *S. aureus* susceptibility or flow cytometry and for driving hemocyte-specific expression. Additionally, the *hmlΔ*GS line (gifted by the Jasper Laboratory at the Buck Institute for Research on Aging) (Ayyaz et al., 2015) line was used to drive hemocyte-specific expression of transgenes. The *UAS-tra*^{F} (BDSC:4590) and the *UAS-bax*/*CyO* (gifted by the Wood Laboratory at the Institute for Generation and Repair) reporters were used in hemocyte depletion and hemocyte feminisation experiments.

2.1.2 Fly Strains (CHAPTER 4)

In summary, the lines we employed were established in the laboratory of our collaborators by experimental evolution using larvae from an outbred population of wild-caught *D. melanogaster* females. These larvae were housed with a single female wasp of *Leptopilina boulardi* for 24 hours. Following the egg encapsulation in infected individuals, surviving flies with visible capsules, implicating successful encapsulation and melanization of the parasite, were selected to establish the next generation, which

was maintained with the same protocol, establishing a population resistant to wasp parasitism. The control lines were maintained in the same condition but without the infection step, establishing a control population with no parasitism resistance (Leitão et al., 2020). These lines are continually maintained under parasitic selection and were sent to us periodically by our collaborator. We always used F_1 flies for our experiments to ensure that the effect of parasitism was not lost, which may occur in later generations if the extreme pressure of parasitic selection is lifted.

2.2 Fly Husbandry and Maintenance

Stocks of the *hml*Δ-GS, UAS-*bax*/*CyO*, UAS-*tra*^{F} and *w*^{Dah} were maintained at 18±1°C under a 12-hour light and 12-hour dark cycle on Lewis food vials (mix of 93 g agar (ThermoFisher A10752), 1716 g cornmeal (Flystuff 62-101), 310 g Brewer's yeast (Sigma-Aldrich YBD-1KG), 517g of sucrose (Sigma-Aldrich 84097), 1033 g dextrose (Generon HY-B0389), 200 mL phosphoric acid (Merck 7664-38-2) and propionic acid (Merck 79-09-4) per 17 L of water) (Belmonte, 2023). Every five weeks, the flies were transferred to fresh vials. Before setting up crosses, flies were moved to 25±1°C temperature and allowed to expand. The reporter line, *hml*Δ-GAL4, UAS-GFP, was maintained in a population cage with *ad libitum* access to four SYA food bottles (mix of 90 g agar (ThermoFisher A10752), 300 g sucrose (Sigma-Aldrich 84097), 60 g Brewer's yeast (MP Biomedicals 0290331205), 180 mL Nipagin (Merck 99-76-3), 18 mL propionic acid (Merck 79-09-4) per 6L of water) (Belmonte, 2023). The population cage was stored at 25±1°C under a 12-hour light and 12-hour dark cycle, with the provision of fresh SYA food bottles every 10 days. To obtain a new generation for experimental purposes, a 55 mm standard Petri dish filled with apple juice agar (mix

of 9 g agar (ThermoFisher A10752), 10 g sucrose (Sigma-Aldrich 84097), 100 mL apple juice, 2.1 mL Nipagin (Merck 99-76-3) per 300 mL of distilled water) (Monteith, 2018) and yeast paste was placed in the cage, allowing the adult flies to lay eggs overnight. Eggs were collected from the agar surface by first removing the excess yeast and then washing them off with 1 mL of 1 X phosphate-buffered saline (PBS) (Merck 66062) using a paintbrush to dislodge the eggs. The collected egg suspension was poured into a 50 mL Falcon tube and allowed to settle down at the bottom of the tube. Using a 200 µL pipette with a cut-off tip, 20 µL of the suspension was transferred onto SYA food bottles. stored at 25±1°C under a 12-hour light and 12-hour dark cycle.

2.3 Virgin Collection

The *hml*Δ-GS and *hml*Δ>GFP lines were transferred to fresh Lewis and SYA food bottles, respectively, over a three-day period to ensure a well-distributed collection of virgin flies. Parent flies were removed from the bottles and eggs were allowed to develop at 25 ±1°C under a 12-hour light and 12-hour dark cycle for 10-12 days. Upon eclosion, flies were collected twice daily and visually selected under a microscope based on a larger size, lighter colour, the presence of the meconium and female genitalia. Females meeting these criteria were placed into fresh food vials, with 5-10 individuals per vial, and stored at 18±1°C under a 12-hour light and 12-hour dark cycle until a suitable number of virgin females per experiment for genetic crosses was attained.

2.4 Genetic Manipulation

Following virgin collection, females from the *hml*Δ-GS line were crossed with males from the UAS-*bax* line to drive hemocyte ablation and females from the *hml*Δ>GFP reporter line were crossed with UAS-*tra*^{F} males and with *W*^{Dah} males to induce, respectively, hemocyte feminisation and associated control genotype. Each cross was established with a 3:1 ratio of virgin females to males, with approximately 50 individuals in total. The crosses were housed in small embryo collection cages (Flystuff 59-105) supplied with a 35 mm standard Petri dish filled with apple agar that had yeast paste-like smeared around the edges. The cages were placed at 25 ±1°C under a 12-hour light and 12-hour dark cycle for 24 hours before the Petri dish was changed. To collect eggs, excess yeast was initially removed from the agar surface, and the eggs were dislodged with a fine paintbrush and gently washed off with 1 X PBS (Merck 66062). The resulting egg suspension was poured into a 50 mL Falcon tube, allowing the eggs to settle down at the bottom of the tube. Then, 20 µL of the suspension was transferred onto SYA food bottles which were stored at 25 ±1°C under a 12-hour light and 12-hour dark cycle for 10-12 days.

To induce hemocyte ablation, we sorted two-day-old post-eclosion flies, removing those with the *CyO* balancer based on the presence of curly wings. The remaining *hml*Δ-GS, UAS-*bax* flies were subsequently transferred to SYA food bottles containing RU486 (ThermoFisher H11001) the gene-switch system activator. The RU486+/SYA food was prepared by first obtaining working stock, which involved dissolving RU486 powder in 95% ethanol at a concentration of 43 mg per mL, yielding a working solution of 100 mM. Then, 2 mL of this 100 mM stock was added to 998 mL of liquid SYA,

resulting in a final concentration of 200 µM RU486 per 1L of SYA. For the RU486/SYA control treatment, 2 mL of ethanol alone was added to 998 mL of SYA (Rogers). The RU486+/SYA and RU486-/SYA were dispensed into bottles and vials. RU486, commonly known as Mifepristone, is a synthetic steroid with antiprogesterone effects commercially used for pregnancy termination. Consequently, the RU486-infused SYA food was prepared under a fume hood. It was clearly labelled during the drying process and then in storage, and its handling always involved the use of gloves and extreme care.

2.6 Bacterial Infection

We utilised a gram-positive bacterium, *Staphylococcus aureus* (PIG1 strain) (Liu et al., 2005), stored as 200µL frozen aliquots at -70°C. To prepare a liquid bacterial culture, we thawed the aliquot and resuspended it in 50 mL of fresh Lura Bertani (LB) broth. The culture was allowed to grow overnight at 37°C on a shaker at 150 rpm. After reaching saturation, we centrifuged the cultures at 1600 rpm at 4°C for 20 min and resuspended them in 10 mL of 1 x PBS (Merck 66062) to an optical density (OD) of 0.25 (600 nm wavelength). We injected four days post-eclosion flies with 23 nL of the bacterial suspension into each fly abdomen using Nanoject III (Drummond), corresponding to a dose of about 1000 viable bacteria per fly. Injection of the same volume of sterile 1 x PBS was used as a control treatment for t the injection procedure. For injection, we anaesthetized flies with CO_2 for less than 30 min and then observed shortly after to confirm recovery from the injection.

2.7 Monitoring Host Survival

We monitored survival in groups of approximately 50 males and 50 females kept together in 1200 mL plastic boxes with access to two SYA vials on the sides, monitoring survival every 2 to 4 hours and removing dead individuals at each time point with a brush. The survival of individuals was analysed in RStudio (version: 2023.06.0+421) using the package “survival” (Therneau, 2023). Differences in host survival were assessed using a Proportional Hazards Cox Model (coxph) or Mixed Effects Cox Model (coxme), fitting Sex and Genotype (hemocyte feminization, selection lines) or Diet (hemocyte ablation) and their interactions as fixed effects and “Day of Injection” as a random effect. Each genotype was housed in a separate box. Hazard ratios were extracted from these models. The models were specified as follows:

Survival ~ Sex + Genotype (or Diet) + Sex * Genotype (or Diet) + (1|Replicate)

2.8 Bacterial Load Quantification

To monitor bacterial proliferation, each fly was individually homogenized in 250 µL of sterile 1 x PBS (Merck 66062) with two aseptic glass beads in a TissueLyser II (Qiagen) at 30 Hz for 1 minute. Depending on the time the homogenate was sampled at, it was then diluted 1:4, 1:16, 1:64, 1:256 and 1:1024 with 1 x PBS. 4 µL of each dilution was plated on LB agar plates, including undiluted, in duplicate (8- and 12-hour time points) or triplicate (0- and 8-hour time points). Plates were stored at 37°C overnight and then transferred to room temperature and allowed to continue growing until colonies were visible. Images of each plate were captured with iPhone 13 (Apple)

and uploaded into the “CountDrops” software (not published but generously shared by David Duneau and Jean-Baptiste Ferdy, University of Toulouse - Paul Sabatier III) where colonies were counted manually to estimate the number of viable bacterial colonies per fly. Bacterial load was then calculated in RStudio (version: 2023.06.0+421) using the “CFUfit” R package. Differences in bacterial load were compared with a Wilcoxon or Pairwise Wilcoxon test for multiple comparisons. Whenever possible, these bacterial loads were modelled with a weighted mixed-effect generalised linear model using the “spaMM” package (Rousset and Ferdy, 2014) to obtain differences with the individual fly as a mixed effect.

2.9 Flow Cytometry Sample Preparation and Gating Strategy

2.9.1 *S. aureus* Bioparticle Injection

We administered 23 nL (1/mg) of pHrodo bioparticles of S. aureus (ThermoFisher A10010) into the abdominal area of four-day-old adult *Drosophila* individuals. Subsequently, we sorted them by sex, placing 20 flies per vial, and incubated at 25°C for two hours.

2.9.2 Sample Preparation

Pools of 20 four-day-old individuals were anaesthetized with CO_2 and transferred into 1.5 mL microcentrifuge tubes filled with 500 µL of ice-cold 1 x PBS. While keeping the samples on ice, flies were crushed with a pestle. The homogenate was then transferred through a 40 µM filter placed on top of a 50 mL Falcon tube. Any sample residuals on the tube and filter were washed off with an additional 500 µL of ice-cold 1 x PBS. The filtered suspension was transferred to a clean 1.5 mL microcentrifuge

tube and spun at 4°C, 1200 g for 3 minutes. The samples underwent a triple wash procedure, involving the removal of the supernatant, the addition of ice-cold 1 x PBS (Merck 66062) and subsequent centrifugation at 4°C, 1200 g for 3 minutes. The samples were poured through a 50 µm filter to clean 5 mL round-bottom flow cytometry tubes and placed on ice.

2.9.3 Gating Strategy

The samples were run on BD LSRFortessa and obtained data was analysed with FCS Express (version 6.06.0042). First, data was gated using the FSC-A and SSC-A to exclude doublets and debris from the analysis, eliminating cells with low values for both parameters. Then we gated for single cells using FSC-W against FSC-A. To identify green fluorescently labelled hemocytes and red fluorescently labelled *S. aureus* bioparticles, we set the channels to FITC and PE-Texas Red, allowing us to single out cells with overlapping intensity as phagocytic hemocytes. Sex-specific rates of phagocytosis were analysed using a binomial generalized linear model in RStudio (version: 2023.06.0+421) with the “glmer” package, with sex fitted as a fixed effect and the date of the experiment as a random effect.

CHAPTER 3: *Drosophila* phagocytes play a key role in sex-specific susceptibility to *S. aureus* infection

3.1 INTRODUCTION

Staphylococcus aureus is a gram-positive and opportunistic bacterium which commonly colonizes various body parts in approximately 20% to 40% of the general population (Wertheim et al., 2005b), with a primary presence on nasal mucosa. While most staphylococcal infections remain benign, the pathogen may infiltrate deeper tissues, leading to potentially fatal conditions, such as sepsis or endocarditis, making it one of the leading causes of life-threatening bloodstream infections (Kwiecinski and Horswill, 2020). Treatment typically involves antibiotics, whose overuse has led to the emergence of resistant strains, particularly the methicillin-resistant form of *S. aureus* (MRSA), which significantly contributes to high mortality in staphylococcal infections (Yaw et al., 2014). In addition to the challenges posed by antibiotic resistance, sex is also a notable risk factor. Generally, men are more susceptible to various forms of *S. aureus* infection than females and are more frequently carriers of the bacterium (Humphreys et al., 2015). In addition to the clinical observations, the sex-biased susceptibility has been consistently observed in *ex vivo* and *in vivo* laboratory studies (Castleman et al., 2018). Although *S. aureus* is a complex pathogen with several well-studied virulence factors, professional phagocytes have emerged as particularly important disease mediators in different species (Prajsnar et al., 2008, Surewaard et al., 2016). Whilst there is a substantial body of research exploring emerging antibiotic

resistance, the underlying mechanisms of sex-specific susceptibility to the staphylococcal challenge remain poorly explored.

With the observed male-biased susceptibility to *S. aureus* infection and the pivotal role of phagocytes in managing the staphylococcal challenge, we aimed to explore whether the observed differences in disease vulnerability might be linked to sex-specific variations in phagocyte function. Several lines of evidence support such macrophage-focused investigation, such as a study of rodent-derived peritoneal macrophages which have shown enhanced pathogen detection, phagocytosis, and bactericidal activity in female cells (Scotland et al., 2011). Additionally, recombinant chicken-derived female macrophages appear to display greater readiness for infection evidenced by the upregulation of specific IFN-responsive genes at basal conditions (Garcia-Morales et al., 2015). In both the Coxsackievirus B3 and *S. agalactiae* mice infection models, females favoured the M2 activation state, known for promoting tissue repair, which potentially limits the inflammatory damage, a characteristic feature of sepsis (Li et al., 2009, Deny et al., 2022). The emerging evidence for the sex-specific functionality of macrophages in response to various pathogens further supports our rationale for examining macrophages as a potential source of male-biased susceptibility to *S. aureus* challenge.

While there is evidence supporting the sex-specific functionality of mammalian macrophages, it is typically considered through a traditional framework of sex determination, which restricts the role of the chromosome complement to gonadal development and associated hormonal secretions, overlooking the inherent inequality in sex chromosomes present in each cell (Arnold, 2012). The sexual identity of

macrophages at a cellular level may be regulated by genomic sex differences in addition to hormonal signals, potentially playing a role in the dimorphic regulation of pathogenesis and disease outcomes. Such a notion finds partial support in studies on the four core genotypes mice, which enable the evaluation of the sex chromosome effect independent of the gonadal phenotype, revealing that sex chromosome complement contributes to the severity of Coxsackievirus B3 infection (Robinson et al., 2011). However, such manipulation only allows for the assessment of phenotypic differences and does not provide a means to directly manipulate sex-specific regulation at the cellular level.

In this study, we aimed to investigate the role of macrophages in sex-specific susceptibility to *S. aureus* infection, employing *Drosophila melanogaster* as our model organism. *Drosophila* shares many evolutionary conserved features important for immune function with mammals, including disease gene homologs, signalling pathways and notably, the functionality of its phagocytes (Younes et al., 2020). Its immune system relies entirely on innate responses, comprising of humoral and cellular components. The cellular arm is primarily mediated by plasmatocytes, which are macrophage-like cells. These cells are important for development and homeostasis, and are also important for controlling many bacterial infections, including *S. aureus*, acting in parallel with the humoral response (Charroux and Royet, 2009). Sex determination in *Drosophila* is regulated through a well-studied pathway of regulatory genes (Baker and Ridge, 1980), uniquely allowing us to induce female-specific transcriptional regulation of male cells.

In the previous work of our research team, a male-biased susceptibility to *S. aureus* infection was demonstrated, and the single-cell RNA sequencing of adult hemocytes from males and females aided in the identification of sex-specific transcription differences in various genes, including those related to phagocytic pathways, which could contribute to the observed infection dimorphism. In this study, I aimed to further investigate these findings, by replicating the susceptibility pattern at a high resolution, focusing on bacterial proliferation and phagocytosis, and exploring whether the sex-specific outcomes of *S. aureus* infection could be attributed to the sex identity of hemocytes themselves, which is influenced by sex-specific genetic regulation.

3.2 RESULTS

3.3.1 Females demonstrate significantly greater resistance to *S. aureus* infection along with more efficient control of bacterial proliferation

Although *Drosophila melanogaster* is a frequently utilised model host for studying *S. aureus* infection (Lee et al., 2018b, Needham et al., 2004), there is a noticeable scarcity of research comparing the responses of males and females to the challenge posed by this pathogen. In a study using an outbred population derived from the Global Diversity Lines, Duneau, Kondolf *et al*. established that males are significantly more susceptible to *S. aureus* infection than females (Duneau et al., 2017b). However, it is essential to recognize that the direction of sex-biased survival in bacterial infections can be influenced by environmental factors, such as mating or diet, and can vary depending on a host genotype (Belmonte, 2023). Therefore, we opted to confirm the previously observed female-specific survival advantage to *S. aureus* challenge under the conditions of our laboratory, in the genotype, *hmlΔ-GAL4>UAS-GFP*. In this

transgenic line, the *hemolectin* gene promoter drives a hemocyte-specific expression of GFP, whose fluorescent properties we utilise in subsequent stages of our study.

We monitored the survival of four days post-eclosion individuals who were injected with a lethal dose of approximately 1000 viable *S. aureus* cells, resulting in the death of all individuals within 72 hours post-injection. It allowed us to analyse early control of infection but also detect any potential differences in susceptibility. We observed a significant difference in survival between males and females **(Cox-me: Sex: df = 2, $\chi 2 = 51.23$, $P = 0.00002$)** (Fig. 1A). Additionally, we further assessed this difference by extracting hazard ratios from the survival model, which indicated that males are 1.53 times more likely to succumb to the staphylococcal challenge compared to females (Fig. 1B). To explore whether males and females differ in their ability to control *S. aureus* proliferation, we quantified the bacterial load in each individual at specific time points (4, 8, and 12 hours post-injection), while ensuring that both sexes received the same initial pathogen dose. In general, we observed a significant difference in proliferation between males and females **(Wilcoxon test: W = 1358, $P = 0.032$)**. At 4 hours post-injection, there was no detectable increase in bacterial growth, possibly due to it being too early for the pathogen to enter the exponential growth phase. However, a pronounced disparity becomes evident at 8 hours post-injection, where *S. aureus* proliferated significantly more in males than females **(Wilcoxon test: W = 148.5, $P = 0.011$)**, suggesting that females are more effective in limiting the staphylococcal growth during the initial response. At 12 hours post-injection, the sex-specific difference in pathogen loads diminished, possibly due to host-specific increased variability in bacterial growth (Fig. 1C). Therefore, there is a correlation between differences in the survival and bacterial proliferation, where females

demonstrate a reduced likelihood of mortality and maintain a lower pathogen load during the initial stages of infection, but as time progresses, bacterial concentrations become comparable between the two sexes, eventually resulting in the mortality of all individuals (Fig. 1A, 1B, 1C).

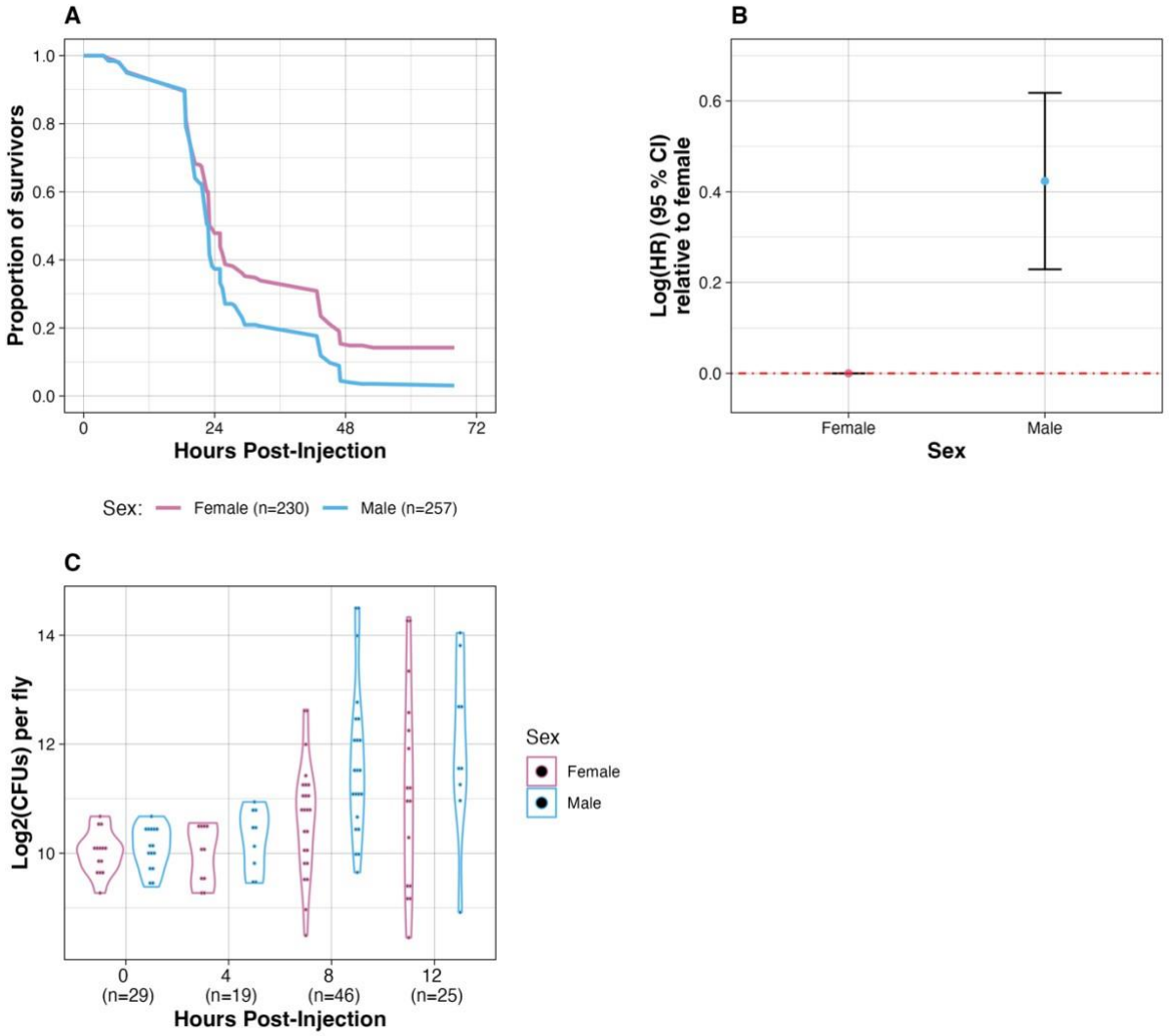

Figure 1. Adult male Drosophila exhibit increased susceptibility to *S. aureus* infection and a diminished ability to control staphylococcal proliferation. A) Survival curves for both males and females following *S. aureus* infection based on pooled survival data from six independent experiment replicates. The replicates were

accounted for in Cox mixed-effects modelling, revealing that females are more susceptible than males **(Cox-me: df = 2, χ2 = 51.23, *P* =0.00002)**. The sample size of each sex is provided in the brackets. B) A graphical representation of the estimated Log(Hazard Ratio) extracted from the Cox mixed-effects model for each sex. A hazard greater than 1 indicates increased susceptibility of males compared to females (red horizontal line) to *S. aureus* infection. C) Bacterial load, presented as Log_2 of *S. aureus* colony forming units (CFUs) in individual flies at 0, 4, 8 and 12 hours post-injection. The number of individuals is indicated below each time point. Overall, *S. aureus* grows more in males than in females **(Wilcoxon test: W = 1358, *P* = 0.032),** with the difference being only detectable at 8 hours post-infection **(Wilcoxon test: W = 148.5, *P* = 0.011)**.

2.3.2 Hemocytes play a crucial role in limiting *S. aureus* growth

The importance of *D. melanogaster* hemocytes in effectively controlling and eliminating *S. aureus* has been documented, often by impairing phagocytosis through methods such as the saturation of phagocytic machinery with latex beads (Nehme et al., 2011) or genetic removal of hemocytes (Defaye et al., 2009). While both of these techniques have consistently resulted in increased susceptibility to staphylococcal challenge in larvae and adults, no studies have directly compared the responses of males and females. To assess the degree of dependence on hemocytes in responding to *S. aureus* infection in both sexes, we employed a genetic ablation strategy. Similar to the approach used by Defaye, Evans *et al*., we induced the hemocyte-specific expression of a pro-apoptotic gene *bax*, known for its role in the efficient activation of *Drosophila* apoptotic pathways (Gaumer et al., 2000). To prevent the adverse effects

of hemocyte depletion during embryonic and postembryonic development (Stephenson et al., 2022), we employed a modified GAL4/UAS system. In this system, GAL4 is coupled with the progesterone-receptor protein called “GeneSwitch” (GS), enabling both spatial and temporal regulation of its expression by administering the synthetic progesterone analogue mifepristone (RU486) (Osterwalder et al., 2001). Therefore, we induced the hemocyte-specific expression of *bax* using *hml*>GS driver by feeding adults the activating drug at 48 hours post-eclosion. This activation time was chosen as during the first two days of adulthood, large individual adipose cells, which are dissociated from the larval fat body during metamorphosis (Nelliot et al., 2006) are removed by hemocytes (Aguila et al., 2007). Henceforth, we will designate the individuals receiving RU486 through dietary intake as “hemocyte-depleted”, while those not exposed to the drug will be referred to as the “control”.

To determine the role of hemocytes in sex-specific outcomes to *S. aureus*, we injected approximately 1000 *S. aureus* cells into each individual and closely monitored their survival. In general, hemocyte-depleted flies displayed a significantly heightened susceptibility, succumbing to infection within 46 hours post-injection, in stark contrast to non-depleted controls who managed to resist the infection for up to 72 hours. Additionally, we found a significant interaction between hemocyte presence or absence and sexually dimorphic susceptibility to *S. aureus* in our model **(Cox-me: df = 4, $\chi 2 = 163.88$, $P = 2.2 \times 10^{-16}$** and **$P = 0.021$)**, underscoring the crucial role of hemocytes in resisting staphylococcal challenge (Fig. 3A). Without hemocytes, both sexes exhibited an increase in susceptibility. However, the sexually dimorphic survival, which was initially observed in non-depleted controls, was notably eliminated in hemocyte-depleted individuals **(Cox-me: df = 2, $\chi 2 = 17.10$, $P = 0.065$)**. Given the

lack of significant difference between hemocyte-depleted males and females, we compared the effect of hemocyte depletion within each sex separately, revealing that hemocyte-depleted males were 2.12 times more likely to succumb to infection compared to control males. In parallel, hemocyte-depleted females were 3.51 times more likely to die of the staphylococcal challenge than control females. Therefore, the magnitude of the increase in hazard ratio is proportionally greater in hemocyte-depleted females compared to hemocyte-depleted males, suggesting that while both sexes rely on hemocytes in resisting *S. aureus* challenge, females may exhibit more efficient hemocyte-regulated control of the infection.

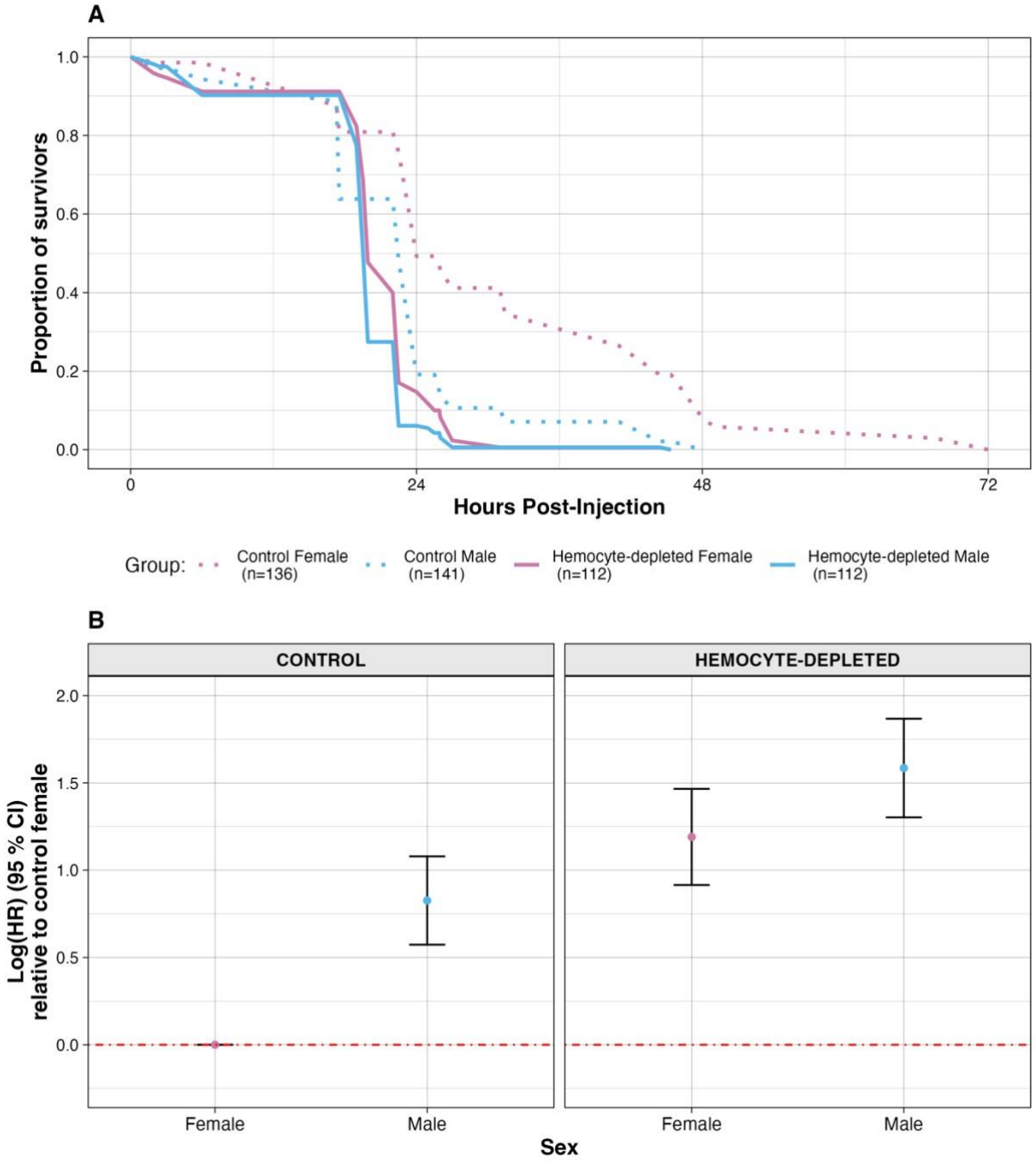

Figure 2. Hemocytes are significant contributors to *S. aureus* survival in both sexes, but their role is more pronounced in enhancing female resistance. A) Survival curves for both sexes in hemocyte-depleted and control groups following *S. aureus* infection based on pooled survival data from three separate experiment repeats. In the Cox mixed-effects modelling, we accounted for the random effect of replication and the interaction between hemocyte depletion and the sex of an

individual, revealing that hemocyte elimination leads to a dramatic increase in *S. aureus* susceptibility and eliminates normally observed differences in survival between the sexes. and that the effect of hemocyte-specific ablation and females are more susceptible than males and that there was an interaction between hemocyte ablation and sex **(Cox-me: df = 4, $\chi 2 = 163.88$, $P = 2.2 \times 10^{-16}$ and $P = 0.021$).**

B) A graphical representation of the estimated Log(Hazard Ratio) extracted from the Cox mixed-effect model. A hazard greater than 1 indicates increased susceptibility compared to the control females (red horizontal line).

To gain deeper insights into the effect of *Drosophila* phagocyte elimination on the dynamics of infection, particularly focusing on the varying impact of hemocyte depletion on the staphylococcal growth in males and females, we injected flies with *S. aureus*. Then, we carefully measured the pathogen load at 4, 8 and 12 hours post-injection, ensuring that both sexes were injected with identical volumes of the bacterial suspension. In general, hemocyte-depleted individuals exhibited significantly higher bacterial proliferation compared to the control genotype **(Wilcox test: $W = 2362.5$, $P = 2.2 \times 10^{-16}$)**. In comparison to the starting load, the ablation of hemocytes led to a substantial increase in pathogen load as early as 4 hours post-infection **(Pairwise Wilcox test: $P = 3.6 \times 10^{-9}$)**. In contrast, for the control genotype, the same timepoint was too early in the bacterial growth trajectory to detect any significant increase, suggesting that hemocyte-depleted individuals lose control over *S. aureus* proliferation in the initial stage of infection. Such observation highlights the pivotal role of hemocytes in limiting staphylococcal dissemination, especially during the early phase of bacterial challenge. Given their ability to synthesize clotting factors, such observation likely implicates hemocytes in wound closure. Furthermore,

bacterial proliferation in hemocyte-depleted individuals was exceptionally rapid, with pathogen load significantly increasing between 4 and 8 hours and between 8 and 12 hours **(Pairwise Wilcox test: *P* = 7.5 x 10^{-13}** and **P = 2.5 x 10^{-5} respectively)**, whereas the only substantial increase in the control individuals was observed between 0 and 8 hours, mirroring the proliferation pattern observed in the *hml*Δ>GFP reporter line. These results suggest that staphylococcal growth is progressing rapidly in the absence of hemocytes. Surprisingly, despite the elimination of dimorphism in the survival, we still observed sex-specific differences in the pathogen load of hemocyte-depleted individuals, with *S. aureus* proliferating significantly more in males at 8 and 12 hours **(Pairwise Wilcox test: *P* = 6.2 x 10^{-06}** and ***P* = 2.4 x 10^{-6} respectively)**. Considering that we had eliminated the primary mediators of cellular immunity, this suggests that the other arm of the *Drosophila* innate system, the humoral response, may in part drive this sex difference in early proliferation, independently of hemocytes. It is conceivable that such systemic response is more upregulated in females, as indicated by their better control of the early pathogen load than males. This does not translate to a dimorphism in susceptibility rates, indicating that the loss of hemocytes transcends this small effect in facilitating the response against this particular bacterium.

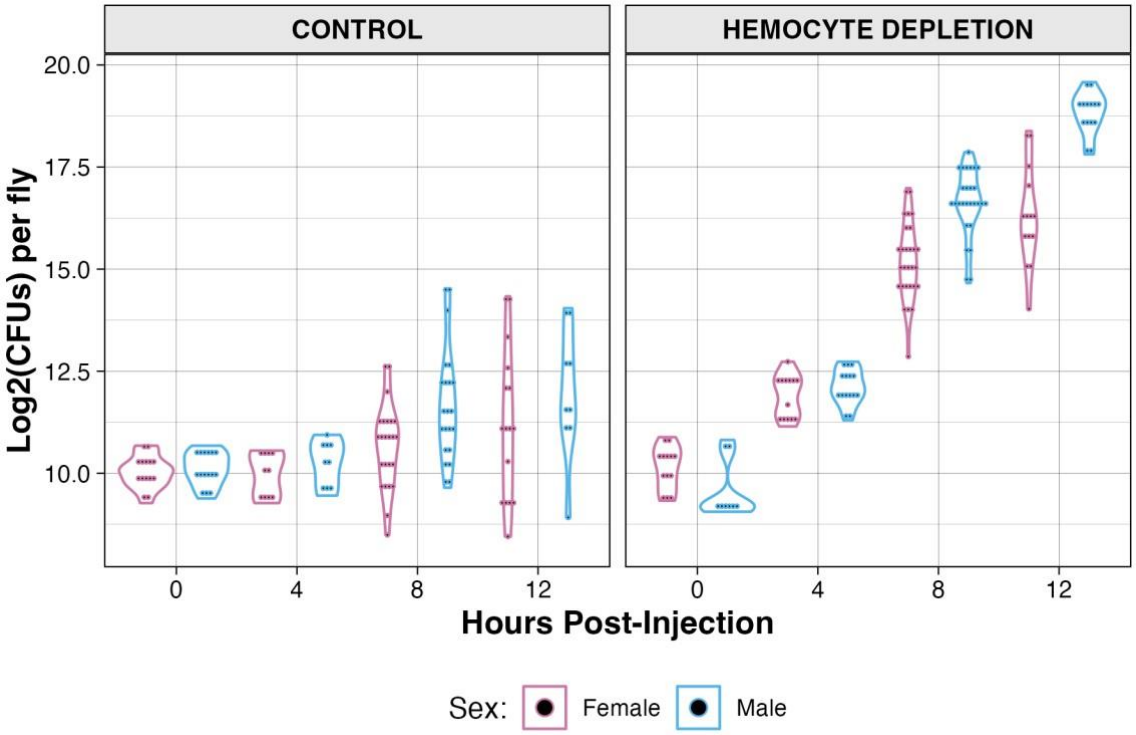

Figure 3. Hemocyte depletion results in a significant increase in *S. aureus* proliferation, highlighting the role of hemocytes in controlling staphylococcal growth, but does not eliminate sex-specific differences in the pathogen load. Bacterial growth, presented as Log_2 of *S. aureus* colony forming units (CFUs) in hemocyte-depleted and control flies at 0, 4, 8 and 12 hours post-injection. Overall, *S. aureus* grows more in hemocyte-depleted individuals compared to controls **(Wilcoxon test: W = 2362.5, *P* = 2.2 x 10^{-16})**, with pathogen load being significantly higher in hemocyte-depleted individuals at every timepoint **(Pairwise Wilcoxon test: *P* = 1.6 x 10^{-8}, *P* = 5.0 x 10^{-16}** and ***P* = 1.7 x 10^{-9} for 4, 8** and **12 hours post-injection respectively)**. Compared to the injected dose, hemocyte-depleted individuals show significant growth as early as 4 hours post-injection, a pattern not otherwise observed in the control genotypes, implying the loss of *S. aureus* control early in the challenge **(Pairwise Wilcoxon test: *P* = 3.6 x 10^{-9})**. There are sex-specific differences in

bacterial proliferation in hemocyte-depleted individuals, with males demonstrating significantly less control over the staphylococcal growth than females at 8 and 12 hours post-infection **(Pairwise Wilcoxon test: $P = 6.2 \times 10^{-6}$ and $P = 2.4 \times 10^{-6}$ respectively)**. Additionally, we modelled the effects of sex and hemocyte depletion using a weighted mixed-effect generalized linear model, revealing significance for both, but notably stronger for the effect of hemocyte elimination (**fitme ~ hemocyte elimination: df = 2, $\chi 2 = 46.12507$, $P = 9.6398 \times 10^{-11}$** and **fitme~ sex: df = 2, $\chi 2 = 12.37728$ P = 0.002)**, suggesting that genetic ablation of *Drosophila* phagocytes overrides the effect of sex. Data was collected over four replicates, with the following number of observations in the **control genotype: n=28, 22, 48, 24** for **0, 4, 8 and 12 hours post-injection** and in the **hemocyte-ablation genotype: n=21, 28, 55, 28** for **0, 4, 8 and 12 hours post-injection**.

2.3.3 Females have a greater proportion of hemocytes engaged in active phagocytosis of *S. aureus* than males

While we have confirmed the male-biased susceptibility to *S. aureus* and the pivotal role of hemocytes in resisting this bacterium, we have yet to explore potential sex-specific differences in the phagocytic functionality of these immune cells. To address this, we implemented a modified protocol (Belmonte, 2023), built on a previously published hemocyte isolation procedure (Krejčová et al., 2019), and examined adult hemocytes using flow cytometry. We injected the previously mentioned *hmlΔ-GAL4>UAS-GFP* flies with *S. aureus* bioparticles. Within two hours post-injection, we quantified the proportion of hemocytes that had successfully engulfed these particles. We identified these phagocytic hemocytes based on the

staphylococcal bioparticles which become fluorescently red upon their uptake by fluorescently green hemocytes.

Overall, there was a marked difference in the proportion of actively phagocytic cells between the sexes, with females having a greater proportion of cells involved in active phagocytosis than males **(glmer: df = 1, χ2 = 346.257, *P* = 2.2 x 10^{-16})**, suggesting that during the initial phase of the infection, female hemocytes are phagocytosing more than male hemocytes, providing females with a survival advantage.

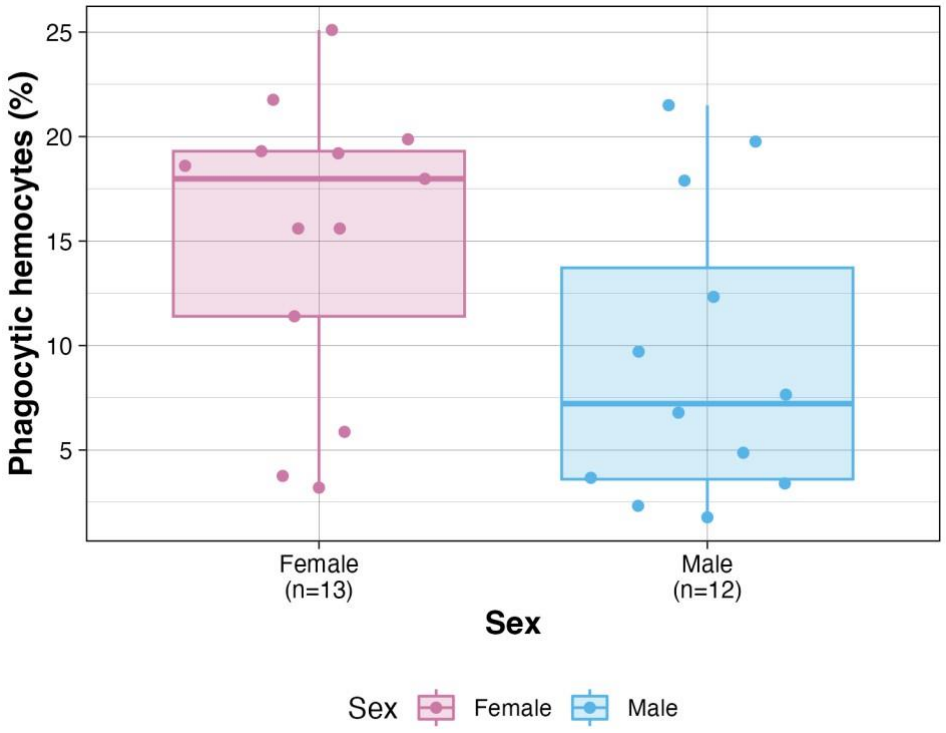

Figure 4. Females demonstrate a significantly greater proportion of actively phagocytosing hemocytes compared to males. Both sexes of *hmlΔ-GAL4>UASGFP* reporter lines were injected with S. aureus particles, revealing that females have a higher percentage of phagocytic hemocytes compared to males **(glmer: df = 1, χ2 = 346.257, *P* = 2.2 x 10^{-16}).** Data was pooled from three replicates with a replicate accounted as a random effect. The number in the bracket denotes the percentage of cells from 20 individuals.

Considering that the hemocyte ablation significantly increased susceptibility to *S. aureus* infection in both sexes and eliminated the dimorphism in survival, we concluded that hemocytes could play a key pivotal role in the differential vulnerability between males and females to staphylococcal challenge. Additionally, a proportionally greater increase in a sex-matched hazard ratio for females, the significant interaction between hemocyte depletion and sex together with a greater proportion of phagocytic cells in females, prompted us to examine whether such contrasting susceptibility is attributed to the impact of sex-specific inductive signalling on *Drosophila* phagocytes or rather to the sex of hemocytes itself.

2.3.4 Hemocyte-specific expression of the feminizing gene, *transformer*, results in a significant enhancement of male survival to *S. aureus*, providing them with greater control over bacterial proliferation.

Sex determination in *Drosophila* is controlled in a cell-autonomous manner, with each cell independently determining its sexual identity through a well-studied splicing and transcriptional pathway. In chromosomally female cells, the presence of two X chromosomes induces transcription of the master sex-determination gene *Sex-lethal.* This results in female-specific splicing of its target, the *transformer* gene, itself a splicing factor. As a result, a functional Tra^{F} protein is produced, which controls alternative splicing of the *doublesex* gene, generating Dsx^{F}. In chromosomal males, Sex-lethal and Tra proteins are non-functional, and *doublesex* is spliced in a default manner, resulting in the expression of male-specific Dsx^{M} (Manolakou et al., 2006). Both Dsx^{F} and Dsx^{M} transcriptionally regulate the expression of many genes in

somatic tissues (Robinett et al., 2010). Therefore, such cellular autonomy of sex determination can be leveraged through *transformer*-specific genetic manipulation of the pathway, enabling the transcriptional transformation of male cells into female cells, or vice versa, which generates tissue-specific sexually mosaic individuals (Regan et al., 2022, Regan et al., 2016). Herein, we aimed to investigate the contribution of the sexual identity of hemocytes to *S. aureus* dimorphic survival. Using the GAL4/UAS system, we modulated *transformer* expression by crossing the *hml*-GAL4 driver with a UAS-regulated expression line driving expression of the active female form of transformer: UAS-*tra*^{Female}, which results in TraF transcript expression within hemocytes. Since UAS-*tra*^{F} has been backcrossed into the *w*^{Dahomey} strain, we crossed *hml*>GFP flies with W^{Dah} flies to obtain a control genotype with a homogenized genetic background. For simplification purposes, we will refer to *w*^{Dah}; *hml*>GFP, UAS-*tra*^{F} individuals as hemocyte-feminized and to *w*^{Dah}; *hml*>GFP as controls.

To determine whether the presence of functional Transformer^{F} protein in male hemocytes would change their response to *S. aureus* infection, we administered approximately 1000 *S. aureus* cells to each individual and closely monitored their survival. Strikingly, individuals with hemocyte-specific feminization demonstrated significantly enhanced resistance to the staphylococcal challenge, extending their survival to as long as 91 hours post-injection. In contrast, all individuals in the control group succumbed to the infection within 70 hours. Remarkably, the survival of hemocyte-feminized males exceeded that of control males, reaching a level comparable to the control females **(Cox-me: df = 4, χ2 = 155.22, *P* = 0. 41)**. When examining the hazard ratios, the beneficial effect of expressing the female form of the

transformer becomes even more evident, with males being 0.92 times as likely to succumb to infection as the control females. In addition to hemocyte-specific feminization in males, we also induced the expression of *tra*^{F} in females, who naturally produce functional Transformer, which potentially amplified its levels. Such hypothesis is supported by our data, as hemocyte-feminized females displayed significantly greater resistance to *S. aureus* challenge compared to control females **(Cox-me: df = 4, χ2 = 155.22, *P* = 0.000012)**, indeed suggesting a dose-dependent effect of the *transformer* (Fig. 6A) . To determine which sex gained a greater advantage from the additional expression of the feminizing gene, we extracted sex-matched hazard ratios from the model. These ratios revealed a relatively comparable benefit, with hemocyte-feminised males being 0.43 times less likely to succumb to the infection compared to control males and hemocyte-feminized females being 0.39 less likely to die from *S. aureus* challenge in comparison to their control counterparts. In our model, we also considered the interaction effect of sex and transcriptional feminization of hemocytes on survival, revealing no significant relationship between these two factors. This indicates that while both sex and feminization significantly influence survival, their effects are independent of each other. Therefore, the effect of hemocyte feminization seems to be consistent across both sexes, further supporting the concept of the dose-dependent effect of Transformer^{F}.

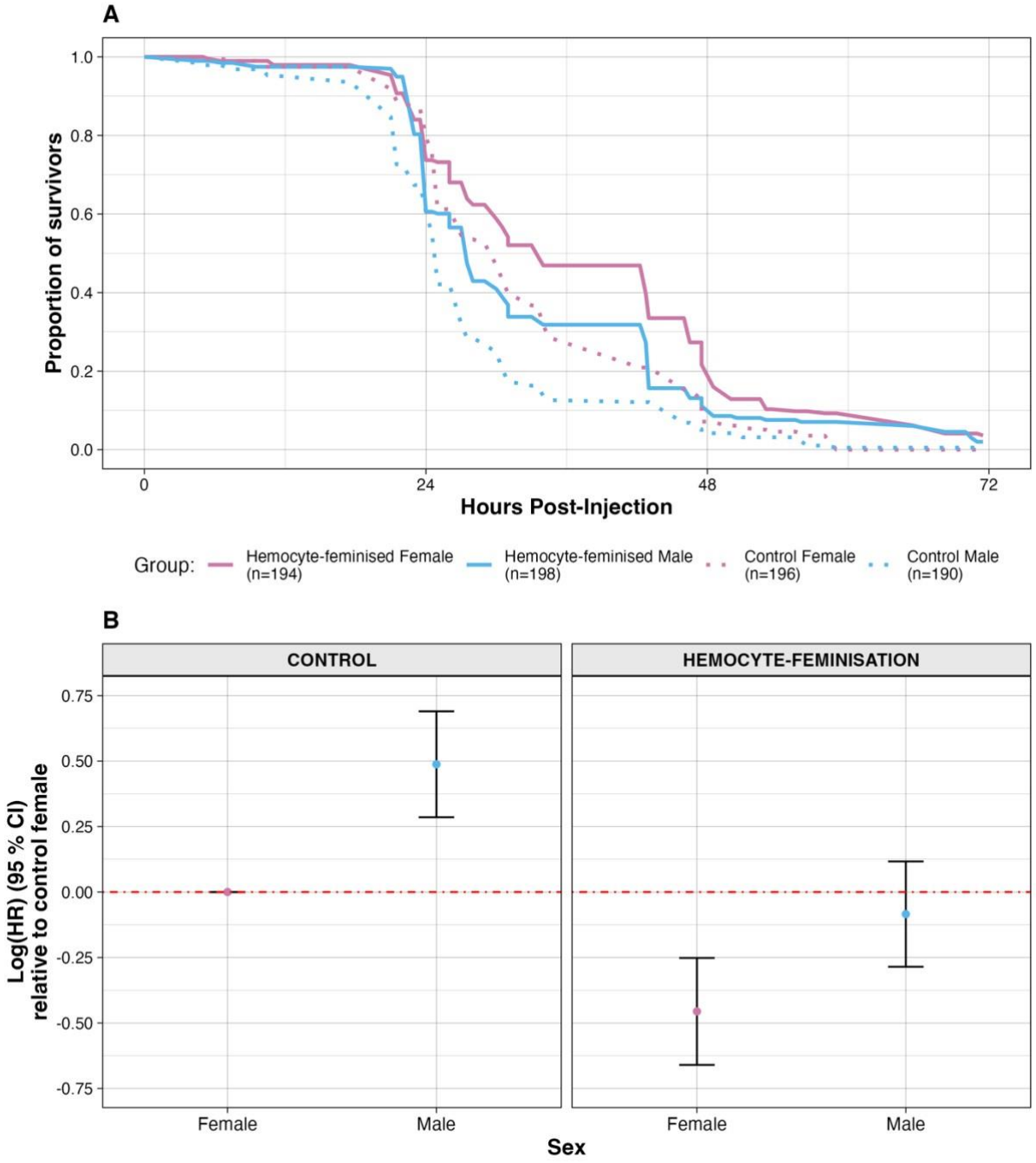

Figure 5. Hemocyte-specific expression of a female form of *transformer* significantly increases survival to *S. aureus* infection in both sexes, with hemocyte-feminized males achieving survival rates comparable to control females. A) Survival curves for both sexes in hemocyte-feminized and control groups following *S. aureus* infection pooled from four independent repeats across different dates. In our Cox mixed effect modelling, the day of injection and the interaction

between sex and transcriptional feminization were accounted for, revealing that hemocyte-feminized individuals show significantly improved survival to *S. aureus* challenge **(Cox-me: df = 4, χ2 = 155.22, *P* = 0.000012)**, yet the interaction between the sex of an individual and expression of *tra*^{F} remains insignificant. The sample size for each group is denoted in the brackets. B) A graphical representation of the estimated Log(Hazard Ratio) extracted from the Cox mixed-effects model for each group. A hazard greater than 1 indicates increased susceptibility of males compared to control females to *S. aureus* infection.

To assess the relationship between survival and the progression of infection in hemocyte-feminized individuals, we again administered *S. aureus* and sampled at 8 and 12 hours post-injection, ensuring equal bacterial dosages. Overall, hemocyte-feminised individuals demonstrated significantly lower staphylococcal growth in comparison to non-feminized controls **(Wilcoxon test: W = 3227.5, *P* = 0.002)**, suggesting that the expression of the female-specific form of *transformer* allows for better control of the pathogen proliferation. Remarkably, we observed a parallel trend in both our survival and bacterial load data. Hemocyte-feminized males matched the survival rates of control females, and this pattern extended to our proliferation analysis, where there was no difference in microbial growth between hemocyte-feminized males and control females at either 8 or 12 hours post-injection **(Pairwise Wilcoxon test: *P* = 0.16541** and ***P* = 0.875 respectively)**. This implies the beneficial effect of the female-specific form of transformer. Remarkably, hemocyte feminization resulted in the elimination of the typical sex-specific differences in *S. aureus* growth at both 8 and 12 hours post-injection. Hence, while both sexes with feminized exhibited significantly improved control of *S. aureus* proliferation, the

expression of the female-specific transcription factor in male hemocytes enabled males to bridge the previously observed gap in proliferation between the sexes. At 12 hours post-infection, bacterial load hemocyte-specific individuals showed a large spread between data points, suggesting that feminization of the hemocytes amplified the usually present increased variation between hosts.

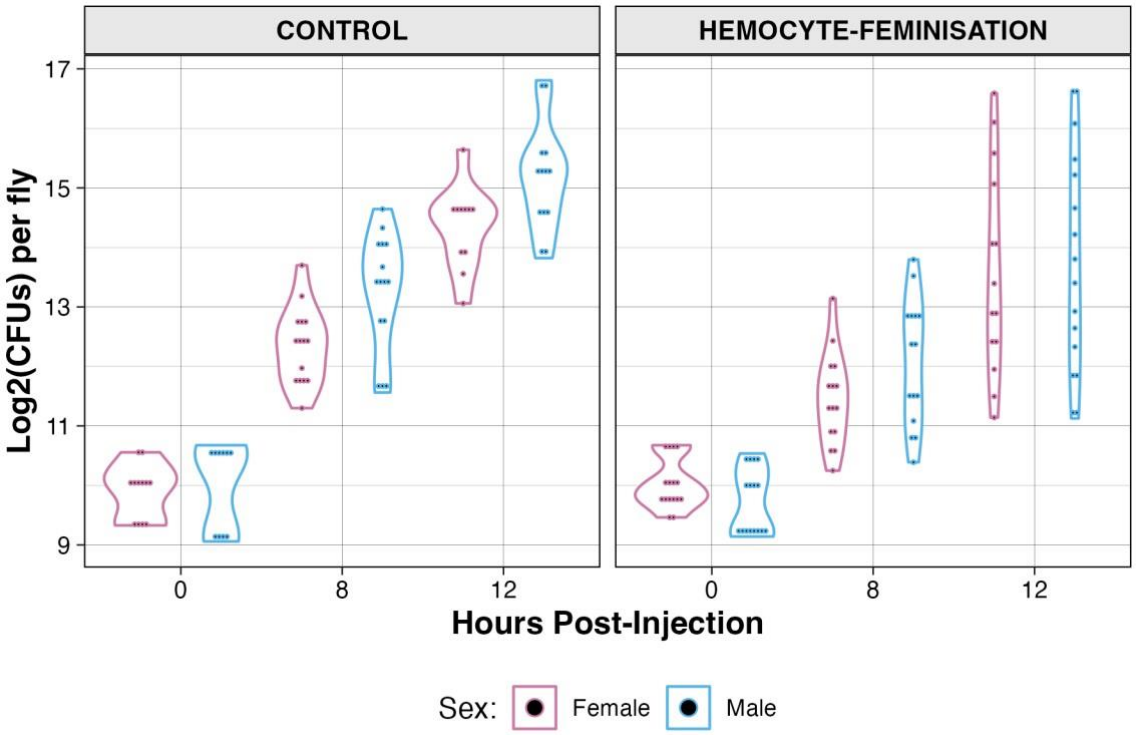

Figure 6. Hemocyte-feminized individuals demonstrated significantly stronger control of *S. aureus* proliferation, with hemocyte-feminized males displaying bacterial growth levels equivalent to those of control females. Bacterial growth presented as Log_2 of *S. aureus* colony forming units (CFUs) per fly at 0, 8 and 12 hours post-injection in hemocyte-feminized and non-feminized control groups. Hemocyte-feminized individuals demonstrated significantly lower *S. aureus* proliferation **(Wilcoxon test: W = 3227.5, *P* = 0.002)**, with hemocyte-feminized males

reaching the same degree of proliferation control as non-feminized females, showing a beneficial effect of *transformer*^{F} expression, specifically for males. While hemocyte-feminized females also demonstrated significantly slower *S. aureus* growth, the sex-specific differences in the staphylococcal load of hemocyte-feminized individuals were eliminated at both 8 and 12 hours post-injection **(Pairwise Wilcoxon test: *P* = 0.165 and *P* = 0.875 respectively)**. Data was collected over three replicates. Each dot represents a single individual, with the corresponding numbers of observations: **n=22, 30 and 24 at 0, 8 and 12 hours post-injection for the control group** and **n=32, 30 and 30 at 0, 8 and 12 hours post-injection for the hemocyte-feminised individuals**.

3.3 DISCUSSION

Our findings confirmed that females are more resistant to *S. aureus* infection, exhibit better control of the staphylococcal proliferation and have a higher proportion of actively phagocytic cells than males. Through hemocyte ablation, we demonstrated the elimination of sexually dimorphic survival in response to *S. aureus*. By expressing the feminizing splicing factor, *transformer*^{Female} (*tra*^{F}) in male hemocytes, we have shown that *tra*^{F} regulation of hemocyte function enhances proliferation control of S. aureus and reduces infection susceptibility. Thus, we conclude that hemocytes are the primary contributors and regulators of sex-specific susceptibility to *S. aureus* in *Drosophila melanogaster*.

3.1 Males are more susceptible than females to *S. aureus* infection

We have demonstrated that males are more susceptible to *S. aureus* infection than females, which was also previously shown by Duneau *et al.*, confirming the male-biased susceptibility of the bacterium across different genotypes. Additionally, we have shown that staphylococcal proliferation is more controlled in females than in males, which can most likely be attributed to the greater proportion of phagocytic cells demonstrated by females. Our results align with previous evidence emphasizing the role of phagocytes in *S. aureus* responses, including mice (Martin et al., 2011, Kitur et al., 2015, Surewaard et al., 2016, Yajjala et al., 2016), zebrafish (Prajsnar et al., 2008, Prajsnar et al., 2021, Pollitt et al., 2018) and *Drosophila* (Charroux and Royet, 2009, Defaye et al., 2009). In addition to the previously attributed role of alpha-Hemolysin, estrogen and neutrophils in mediating sex bias in staphylococcal infection (Castleman et al., 2018), we now suggest macrophages as another source of such dimorphism.

3.2 Hemocytes are indispensable in the response to *S. aureus* in both sexes

Our experiments have conclusively shown a significant reduction in resistance to *S. aureus* infection in both sexes. This points to the crucial role played by hemocytes in responding to the staphylococcal challenge, which aligns with similar results obtained in other studies involving hemocyte ablation in *Drosophila*, but also in other species like zebrafish or mice. Additionally, we observed that the elimination of hemocytes led to the loss of sex-specific survival differences, further validating *Drosophila* as a model organism to study how macrophages contribute to sex-specific susceptibility to *S. aureus*. Our data clearly illustrates a significant interaction between the sex of an

individual and the presence of hemocytes, which jointly influence resistance to staphylococcal challenge. Additionally, we found that the increase in a hazard ratio is proportionally greater in hemocyte-depleted females compared to their control counterparts, in comparison to the effect seen in hemocyte-depleted males versus healthy males. Such observation suggests that females may possess some hemocyte-specific advantage, normally making them more resistant to *S. aureus*, which elimination makes females drop more in survival than males.

We also assessed the impact of hemocyte depletion on the dynamics of the staphylococcal infection, revealing that the absence of hemocytes significantly reduces control over bacterial proliferation in both sexes, resulting in unrestricted growth, as previously observed in larvae (Charroux and Royet, 2009). Interestingly, the sex-specific differences in *S. aureus* growth persisted, with hemocyte-depleted females continuing to limit the proliferation more efficiently than hemocyte-depleted males. This suggests that the absence of the main mediators of the cellular immune response exposed the generally weaker immune response in males or implicated a more efficient involvement of humoral response in females.

Which hemocyte-related factors could render females less susceptible to S. aureus challenge?

3.2.1 Transcriptional control of hemocyte function

Data from our laboratory has previously shown sex-specific transcriptomes of adult hemocytes before and after *S. aureus* exposure, supporting our hypothesis that hemocytes demonstrate sexually dimorphic responses to bacterial challenge. Before infection, males exhibit higher expression of genes from the Toll and IMD pathways,

such as Dpt and Drsl5, along with a more robust induction of certain antimicrobial genes, e.g. GNBP3. In contrast, females display higher expression of genes related to the endocytosis pathway, specifically those linked with particle internalization and phagosome maturation. However, following infection, the differential induction of many antimicrobial peptides diminishes, resulting in comparable expression levels between the sexes (Belmonte, 2023). Hence, before the bacterial challenge, it appears that females possess pre-existing phagocytic machinery, likely allowing them to mount a more rapid and effective immune response and gain better control of the pathogen growth. The mechanistic immune advantage is also evident in my flow cytometry data, which shows that females displayed higher rates of phagocytosis of staphylococcal bioparticles than males, providing experimental validation. However, such a heightened phagocytic response may render females more exposed to its loss or impairment, as indicated by a proportionally greater increase in the hazard ratio for females following hemocyte ablation. Despite male hemocytes appearing better equipped for AMP production before infection, such advantage somehow diminishes, as both sexes upregulate the systemic responses to similar levels during the challenge, preventing males from matching the significantly more efficient cellular responses exhibited by females.

3.2.2 Sex differences in hemocyte functional subtypes

Recent advances in single-cell RNA sequencing uncovered various new plasmatocyte subtypes with differing functionalities in larvae. Several independent studies have identified a cluster of plasmatocytes expressing antimicrobial peptide encoding genes, which mainly emerge in wounding (Cattenoz et al., 2020, Tattikota et al., 2020, Leitão et al., 2020). These clusters are also enriched in the IMD and to a lesser extent Toll

signalling pathways, suggesting that plasmatocytes can elicit a humoral response against a wide variety of pathogens. Additionally, two minor phagocytic subtypes enriched in peroxidase enzyme-encoding genes were identified. Peroxisomes, small membrane-enclosed organelles, are known to be crucial for phagocytosis in *Drosophila*, and notably, for survival and the Toll-mediated immune response to *S. aureus* (Di Cara et al., 2017, Cattenoz et al., 2020). Belmonte et al. examined data from these studies to investigate sex-based differences in larval functional clusters, revealing sex-specific disparities in the relative size of nearly all clusters (Belmonte, 2023). However, it is essential to note hemocytes from unchallenged larvae do not encompass lymph-gland-derived hemocytes, which constitute the main source of adult hemocytes (Lanot et al., 2001). So while these are informative to an extent, they do not precisely reflect the situation in adults, and the link between the larval functional clusters and adult plasmatocyte specialization remains uncertain. However, it is plausible that adult females possess more antimicrobial and phagocytic plasmatocytes than adult males, granting them with additional survival advantage alongside the upregulated phagocytic responses.

3.2.3 Non-cell autonomous roles for hemocytes

Besides differences in hemocyte subpopulation composition, it is important to consider the possibility of hemocytes signalling to other immunocompetent *Drosophila* tissues, like the fat body. While the signalling pathways governing AMP expression are present and activated within hemocytes (Vlisidou and Wood, 2015), supported by the sex-specific transcriptome adult of hemocytes (Belmonte, 2023), the exact nature of signalling between hemocytes and the fat body remains to be elucidated. Despite *S. aureus* displaying resistance to the Toll-dependent response, as evidenced by the

lack of protection from Defensin overexpression or constitutive Toll pathway activation (Nehme et al., 2011), several AMPs, such as Diptericin, Metchnikowin and Bomanins were upregulated in larval and in adult hemocytes (Ramond et al., 2020, Belmonte, 2023), suggesting that AMPs may, to some extent, contribute to the response to this bacterium. Hemocyte-derived AMPs are likely only a small percentage of total AMPs but may have local effects. Previous studies have shown that even with the elimination of hemocytes, AMP production remained at wild-type levels in hemocyte-depleted individuals, although they became more susceptible to infection (Defaye et al., 2009, Charroux and Royet, 2009), indicating that plasmatocytes are not strictly required for the activation of systemic AMP production. While we did not quantify the expression of AMPs or their hemocyte-specific activation, hemocyte-depleted females showed slower *S. aureus* proliferation than males, suggesting that the humoral response was still functional, but its exact activity levels remain uncertain. Therefore, while hemocytes likely serve the same function in both sexes, they may trigger the systemic response at different rates.

In addition to resistance factors like AMPs, *Drosophila* hemocytes express Unpaired cytokine-like molecules, which can induce fat body responses such as AMP release. For instance, *upd3*, which has shown increased activation following ablation of *hml*+ hemocytes in larvae, possibly due to associated expansion of *pxn*+ hemocytes, the effect which is even strengthened Such upregulation resulted in enhanced JAK-STAT pathway activation and subsequent stimulation of the fat body (Shin et al., 2020). While the role of the JAK-STAT pathway in AMP production is relatively limited, it may have other implications, such as facilitating phagosome maturation with *S. aureus* as demonstrated in mammalian cell culture (Zhu et al., 2015).

3.2.4 Hemocyte metabolic regulation

Sex-specific disparities in energy allocation for immune responses stem from the differing priorities between the sexes. Males invest in traits for mate attraction, limiting their expenditure in immunity. Conversely, females prioritize extended reproductive lifespan, requiring a stronger immune response and greater energy investment (Rolff, 2002). The differential energy allocation to the immune system between the sexes likely leads to sex-specific metabolic regulation of immune cells. The ablation of *hml*+ hemocytes, which paradoxically increases their Upd3 expression (Shin et al., 2020), also downregulates insulin signalling, reducing triacyl glycerides storage in the fat body. This suggests metabolic distinctions within the plasmatocyte population, with specialized hemocytes, e.g. *pxn*+ *hml* responding to metabolic changes (Shin et al., 2020). This could parallel the M1/M2 macrophage paradigm in *Drosophila* and imply conservation of alternatively activated macrophages. Additional support for such concept comes from the plasmatocyte-specific metabolic reprogramming in response to bacterial infection, shifting towards aerobic glycolysis while depleting glucose from the other body parts (Krejčová et al., 2019). This metabolic change mimics the M1 macrophage activation, enhancing phagocytic activity and promoting a proinflammatory phenotype. Hence, females may mount a more efficient immune response to *S. aureus* due to more efficient metabolic remodeling within their plasmatocytes which has also been supported by sex-specific transcriptional regulation of metabolic genes observed in our laboratory (Belmonte, 2023).

3.3 The consequences of expressing the female-specific splicing factor in hemocytes

Our experiments showed that expressing the female-specific form of the *Drosophila* sex determination gene, *transformer*^{Female} (*tra*^{F}), in hemocytes significantly enhances resistance to *S. aureus* challenge in both males and females. Remarkably, this effect was particularly beneficial for males, as it allowed them to nearly match the survival of control females. We also observed a similar trend in our proliferation data, where hemocyte activation of *tra*^{F} led to substantially greater control of *S. aureus* growth in both sexes. Interestingly, males achieved the same level of control over staphylococcal growth as control females. Hence, the expression of *tra*^{F} likely induces female-specific regulation of male hemocytes, providing them with an advantage similar to that of females. This could result in more efficient phagocytosis, which subsequently limits proliferation. However, the improvement was not exclusive to males but was also seen in females, who naturally express the female form of *transformer*. Consequently, driving its expression in hemocytes may have artificially amplified *tra*^{F} levels within these cells. Therefore, the enhancement in survival and better control of bacterial proliferation in females additionally expressing *tra*^{F} could implicate the dose-dependent effect of the gene. Such a notion is further supported by the lack of interaction between sex and hemocyte feminization. Interestingly, we have observed the elimination of sex-specific differences in the staphylococcal proliferation, further supporting the idea that the expression of *tra*^{F} in male hemocytes improved their functionality, possibly allowing them to match the phagocytic activity of female hemocytes.

The beneficial effects observed when expressing *tra*^{F} in both male and female hemocytes should be interpreted as a female-specific transcriptional regulation of male cells rather than changes in the chromosome complement itself or cell sex during development, as the *hml* marker used to drive *tra*^{F} is only expressed in mature, fully differentiated cells. Therefore, for now, we should identify *tra*^{F} as an important regulator enhancing the more effective hemocyte-specific response to *S. aureus* in females. To definitively establish Tra^{F} as the key effector protein modulating sexspecific hemocyte responses, we must demonstrate its role in a reverse scenario by inducing the loss of *tra*^{F} in females.

The *transformer* gene occupies the second position in the regulatory hierarchy that determines the sex of each *Drosophila* cell, with an upstream gene, *Sex-lethal*, and two downstream genes, *doublesex* and *fruitless*. These genes, depending on the presence of functional *transformer*, contribute to sex-specific splicing of *doublesex* and *fruitless*, with the latter one primarily regulating male courtship behaviour (Demir and Dickson, 2005) and female receptivity (Chowdhury et al., 2020). These genes yield male-specific forms of Dsx^{M} and Fru^{M} or female-specific Dsx^{F} and Fru^{F}. Both, Dsx^{F} and Dsx^{M} are expressed in various somatic tissues, including those with no reported sexual dimorphism, such as leg muscles or the digestive system (Robinett et al., 2010). Given the improved survival and proliferation control following hemocyte-specific expression of *tra*^{F}, such transcriptional regulation is likely important for adult hemocyte function, e.g. targeting genes implicated in the endocytosis whose sex-specific hemocyte expression has been shown before (Belmonte, 2023). It is possible that the hemocyte-specific expression of *tra*^{F} also regulates some of the sex-

specific differences discussed above, e.g. enhancing hemocyte signalling to the fat body.

However, the extent of *tra*^{F}, and consequently its two downstream targets, on the expression of hemocyte-specific genes remains to be elucidated. For instance, most genes with significant sex-biased expression in *Drosophila* head tissue are regulated downstream of *tra*, and some genes are not even under its direct control, e.g. the male-specific lethal 2, which plays a major role in the dosage compensation (Chang et al., 2011). Additionally, many genes with male-biased expression are enriched on the X chromosome and located close to the dosage compensation gene entry sites, suggesting that differences in sex chromosome composition contribute to the dimorphism in gene expression, which is something we could not manipulate. (Abdullah et al., 2012). Therefore, the results of *transformer* manipulation in male and female hemocytes should be interpreted with caution, as there is still a lot to be determined about the regulation of its targets both upstream and downstream.

3.4 Experimental limitations of our genetic system

While our experiments revealed genotype- and sex-specific significant differences, it is essential to acknowledge the limitations of the hemocyte genetic marker, *hemolectin (hml)*, we employed to induce transgene expression (Goto et al., 2001). At the larval stage, *hml* is activated only in a subpopulation of fully differentiated plasmatocytes and lamellocytes some studies report that *hml* is found only in 65% of adult cells (Boulet et al., 2021a). While *hml* is the most pervasive hemocyte marker, circulating plasmatocytes also express other markers, such as *peroxidasin (pxn)* (Nelson et al., 1994), which we did not consider in our experiments. Consequently, the *hml* marker alone might have been not sufficient to induce cell killing and

feminization in all hemocytes. This notion is further supported by the *hml*-driven expression of *bax* during adulthood, which resulted in the ablation of almost all *hml*+ cells. Nonetheless, some *pxn*+ blood cells persisted, giving rise to two distinct hemocyte subpopulations, *pxn*+ *hml*+ and *hml-pxn*+ (Boulet et al., 2021a).

Apart from the dose-dependent effect of *transformer*^{F}, another explanation for the enhancement of survival and load control in hemocyte-feminized females could stem from genetic factors directly associated with the GAL4/UAS system in both hemocyte-feminised and control lines, which might be regulating these differences. To eliminate such a possibility, we would need further testing in another genetic context e.g. using the GeneSwitch system.

3.5 Parasite-mediated effects

Finally, while all of our experiments primarily focused on *Drosophila* responses to *S. aureus*, it is important to recognize that bacteria can also influence host phenotypes and evade its immune responses. Although in humans, *S. aureus* has been demonstrated to evolve various evasion strategies, little is known about the mechanisms employed by the bacterium to subvert the immune responses in *Drosophila*, a widely used model host for *S. aureus* infection. One notable mechanism involves wall teichoic acids, which limit the access of innate immune receptors to peptidoglycan, impairing the binding of PGRP-SA (Vaz et al., 2019, Atilano et al., 2011). As a result, staphylococci are allowed to go undetected and multiply within the fly. Additionally, wall teichoic acid-mediated inhibition of PGRP-SA attachment to *S. aureus* has been shown to impair subsequent Toll pathway activation (Tabuchi et al.,

2010). Therefore, it is plausible that *Drosophila* females may possess a superior ability to combat the interference of wall teichoic acids on PGRP-SA, making them more efficient at pathogen recognition, which then translates to higher rates of phagocytosis, and increased Toll activation, ultimately leading to more controlled staphylococcal proliferation.

While we know that females have a higher proportion of hemocytes that phagocytose *S. aureus* bioparticles, it is important to note that this may not necessarily transfer to the same outcome with live bacterium, as phagocytic activity can be influenced by the characteristics of phagocytosed particles (Underhill and Goodridge, 2012). Additionally to the complex surface proteins, *S. aureus* is known for its ability to target the integral components of the host immune response, particularly the professional phagocytes. For instance, *S. aureus* can evade and manipulate macrophages, allowing for its intracellular survival within these cells and serving as a potential source for further dissemination (Kubica et al., 2008). However, thus far such a phenomenon has not been demonstrated in *Drosophila*.

3.6 Future work

In future investigations, it would be valuable to determine sex differences in adult plasmatocyte composition, i.e. determine if females have a higher proportion of antimicrobial and phagocytic plasmatocytes than males. Identifying appropriate genetic markers for these plasmatocyte subtypes would allow their specific manipulation, advancing our understanding. Further clarification on the role of the sex determination cascade in regulating hemocyte responses is needed, beginning with

tra^{F} validation in females through its RNAi-mediated knock-down. Assessing the expression of previously identified genes with sex-specific patterns, such as those related to the endocytosis pathway in hemocytes of feminised males would shed light on the extent of this cell-autonomous regulation and its role in *S. aureus* dimorphism. To explore if developing with female cells provides additional advantages, it is worth expressing *tra*^{F} under the control of GATA protein Serpent (Srp) (Rehorn et al., 1996), creating sexual chimaeras during haematopoiesis.

Finally, we have not tested any sex differences in functionality of the hemocytes *ex vivo*. It is crucial to determine how effective *Drosophila* hemocytes are at recognition, uptake and subsequent degradation of *S. aureus*. For instance, we should determine if there is a differential ability between the sexes in overcoming peptidoglycan inaccessibility. To assess that, I would induce *hml*-driven fluorescence of PGRP-SA. Subsequently, based on the endogenous fluorescence, hemocytes would be extracted using FACS sorting and transferred with wall teichoic acid-deficient and parental strains of *S. aureus* labelled with a fluorescent reporter, e.g. mCherry. Then, microscopic imagining would allow us to determine which bacterial cells were internalized, which were bound to the membrane, and which remained free-floating. A similar technique could be employed to establish a killing assay which would allow us to determine the killing capacity of *Drosophila* phagocytes.

3.7 Conclusion

We have demonstrated that *Drosophila* females are less susceptible to *S. aureus* infection than males, highlighting a conserved susceptibility observed in humans and

other model organisms. It appears that phagocytic cells play a crucial role in the differential *S. aureus* susceptibility between the sexes, with females relying more on them to maintain resistance. This difference is most likely driven by hemocyte-specific disparities in gene expression, with females exhibiting higher levels of molecular machinery even before bacterial challenge. This initial advantage probably results in slower pathogen proliferation and reduced susceptibility. Additionally, our research has shown that to some extent, this dimorphism is mediated by the cell-autonomous regulation of hemocyte responses, with female sex determination factors providing a significant survival advantage and more controlled bacterial growth.

Our findings not only shed light on broadly observed sex differences in the context of *S. aureus* infection but also pinpoint the existence of sex-specific regulation at the single-cell level, highlighting the vital biological paradigm that every cell is influenced directly by its sex chromosome complement.

CHAPTER 4: Adaptation to *L. boulardi* parasitism at larval stages trades off with resistance to *S. aureus* infection during adulthood

4.1 INTRODUCTION

Throughout its life, *Drosophila* encounters various microorganisms and possesses a robust innate immune response, comprising both cellular and humoral components, allowing it to effectively combat pathogens with diverse virulence strategies. These responses can be further classified based on their state of readiness. Constitutive immune responses are perpetually active, even in the absence of infectious stimuli, acting promptly to prevent the infection. Whereas inducible responses are triggered only in response to pathogenic attacks, typically intensifying the already present constitutive factors (Boots and Best, 2018). Mounting an immune response is energetically costly, and often requires a trade-off with other vital biological functions, such as development, due to the reallocation of a finite pool of resources (Bajgar et al., 2015). Different states of readiness impose distinct energy costs, with inducible responses incurring costs only after infection and constitutive immune responses are continually active, requiring a constant energy investment. Organisms strive to strike a balance between investing in constitutive and inducible factors and achieving an optimal response (Martin and Tate, 2023). However, selective pressures, such as the frequency of infection may necessitate differential allocation of energy.

In the wild, *Drosophila* is at risk of attack from parasitoid wasps, such as *Leptopilina boulardi*, which lay their eggs in fly larvae or pupae. Under normal conditions, there are only two classes of hemocytes present in larvae, plasmatocytes and crystal cells.

However, upon wasp egg oviposition, these circulating hemocytes together with prohemocytes and plasmatocytes from the lymph gland (Cho et al., 2020, Honti et al., 2014) differentiate into lamellocytes, which are key effector cells in the anti-parasitoid immune response. Once plasmatocytes surround the parasitoid eggs, lamellocytes adhere to them, forming a multilayered capsule that is melanized by the activity of phenyloxidases (Dudzic et al., 2015), whose by-products contribute to parasite-killing (Nappi et al., 2009). Recent studies have demonstrated that when *Drosophila* is subjected to repeated selection for survival to *L. boulardi* parasitism, the inducible production of immature lamellocytes becomes constitutive, even in the lack of wasp infestation. Such adaptation allows the host to mount quicker and more effective immune responses, resulting in a survival rate of almost 80% in the lines with evolved wasp resistance as opposed to the 10% survival rate observed in the control population (Leitão et al., 2020). Nonetheless, wasp parasitism is not the sole pathogenic threat *Drosophila* may encounter throughout their lifespan, and little is known about how adaptation to one parasite early in life affects the immune response to a different pathogen later on, particularly if it requires a distinct immune response from the same immune tissue – in this case, hemocytes. In addition to the limited energy resources, type of pathogen and its infection frequency, the allocation of host investment into constitutive and induced immunity may be also differentially regulated between the sexes as males and females are subjected to different selective pressures.

We utilised these valuable populations of experimentally evolved *Drosophila*, exposed to high levels of parasitism, to determine whether selection for survival to one parasite at juvenile stages affects the response to a different pathogen, such as *S. aureus*,

during adulthood. We also investigated how these pathogen-related selective pressures differ between males and females. Since *S. aureus* is primarily controlled by plasmatocytes, we aimed to determine how the changes in hemocyte composition at the larval stage, favouring lamellocytes, influence the immune response to a pathogen that is primarily controlled by a distinct blood cell type from the hemocyte population – plasmatocytes. These results form part of a larger study that aims to understand the costs to immune responses across the life course of adapting to a stage-specific parasite.

4.2 RESULTS

To explore how multiple generations of selection for parasitism at larval stages (selection lines), along with associated changes in hemocyte composition, influence the susceptibility to *S. aureus* infection during adulthood, we infected them with approximately 1500 cells of the bacterium and closely monitored their survival. In general, the selection lines show significantly increased susceptibility to the staphylococcal challenge when compared to the control lines, with individuals possessing evolved parasite resistance succumbing to infection within 70 hours **(Cox-ph: df = 3, $\chi 2 = 73.65$, $P = 2.2 \times 10^{-16}$)**. While in control individuals, the typical sex-based survival was observed **(Cox-ph: df=3, $\chi 2 = 73.65$, $P = 0.003$)**, in flies with developed parasite resistance the sexually dimorphic response to *S. aureus* was eliminated **(Cox-ph: df = 2, $\chi 2 = 1$, $P = 0.148$)**. To understand how each sex is affected by the early-life parasite resistance, we extracted hazard ratios from our model, revealing that selection-line females are 2.2 times more likely to succumb to *S. aureus* infection compared to their control counterparts, whereas selection-line

males are 1.7 times more likely to perish from the staphylococcal challenge compared to control males. Such observation suggests that the proportional increase in hazard is greater for females than males.

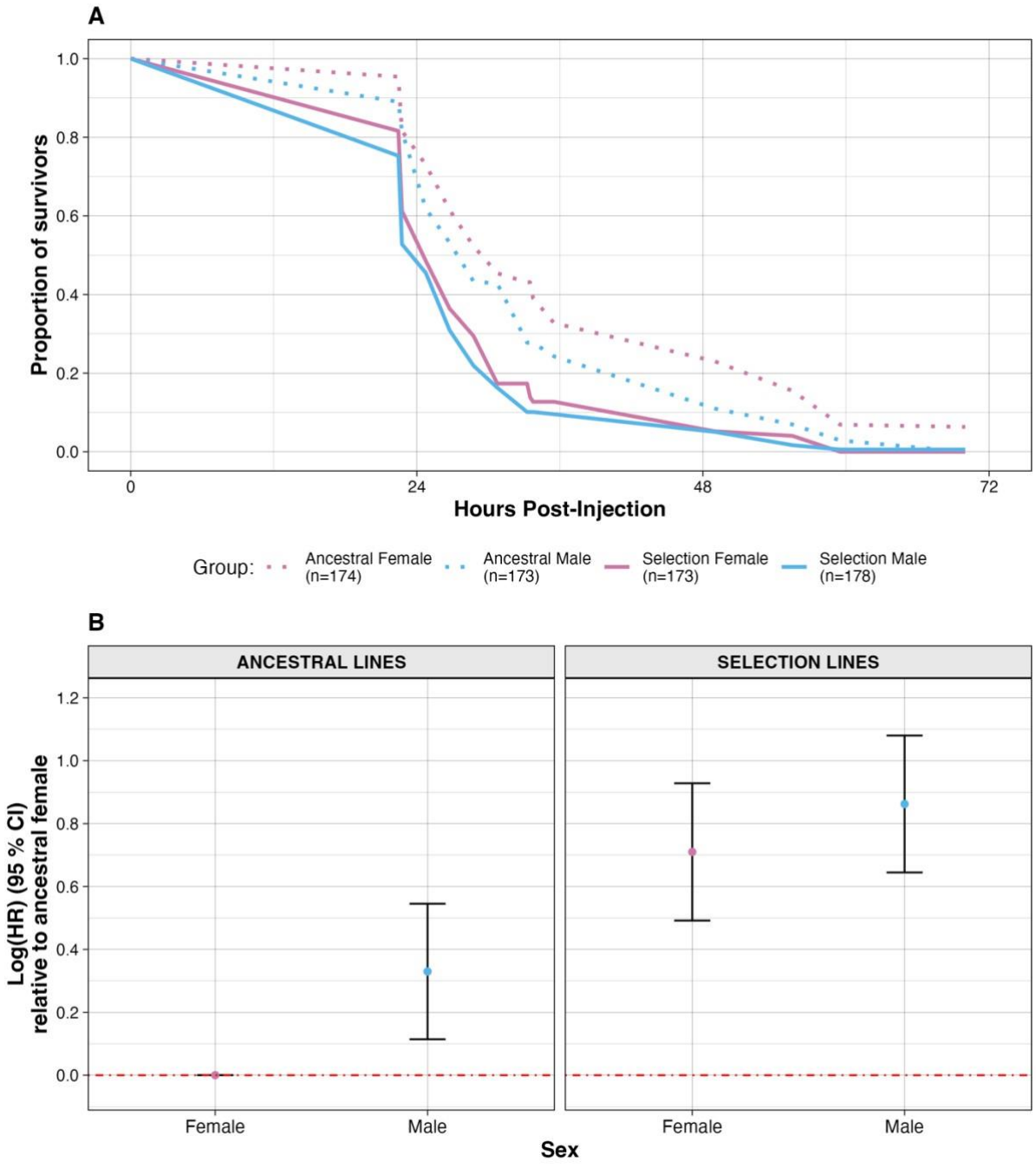

Figure 7. *S. aureus* challenge of selection line individuals results in significantly greater susceptibility to the infection and the loss of dimorphism. A) Survival curves for both sexes in the selection and ancestral lines following *S. aureus* infection based on survival data from a single experiment. In the Cox proportional hazards modelling, we accounted for the interaction between selection and the sex of an individual, revealing that juvenile adaptation to wasp parasitism leads to a dramatic increase in *S. aureus* susceptibility in both sexes, eliminating the usually observed dimorphism in survival **(Cox-ph: df = 3, $\chi 2 = 73.65$, $P < 0.0001$)** and **(Cox-ph: df = 2, $\chi 2 = 1$, $P = 0.148$)** respectively. The sample size of each sex is provided in the brackets. B) A graphical representation of the estimated Log(Hazard Ratio) extracted from the Cox proportional hazards model. A hazard greater than 1 indicates increased susceptibility compared to the ancestral females (red horizontal line).

To further assess how parasitism selection affects within-host proliferation of *S. aureus*, we injected selection and control lines with a precise inoculum and sampled at 8 and 12 hours post-injection, ensuring equal loads were administered. Overall, bacterial proliferation was greater in the selection lines **(Wilcoxon test: W = 11996, $P = 0.038$)** compared to the control lines, suggesting that the adaptation for wasp parasitism results in less efficient control of bacterial proliferation. Contrary to our expectations based on the survival rates, the sex-specific differences in selection lines were not evident at 8 hours post-injection but did appear at 12 hours post-injection, with selection line females showing significantly poorer control over pathogen growth than selection line males **(Pairwise Wilcoxon test: $P = 0.003$)**, possibly implicating differential regulation of humoral response or development of bacterial tolerance.

Another surprising observation comes from the control lines, where we did not observe sex-specific differences in bacterial load at either 8 or 12 hours, which had not been observed before in other genotypes in use in the laboratory.

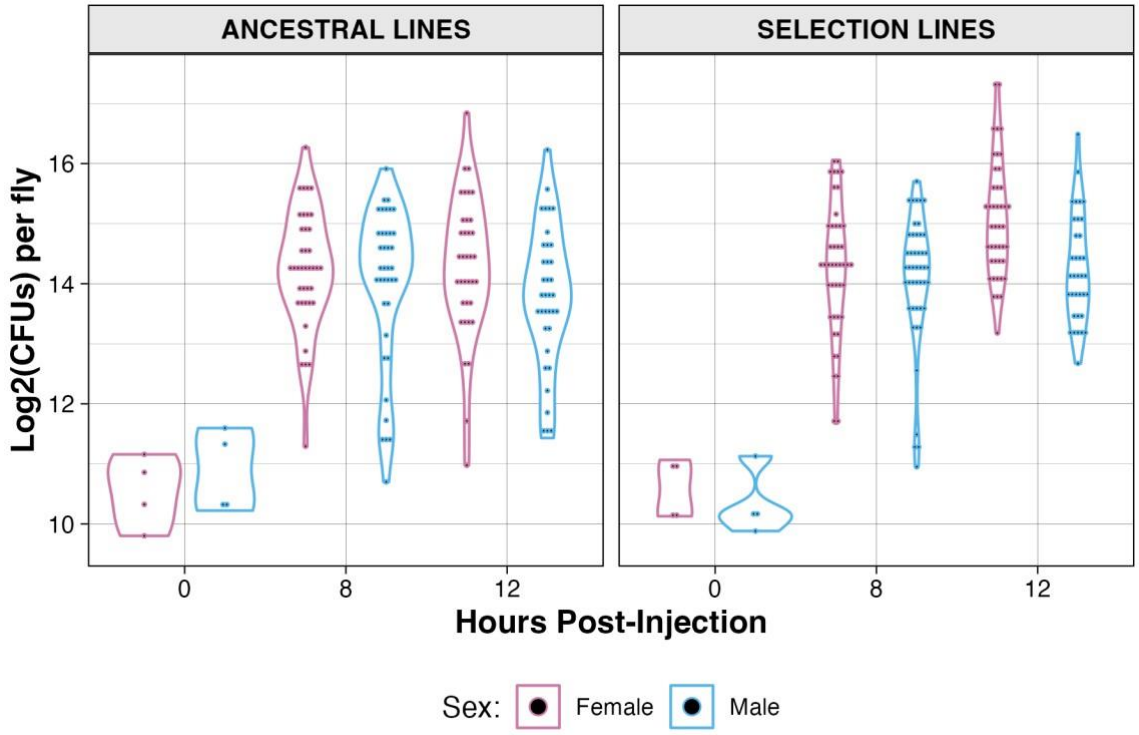

Figure 8. Selection lines demonstrated significantly worse control of S. aureus proliferation. Bacterial growth, presented as Log_2 of *S. aureus* colony forming units (CFUs) in ancestral and selection line individuals at 0, 8 and 12 hours post-injection. Overall, selection line flies demonstrated greater staphylococcal proliferation compared to ancestral line flies **(Wilcoxon test: W = 11996, *P* = 0.03801)**, showing the harmful effect of wasp parasitism adaption on proliferation control. The only sex-specific difference in bacterial load was observed at 12 hours post-injection **(Pairwise Wilcoxon test: *P* = 0.00315)**, which was not in line with the survival analysis. Data was collected over three replicates. Each dot represents a single individual, with the following number of observations in the **ancestral lines: n=8, 79,**

78 for **0, 8** and **12 hours post-injection** and in the **selection lines: n=8, 92, 79** for **0, 8** and **12 hours post-injection**.

4.3 DISCUSSION

Our collaborators have successfully generated a valuable collection of experimentally evolved *Drosophila* lines derived from wild-caught specimens (Leitão et al., 2020). These lines underwent parasitation by *L. boulardi*, a wasp that deposits eggs within the bodies of larvae, subsequently developing at the expense of the host. *Drosophila* has strong physiological defences, such as encapsulation, preceded by lamellocyte differentiation, and melanization, allowing some of them to combat such a threat. The surviving individuals were selectively bred to establish the next generations, eventually creating a population with enhanced resistance to wasp infestation. Single-cell RNA sequencing revealed that the resistance observed in selection line larvae is attributed to the constitutive differentiation of lamellocyte precursors, which were previously usually produced only in response to infection, leading to a state of persistently primed immune response and the altered composition of the hematopoietic system (Leitão et al., 2020). We were particularly interested in examining how such hemocyte-specific adaptation to one parasite early in life influences the response to a different parasite, *S. aureus*, whose proliferation is primarily controlled by *Drosophila* hemocytes.

4.3.1 Constitutive immunity to wasps comes at the cost of resistance to *S. aureus* infection

Our results indicate that lines subjected to multiple generations of wasp parasitism (selection lines) exhibit increased susceptibility to *S. aureus* infection compared to the lines maintained without repeated parasite infection (ancestral lines). Such a heightened susceptibility can be traced back to the shift from inducible lamellocyte differentiation to constitutive lamellocyte presence, which is the observed consequence of continuous selection for parasitism survival. Significant amplification of lamellocytes has been shown to alter the composition of the hematopoietic system and transcriptional states of other cells. Single-cell sequencing of unchallenged larval hemocytes from the ancestral lines revealed nine transcriptionally distinct hemocyte clusters, primarily composed of self-cycling plasmatocytes (PLASM1 and PLASM2), which upon parasitic wasp challenge, differentiate into immature lamellocyte precursors (LAM1 and LAM2) before maturing into mature LAM3. Within the selection lines, a constitutive increase in the presence of LAM1 and LAM2 is observed, indicating the fixation of inducible lamellocytes, a phenomenon that occurs at the expense of circulating plasmatocytes (Leitão et al., 2020). Therefore, the increased susceptibility of selection lines to *S. aureus* is likely attributed to the persistent presence of lamellocytes and a corresponding loss of plasmatocytes in larvae, which carries over into adulthood. This suggests that investment in larval lamellocyte production comes at the expense of adult plasmatocyte responses, making selection lines particularly ineffective at fighting *S. aureus*, which primarily triggers plasmatocyte-mediated immune responses.

4.3.2 Heavy selection pressure on both sexes compresses adult immune dimorphism

In the ancestral adult lines, females displayed lower susceptibility to *S. aureus* infection than males, consistent with our observations in other genotypes (e.g. CHAPTER 2). We observed that sex differences in survival to *S. aureus* challenge are not detectable in the selection lines. When it comes to sex-specific transcriptomic differences in single-cell sequencing data of these lines, Belmonte (2023) revealed that both sexes in the selection lines experienced a decrease in the proportion of self-cycling plasmatocytes (PLASM1) in favour of more immature lamellocytes (LAM1 and LAM2), both at baseline and upon parasitation. However, selection line females experienced a more substantial loss of self-cycling plasmatocytes (PLASM1) compared to males (Belmonte, 2023). Consequently, the hemocyte-specific advantage of females, such as a higher proportion of actively phagocytic hemocytes and more primed phagocytic response (CHAPTER 2) is most likely permanently lost due to the general decrease of self-cycling plasmatocytes, which are primarily involved in phagocytosis. While the population of self-cycling plasmatocytes also decreased in the selection line males, they do not show as much hemocyte-specific advantage as females. This is further supported by a proportionally greater increase in the hazard ratio in females than in males. Therefore, while both sexes lose phagocytic activity, resulting in the elimination of dimorphism, the loss is probably more pronounced in females.

The induction of resistance to parasitism is also recognized as a costly process (Dias et al., 2022) and both males and females bear these costs This is evidenced by the

elimination of the dimorphism, which results from the diversion of the energy resources, which would normally be allocated to maintaining the pool of plasmatocytes capable of responding to bacterial infections, to resist the wasp parasitism. In the context of the staphylococcal challenge, this juvenile-derived reallocation of energy resources, results in a proportionally greater increase in sex-matched hazard ratio in females compared to males, suggesting that the cost of resistance to wasps might be greater for females. However, as both sexes are very strongly affected by the adaptations to wasp parasitism, resulting in no difference in susceptibility between males and females, this suggests the pressures associated with adapting to wasp parasitism override any sex-specific pressures on developing or maintaining the immune system.

4.3.3 Adaptation to juvenile parasitism reduces control over *S. aureus* proliferation in adulthood

Our results reveal significantly greater *S. aureus* proliferation in the selection lines compared to the ancestral lines, likely due to a limited plasmatocyte response, a consequence of constitutive lamellocyte differentiation, resulting in decreased bacterial clearance. While we did not detect sex differences in bacterial load at 8 hours, in line with the survival analysis and loss of dimorphism, to our surprise, selection line females exhibited higher *S. aureus* growth at 12 hours post-injection, despite no difference in survival between the sexes. Several theories could account for such observation.

Females are thought to invest more energy in immunity, enhancing immunocompetence for extended breeding time (Rolff, 2002), which likely leads them to prioritize resource allocation toward resistance mechanisms over males (Vincent and Sharp, 2014). However, investment in parasite-specific resistance at juvenile stages, a process known to be resource-intensive (Dias et al., 2022), likely limits energy allocation into an effective immune response against different parasites in adulthood. Supporting this, we see similar effects on susceptibility in males and females from selected lines. Although males and females do not show different encapsulation rates either before or after selection (unpublished data from our collaborators), males and females may have taken slightly different routes to adapt to wasp parasitism, leading to subtle differences, such as those seen in early load, that are exposed by our bacterial infection paradigm.

One source of variation could be other immune tissues. The wasp selection, applied to the entire fly and not just hemocytes, likely affected the fat body as well, known to respond to wasp challenges through the Toll and JAK/STAT pathways (Schlenke et al., 2007, Yang and Hultmark, 2016). Therefore, a possible explanation for the slightly increased bacterial load at 12 hours in selection line females may involve the fat body and its signalling cascades. Most genes altered upon parasitoid challenge differ from those induced in antimicrobial responses, but two PGRPs and three AMPs were significantly upregulated shortly after the attack, likely due to cuticle puncturing which increases exposure to low levels of microorganisms (Wertheim et al., 2005a). In another study, *L. boulardi* eggs triggered the expression of AMP-encoding genes in susceptible *Drosophila* larvae, but not in resistant strains, suggesting that parasitism resistant individuals may favour cellular responses over humoral ones, overcoming

the need for wound-related protection (Coustau et al., 1996). Additionally, a more marked reduction in phagocytic plasmatocytes of selection line female larvae (Belmonte, 2023) may exacerbate the effect of reduced AMP-producing plasmatocytes, resulting in elevated bacterial loads. Hence, the potential impairment of the humoral response in selection lines, normally aiding in wound closure, might disproportionately affect females, who are already weakened by a greater loss of phagocytic plasmatocytes, resulting in higher bacterial load at a later stage.

Finally, the decrease in the proportion of self-cycling plasmatocytes could also impair their role in activating the JAK/STAT signalling in the fat body mediated through plasmatocyte-secreted cytokines Upd2 and Upd3 (Woodcock et al., 2015, Shin et al., 2020), and in the expression of *tep* and *tot* genes Plasmatocyte-secreted cytokines. Therefore, it is plausible that selection line females have a limited expression of *tep* genes, which have been shown to facilitate efficient phagocytosis of *S. aureus* (Dostálová et al., 2017), potentially leading to higher bacterial loads.

4.3.4 Experimental limitations and future work

Contrary to our observations in survival monitoring, there was no difference in *S. aureus* proliferation between the sexes of the ancestral populations, which contradicts the usual bacterial growth pattern seen in the control genotypes. Since the flies in the ancestral lines are essentially wild-type, with no artificially selected traits or genetic modifications, the chosen optical density (OD) of 0.25 might have been too overwhelming for them to detect subtle sex-specific differences in bacterial load that otherwise would have been optimal to uncover such disparities in mutants.

Additionally, the only trait considered for selection was survival to *L. boulardi*, without accounting for potential variations that might emerge in individuals later in life. Given that adaptation to wasp parasitism at larval stages affects susceptibility to *S. aureus* infection in adulthood, it would be interesting to see how such adjustment and associated changes in the hematopoietic system affect lifespan. Therefore, adding another trait for selection, such as a long lifetime, could provide insight into the connection between wasp parasitism adaptation and longevity. While all of the transcriptome information comes from larval stages, how adult hemocyte subpopulations exactly react to such adaptation and how it influences their activity remains unknown.

4.4 CONCLUSION

In our study, we successfully demonstrated that the early development of resistance to one pathogen imposes a cost on the effective immune response to a different pathogen later in life. Our findings are further substantiated by the results of single-cell RNA analyses of larval hemocytes, which help pinpoint the underlying cause of this immune trade-off in adulthood: persistent changes in the composition of the hematopoietic system. In the context of our *S. aureus* survival monitoring, we observed that adaptation to juvenile parasitism results in the loss of sex-specific differences in *S. aureus* susceptibility during adulthood, clearly indicating that parasitic selective pressures override any positive implications of sex-specific influences. It becomes evident that under certain conditions, a more potent but also more resource intensive immune response in females can make them more exposed to its eventual impairment, e.g. through evolutionary adaptation to a different pathogen. While there

are numerous examples of infectious diseases that have acted on human populations over many generations, allowing them to develop resistance alleles, there are relatively few studies that explore how the adaptation of resistance to one infectious agent may render individuals more susceptible to another. However, given our observations, this could be the case. Our results underscore the concept of a finite pool of resources available to organisms, which must be strategically allocated to various aspects of their survival and immune defence throughout their lifetime.

5. CONCLUDING REMARKS

Using *Drosophila melanogaster*, we have successfully and with robust statistical power confirmed the male-biased susceptibility to *S. aureus* infection, with a particular focus on phagocytes as the primary mediators of the dimorphism. In addition to survival analysis, we have examined bacterial proliferation and phagocytic activity, adding further reliability to the findings previously obtained by our research group. Furthermore, we have demonstrated a very important, yet often overlooked, biological principle, highlighting that the specific chromosome complement, either female or male, regulates not only the development of our gonads but is in fact, present in every single cell of our organism. The consequences of inherent chromosome imbalance between the sexes influence regulation at a cellular level, influencing immune responses. We have explored how the body is a closed system, where energy allocated to one process, such as adaptation for wasp parasitism at juvenile stages, cannot be recycled, trading off with another process, such as resistance to *S. aureus* infection. In the case of parasitic pressures and associated energy investments, sex-

related pressures are overridden, suggesting that while sex is a very important factor in mediating disease susceptibility, it is not the only one.

Our findings bring implications for research, encouraging the inclusion of sex as a factor in experimental design, statistical analysis and reporting. Additionally, we postulate for more awareness regarding the sex of the cell line used, as we have clearly shown that cells exhibit sex-specific regulation. Further research could bring new advancements in personalized treatment strategies, targeting the sex-specific differences not only in terms of systemic regulation but also at a cellular level.

6. REFERENCES

• ABDULLAH, M., CHAI, P. S., CHONG, M. Y., TOHIT, E. R., RAMASAMY, R., PEI, C. P. & VIDYADARAN, S. 2012. Gender effect on *in vitro* lymphocyte subset levels of healthy individuals. Cellular Immunology, 272, 214-9.

• AGAISSE, H., BURRACK, L. S., PHILIPS, J. A., RUBIN, E. J., PERRIMON, N. & HIGGINS, D. E. 2005. Genome-wide RNAi screen for host factors required for intracellular bacterial infection. Science, 309, 1248-51.

• AGAISSE, H., PETERSEN, U. M., BOUTROS, M., MATHEY-PREVOT, B. & PERRIMON, N. 2003. Signalling role of hemocytes in *Drosophila* JAK/STAT-dependent response to septic injury. Developmental Cell, 5, 441-50.

• AGUILA, J. R., SUSZKO, J., GIBBS, A. G. & HOSHIZAKI, D. K. 2007. The role of larval fat cells in adult *Drosophila melanogaster*. Journal of Experimental Biology, 210, 956-963.

• ALBRECHT, V. S., LIMBAGO, B. M., MORAN, G. J., KRISHNADASAN, A., GORWITZ, R. J., MCDOUGAL, L. K. & TALAN, D. A. 2015. *Staphylococcus aureus* Colonization and Strain
Type at Various Body Sites among Patients with a Closed Abscess and Uninfected Controls at U.S. Emergency Departments. Journal of Clinical Microbiology, 53, 3478-84.

• ALLARD, C., CARIGNAN, A., BERGEVIN, M., BOULAIS, I., TREMBLAY, V., ROBICHAUD, P., DUPERVAL, R. & PEPIN, J. 2008. Secular changes in incidence and mortality associated with *Staphylococcus aureus* bacteraemia in Quebec, Canada, 1991-2005. Clinical Microbiology and Infection, 14, 421-8.

• AOMATSU, M., KATO, T., KASAHARA, E. & KITAGAWA, S. 2013. Gender difference in tumour necrosis factor-α production in human neutrophils stimulated by lipopolysaccharide and interferon-γ. Biochemical and Biophysical Research Communications, 441, 220-5.

• ARNOLD, A. P. 2012. The end of gonad-centric sex determination in mammals. Trends in Genetics, 28, 55-61.

• ARNOLD, A. P. 2019. Rethinking sex determination of non-gonadal tissues. Current Topics in Developmental Biology, 134, 289-315.

• ASAI, A., TSUDA, Y., KOBAYASHI, M., HANAFUSA, T., HERNDON, D. N. & SUZUKI, F. 2010. Pathogenic role of macrophages in intradermal infection of methicillin-resistant *Staphylococcus aureus* in thermally injured mice. Infection and Immunity, 78, 4311-9.

• ATILANO, M. L., YATES, J., GLITTENBERG, M., FILIPE, S. R. & LIGOXYGAKIS, P. 2011. Wall teichoic acids of *Staphylococcus aureus* limit recognition by the *Drosophila* peptidoglycan recognition protein-SA to promote pathogenicity. PLOS Pathogens, 7, e1002421.
• AYYAZ, A., LI, H. & JASPER, H. 2015. Haemocytes control stem cell activity in the *Drosophila* intestine. Nature Cell Biology, 17, 736-748.

• BAIN, B. J. & ENGLAND, J. M. 1975. Normal haematological values: sex difference in neutrophil count. British Medical Journal, 1, 306-9.

• BAIN, C. C., GIBSON, D. A., STEERS, N. J., BOUFEA, K., LOUWE, P. A., DOHERTY, C., GONZÁLEZ-HUICI, V., GENTEK, R., MAGALHAES-PINTO, M., SHAW, T., BAJÉNOFF, M., BÉNÉZECH, C., WALMSLEY, S. R., DOCKRELL, D. H., SAUNDERS, P. T. K., BATADA, N. N. & JENKINS, S. J. 2020. Rate of replenishment and microenvironment contribute to the sexually dimorphic phenotype and function of peritoneal macrophages. Science Immunology, 5.

• BAJGAR, A., KUCEROVA, K., JONATOVA, L., TOMCALA, A., SCHNEEDORFEROVA, I., OKROUHLIK, J. & DOLEZAL, T. 2015. Extracellular Adenosine Mediates a Systemic Metabolic Switch during Immune Response. PLOS Biology, 13, e1002135.

• BAKER, B. S. & RIDGE, K. A. 1980. Sex and the single cell. I. On the action of major loci affecting sex determination in *Drosophila melanogaster*. Genetics, 94, 383-423.

• BEERY, A. K. & ZUCKER, I. 2011. Sex bias in neuroscience and biomedical research. Neuroscience and Biobehavioral Reviews, 35, 565-72.

• BELL, L. R., HORABIN, J. I., SCHEDL, P. & CLINE, T. W. 1991. Positive autoregulation of Sex-lethal by alternative splicing maintains the female-determined state in *Drosophila*. Cell, 65, 229-239.

• BELMONTE, R. 2023. Sex differences in immune tissue function and infection outcomes in *Drosophila melanogaster*. Doctor of Philosophy, University of Edinburgh.

• BELMONTE, R. L., CORBALLY, M. K., DUNEAU, D. F. & REGAN, J. C. 2019. Sexual Dimorphisms in Innate Immunity and Responses to Infection in *Drosophila melanogaster*. Frontiers in Immunology, 10, 3075.

• BENOIT, M., DESNUES, B. & MEGE, J.-L. 2008. Macrophage polarization in bacterial infections. The Journal of Immunology, 181, 3733-3739.

• BILEN, J., ATALLAH, J., AZANCHI, R., LEVINE, J. D. & RIDDIFORD, L. M. 2013. Regulation of onset of female mating and sex pheromone production by juvenile hormone in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences USA, 110, 18321-18326.

• BOGGS, R. T., GREGOR, P., IDRISS, S., BELOTE, J. M. & MCKEOWN, M. 1987. Regulation of sexual differentiation in *D. melanogaster* via alternative splicing of RNA from the transformer gene. Cell, 50, 739-47.

• BOOTS, M. & BEST, A. 2018. The evolution of constitutive and induced defences to infectious disease. Proceedings of the Royal Society B: Biological Sciences, 285, 20180658.

• BOU AOUN, R., HETRU, C., TROXLER, L., DOUCET, D., FERRANDON, D. & MATT, N. 2011. Analysis of thioester-containing proteins during the innate immune response of *Drosophila melanogaster*. Journal of Innate Immunity, 3, 52-64.

• BOULET, M., RENAUD, Y., LAPRAZ, F., BENMIMOUN, B., VANDEL, L. & WALTZER, L. 2021a. Characterization of the *Drosophila* Adult Hematopoietic System Reveals a Rare Cell Population with Differentiation and Proliferation Potential. Frontiers in Cell and Developmental Biology, 9.

• BOUMAN, A., HEINEMAN, M. J. & FAAS, M. M. 2005. Sex hormones and the immune response in humans. Human Reproduction Update, 11, 411-23.

• BRAUN, A., HOFFMANN, J. A. & MEISTER, M. 1998. Analysis of the *Drosophila* host defence in domino mutant larvae, which are devoid of hemocytes. Proceedings of the National Academy of Sciences USA, 95, 14337-14342.

• BRENNAN, C. A., DELANEY, J. R., SCHNEIDER, D. S. & ANDERSON, K. V. 2007. Psidin Is Required in *Drosophila* Blood Cells for Both Phagocytic Degradation and Immune Activation of the Fat Body. Current Biology, 17, 67-72.

• BRETSCHER, A. J., HONTI, V., BINGGELI, O., BURRI, O., POIDEVIN, M., KURUCZ, É., ZSÁMBOKI, J., ANDÓ, I. & LEMAITRE, B. 2015. The Nimrod

transmembrane receptor Eater is required for hemocyte attachment to the sessile compartment in *Drosophila melanogaster*. Biology Open, 4, 355-363.

• BROWN, S., HU, N. & HOMBRÍA, J. C.-G. 2003. Novel level of signalling control in the JAK/STAT pathway revealed by *in situ* visualisation of protein-protein interaction during *Drosophila* development. 130, 3077–3084.

• BROWN, S., HU, N. & HOMBRÍA, J. C.-G. 2001. Identification of the first invertebrate interleukin JAK/STAT receptor, the *Drosophila* gene domeless. Current Biology, 11, 1700-1705.

• BUCHANAN, J. L., MEIKLEJOHN, C. D. & MONTOOTH, K. L. 2018. Mitochondrial Dysfunction and Infection Generate Immunity–Fecundity Trade-offs in *Drosophila*. Integrative and Comparative Biology, 58, 591-603.

• BUCHON, N., SILVERMAN, N. & CHERRY, S. 2014. Immunity in *Drosophila melanogaster* — from microbial recognition to whole-organism physiology. Nature Reviews Immunology, 14, 796-810.

• BULET, P., URGE, L., OHRESSER, S., HETRU, C. & OTVOS JR, L. 1996. Enlarged scale chemical synthesis and range of activity of drosocin, an O-glycosylated antibacterial peptide of *Drosophila*. European Journal of Biochemistry, 238, 64-69.

• BURTIS, K. C. & BAKER, B. S. 1989. *Drosophila* doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell, 56, 997-1010.

• CAPUANO, A., ROSSI, F. & PAOLISSO, G. 2020. Covid-19 Kills More Men Than Women: An Overview of Possible Reasons. Frontiers in Cardiovascular Medicine, 7, 131.

• CARTON, Y., BOULETREAU, M., ALPHEN, J. & VAN LENTEREN, J. 1986. The *Drosophila* parasitic wasps.

• CASE, L. K., TOUSSAINT, L., MOUSSAWI, M., ROBERTS, B., SALIGRAMA, N., BROSSAY, L., HUBER, S. A. & TEUSCHER, C. 2012. Chromosome Y regulates survival following murine Coxsackievirus B3 infection. G3: Genes| Genomes| Genetics, 2, 115-121.

• CASE, L. K., WALL, E. H., DRAGON, J. A., SALIGRAMA, N., KREMENTSOV, D. N., MOUSSAWI, M., ZACHARY, J. F., HUBER, S. A., BLANKENHORN, E. P. & TEUSCHER, C. 2013. The Y chromosome as a regulatory element shaping immune

cell transcriptomes and susceptibility to autoimmune disease. Genome Research, 23, 1474-85.

• CASTLEMAN, M. J., POKHREL, S., TRIPLETT, K. D., KUSEWITT, D. F., ELMORE, B. O., JOYNER, J. A., FEMLING, J. K., SHARMA, G., HATHAWAY, H. J., PROSSNITZ, E. R. & HALL, R. 2018. Innate Sex Bias of *Staphylococcus aureus* Skin Infection Is Driven by α-Hemolysin. Journal of Immunology, 200, 657-668.

• CATTENOZ, P. B., SAKR, R., PAVLIDAKI, A., DELAPORTE, C., RIBA, A., MOLINA, N., HARIHARAN, N., MUKHERJEE, T. & GIANGRANDE, A. 2020. Temporal specificity and heterogeneity of *Drosophila* immune cells. EMBO Journal, 39, e104486.

• CHANG, P. L., DUNHAM, J. P., NUZHDIN, S. V. & ARBEITMAN, M. N. 2011. Somatic sex-specific transcriptome differences in *Drosophila* revealed by whole transcriptome sequencing. BMC Genomics, 12, 364.

• CHARROUX, B. & ROYET, J. 2009. Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the *Drosophila* immune response. Proceedings of the National Academy of Sciences USA, 106, 9797-802.

• CHENG, L. W., VIALA, J. P. M., STUURMAN, N., WIEDEMANN, U., VALE, R. D. & PORTNOY, D. A. 2005. Use of RNA interference in *Drosophila* S2 cells to identify host pathways controlling compartmentalization of an intracellular pathogen. Proceedings of the National Academy of Sciences USA, 102, 13646-13651.

• CHO, B., YOON, S.-H., LEE, D., KORANTENG, F., TATTIKOTA, S. G., CHA, N., SHIN, M., DO, H., HU, Y. & OH, S. Y. 2020. Single-cell transcriptome maps of myeloid blood cell lineages in *Drosophila*. Nature Communications, 11, 4483.

• CHOWDHURY, M., LI, C.-F., HE, Z., LU, Y., LIU, X.-S., WANG, Y.-F., IP, Y. T., STRAND, M. R. & YU, X.-Q. 2019. Toll family members bind multiple Spätzle proteins and activate antimicrobial peptide gene expression in *Drosophila*. Journal of Biological Chemistry, 294, 10172-10181.

• CHOWDHURY, T., CALHOUN, R. M., BRUCH, K. & MOEHRING, A. J. 2020. The fruitless gene affects female receptivity and species isolation. Proceedings of the Royal Society B: Biological Sciences, 287, 20192765.

• CLEMMONS, A. W., LINDSAY, S. A. & WASSERMAN, S. A. 2015. An effector peptide family required for *Drosophila* Toll-mediated immunity. PLOS Pathogens, 11, 4876.

• CLINE, T. W. 1988. Evidence that sisterless-a and sisterless-b are two of several discrete "numerator elements" of the X/A sex determination signal in *Drosophila* that switch Sxl between two alternative stable expression states. Genetics, 119, 829-62.

• COCIANCICH, S., GHAZI, A., HETRU, C., HOFFMANN, J. A. & LETELLIER, L. 1993. Insect defensin, an inducible antibacterial peptide, forms voltage-dependent channels in *Micrococcus luteus*. Journal of Biological Chemistry, 268, 19239-19245.

• CORREA-DE-ARAUJO, R. 2006. Serious gaps: how the lack of sex/gender-based research impairs health. Journal Women's Health (Larchmt), 15, 1116-22.

• COUSTAU, C., CARTON, Y., NAPPL, A., SHOTKOSKI, F. & FFRENCH-CONSTANT, R. 1996. Differential induction of antibacterial transcripts in *Drosophila* susceptible and resistant to parasitism by *Leptopilina boulardi*. Insect Molecular Biology, 5, 167-172.

• COX, K. H., BONTHUIS, P. J. & RISSMAN, E. F. 2014. Mouse model systems to study sex chromosome genes and behaviour: relevance to humans. Frontiers in Neuroendocrinology, 35, 405-19.

• CUI, C., YANG, W., SHI, J., ZHOU, Y., YANG, J., CUI, Q. & ZHOU, Y. 2018. Identification and analysis of human sex-biased microRNAs. Genomics, proteomics & bioinformatics, 16, 200-211.

• CUTTELL, L., VAUGHAN, A., SILVA, E., ESCARON, C. J., LAVINE, M., VAN GOETHEM, E., EID, J.-P., QUIRIN, M. & FRANC, N. C. 2008. Undertaker, a *Drosophila* Junctophilin, Links Draper-Mediated Phagocytosis and Calcium Homeostasis. Cell, 135, 524-534.

• D'AGOSTINO, P., MILANO, S., BARBERA, C., DI BELLA, G., LA ROSA, M., FERLAZZO, V., FARRUGGIO, R., MICELI, D. M., MIELE, M., CASTAGNETTA, L. & CILLARI, E. 1999. Sex hormones modulate inflammatory mediators produced by macrophages. Annals of New York Academy of Sciences, 876, 426-9.

• DE GREGORIO, E., HAN, S. J., LEE, W. J., BAEK, M. J., OSAKI, T., KAWABATA, S., LEE, B. L., IWANAGA, S., LEMAITRE, B. & BREY, P. T. 2002. An immune-responsive Serpin regulates the melanization cascade in *Drosophila*. Dev Cell, 3, 581-92.

• DE GREGORIO, E., SPELLMAN, P. T., RUBIN, G. M. & LEMAITRE, B. 2001. Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. Proceeding of the National Academy of Sciences USA, 98, 12590-5.

• DEFALCO, T., CAMARA, N., LE BRAS, S. & VAN DOREN, M. 2008. Nonautonomous Sex
Determination Controls Sexually Dimorphic Development of the *Drosophila* Gonad. Developmental Cell, 14, 275-286.

• DEFAYE, A., EVANS, I., CROZATIER, M., WOOD, W., LEMAITRE, B. & LEULIER, F. 2009. Genetic ablation of *Drosophila* phagocytes reveals their contribution to both development and resistance to bacterial infection. Journal of Innate Immunology, 1, 322-34.

• DEMIR, E. & DICKSON, B. J. 2005. fruitless splicing specifies male courtship behaviour in *Drosophila*. Cell, 121, 785-94.

• DENY, M., ARROBA NUÑEZ, L. A., ROMANO, M., DENIS, O., CASIMIR, G. & CHAMEKH, M. 2022. Sex difference in innate inflammatory response and macrophage polarization in *Streptococcus agalactiae*-induced pneumonia and potential role of microRNA-2233p. Scientific Reports, 12, 17126.

• DI CARA, F., SHESHACHALAM, A., BRAVERMAN, N. E., RACHUBINSKI, R. A. & SIMMONDS, A. J. 2017. Peroxisome-Mediated Metabolism Is Required for Immune Response to Microbial Infection. Immunity, 47, 93-106.e7.

• DIAS, S. P., BROUWER, M. C. & BEEK, D. V. D. 2022. Sex and Gender Differences in Bacterial Infections. Infection and Immunity, 90, e00283-22.

• DOSTÁLOVÁ, A., ROMMELAERE, S., POIDEVIN, M. & LEMAITRE, B. 2017. Thioester-containing proteins regulate the Toll pathway and play a role in *Drosophila* defence against microbial pathogens and parasitoid wasps. BMC Biology, 15, 79.

• DUDZIC, J. P., KONDO, S., UEDA, R., BERGMAN, C. M. & LEMAITRE, B. 2015. *Drosophila* innate immunity: regional and functional specialization of prophenoloxidases. BMC Biology, 13, 1-16.

• DUNEAU, D. F., KONDOLF, H. C., IM, J. H., ORTIZ, G. A., CHOW, C., FOX, M. A., EUGÉNIO, A. T., REVAH, J., BUCHON, N. & LAZZARO, B. P. 2017b. The Toll pathway underlies host sexual dimorphism in resistance to both Gram-negative and Gram-positive bacteria in mated *Drosophila*. BMC Biology, 15, 1-17.

• EDWARDS, D. P. 2005. Regulation of signal transduction pathways by estrogen and progesterone. Annual Review of Physiology, 67, 335-376.

• EKENGREN, S. & HULTMARK, D. 1999. *Drosophila* cecropin as an antifungal agent. Insect Biochemistry and Molecular Biology, 29, 965-972.

• EKENGREN, S. & HULTMARK, D. 2001. A Family of Turandot-Related Genes in the Humoral Stress Response of *Drosophila*. Biochemical and Biophysical Research Communications, 284, 998-1003.

• FEHLBAUM, P., BULET, P., MICHAUT, L., LAGUEUX, M., BROEKAERT, W. F., HETRU, C. & HOFFMANN, J. A. 1994. Insect immunity. Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. Journal of Biological Chemistry, 269, 33159-33163.

• FLANNAGAN, R. S., HEIT, B. & HEINRICHS, D. E. 2015. Antimicrobial Mechanisms of Macrophages and the Immune Evasion Strategies of *Staphylococcus aureus*. Pathogens, 4, 826-68.

• FLATT, T., HEYLAND, A., RUS, F., PORPIGLIA, E., SHERLOCK, C., YAMAMOTO, R., GARBUZOV, A., PALLI, S. R., TATAR, M. & SILVERMAN, N. 2008. Hormonal regulation of the humoral innate immune response in *Drosophila melanogaster*. Journal of Experimental Biology, 211, 2712-2724.

• FRANC, N. C., DIMARCQ, J.-L., LAGUEUX, M., HOFFMANN, J. & EZEKOWITZ, R. A. B. 1996. Croquemort, A Novel *Drosophila* Hemocyte/Macrophage Receptor that Recognizes Apoptotic Cells. Immunity, 4, 431-443.

• FREEMAN, M. R., DELROW, J., KIM, J., JOHNSON, E. & DOE, C. Q. 2003. Unwrapping Glial Biology: Gcm Target Genes Regulating Glial Development, Diversification, and Function. Neuron, 38, 567-580.

• FURUKAWA, K., ITOH, K., OKAMURA, K., KUMAGAI, K. & SUZUKI, M. 1984. Changes in NK cell activity during the estrous cycle and pregnancy in mice. Journal of Reproductive Immunology, 6, 353-63.

• GALLI, G. & SALEH, M. 2021. Immunometabolism of macrophages in bacterial infections. Frontiers in Cellular and Infection Microbiology, 10, 607650.

• GARCÍA-GÓMEZ, E., GONZÁLEZ-PEDRAJO, B. & CAMACHO-ARROYO, I. 2013. Role of sex steroid hormones in bacterial-host interactions. Biomed Research International, 2013, 928290.

GARCÍA-LARA, J., NEEDHAM, A. J. & FOSTER, S. J. 2005. Invertebrates as animal models for *Staphylococcus aureus* pathogenesis: a window into host-pathogen interaction. FEMS Immunology and Medical Microbiology, 43, 311-23.

• GARCIA-MORALES, C., NANDI, S., ZHAO, D., SAUTER, K. A., VERVELDE, L., MCBRIDE, D., SANG, H. M., CLINTON, M. & HUME, D. A. 2015. Cell-autonomous sex differences in gene expression in chicken bone marrow-derived macrophages. Journal of Immunology, 194, 2338-44.

• GARG, A. & WU, L. P. 2014. *Drosophila* Rab14 mediates phagocytosis in the immune response to Staphylococcus aureus. Cellular Microbiology, 16, 296-310.

• GARVER, L. S., WU, J. & WU, L. P. 2006. The peptidoglycan recognition protein PGRP-
SC1a is essential for Toll signalling and phagocytosis of *Staphylococcus aureus* in *Drosophila*. Proceedings of the National Academy of Sciences USA, 103, 660-5.

• GAUMER, S., GUÉNAL, I., BRUN, S., THÉODORE, L. & MIGNOTTE, B. 2000. Bcl-2 and Bax mammalian regulators of apoptosis are functional in *Drosophila*. Cell Death & Differentiation, 7, 804-14.

• GEORGEL, P., NAITZA, S., KAPPLER, C., FERRANDON, D., ZACHARY, D., SWIMMER, C., KOPCZYNSKI, C., DUYK, G., REICHHART, J. M. & HOFFMANN, J. A. 2001. *Drosophila* immune deficiency (IMD) is a death domain protein that activates antibacterial defence and can promote apoptosis. Developmental Cell, 1, 503-14.

• GHORAI, A. & GHOSH, U. 2014. miRNA gene counts in chromosomes vary widely in a species and biogenesis of miRNA largely depends on transcription or posttranscriptional processing of coding genes. Frontiers in Genetics, 5, 100.

• GHOSH, S., SINGH, A., MANDAL, S. & MANDAL, L. 2015. Active hematopoietic hubs in *Drosophila* adults generate hemocytes and contribute to immune response. Developmental Cell, 33, 478-88.

• GILBERT, S. F. 2000. Chromosomal Sex Determination in Mammals. Developmental Biology. Sunderland: Sinauer Associates.

• GOTO, A., KUMAGAI, T., KUMAGAI, C., HIROSE, J., NARITA, H., MORI, H., KADOWAKI, T., BECK, K. & KITAGAWA, Y. 2001. A *Drosophila* haemocyte-specific protein, hemolectin, similar to human von Willebrand factor. Biochemical Journal, 359, 99-108.

• HANNAH, M. F., BAJIC, V. B. & KLEIN, S. L. 2008. Sex differences in the recognition of and innate antiviral responses to Seoul virus in Norway rats. Brain, Behaviour, Immunity, 22, 503-16.

• HASHIMOTO, Y., TABUCHI, Y., SAKURAI, K., KUTSUNA, M., KUROKAWA, K., AWASAKI, T., SEKIMIZU, K., NAKANISHI, Y. & SHIRATSUCHI, A. 2009. Identification of lipoteichoic acid as a ligand for draper in the phagocytosis of *Staphylococcus aureus* by *Drosophila* hemocytes. Journal of Immunology, 183, 7451-60.

• HEDENGREN, M., BORGE, K. & HULTMARK, D. 2000. Expression and evolution of the *Drosophila* attacin/diptericin gene family. Biochemical and biophysical research communications, 279, 574-581.

• HILL-BURNS, E. M. & CLARK, A. G. 2009. X-linked variation in immune response in *Drosophila melanogaster*. Genetics, 183, 1477-91.

• HIRAYAMA, D., IIDA, T. & NAKASE, H. 2017. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. International Journal of Molecular Sciences, 19.

• HONTI, V., CSORDÁS, G., KURUCZ, É., MÁRKUS, R. & ANDÓ, I. 2014. The cell-mediated immunity of *Drosophila melanogaster*: hemocyte lineages, immune compartments, microanatomy and regulation. Developmental & Comparative Immunology, 42, 47-56.

• HORI, A., KURATA, S. & KURAISHI, T. 2018. Unexpected role of the IMD pathway in *Drosophila* gut defence against *Staphylococcus aureus*. Biochemical and Biophysical Research Communications, 495, 395-400.

• HORN, L., LEIPS, J. & STARZ-GAIANO, M. 2014. Phagocytic ability declines with age in adult *Drosophila* hemocytes. Aging Cell, 13, 719-728.

• HORSBURGH, M. J., CLEMENTS, M. O., CROSSLEY, H., INGHAM, E. & FOSTER, S. J. 2001. PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. Infection and Immunity, 69, 3744-54.

• HORSBURGH, M. J., WILTSHIRE, M. D., CROSSLEY, H., INGHAM, E. & FOSTER, S. J. 2004. PheP, a putative amino acid permease of *Staphylococcus aureus*, contributes to survival *in vivo* and during starvation. Infection and Immunity, 72, 3073-6.

• HORTON, K. C., MACPHERSON, P., HOUBEN, R. M. G. J., WHITE, R. G. & CORBETT, E. L. 2016. PLOS Medicine, 13, e1002119.

• HOU, J. & ZHENG, W. F. 1988. Effect of sex hormones on NK and ADCC activity of mice. International Immunopharmacology, 10, 15-22.

• HUMPHREYS, H., FITZPATICK, F. & HARVEY, B. J. 2015. Gender Differences in Rates of Carriage and Bloodstream Infection Caused by Methicillin-Resistant *Staphylococcus aureus*: Are They Real, Do They Matter and Why? Clinical Infectious Diseases, 61, 17081714.

• IKUTA, K. S., SWETSCHINSKI, L. R., ROBLES AGUILAR, G., SHARARA, F., MESTROVIC, T., GRAY, A. P., DAVIS WEAVER, N., WOOL, E. E., HAN, C., GERSHBERG HAYOON, A., AALI, A., ABATE, S. M., ABBASI-KANGEVARI, M., ABBASI-KANGEVARI, Z., ABD-ELSALAM, S., ABEBE, G., ABEDI, A., ABHARI, A. P., ABIDI, H., ABOAGYE, R. G., ABSALAN, A., ABUBAKER ALI, H., ACUNA, J. M., ADANE, T. D., ADDO, I. Y., ADEGBOYE, O. A., ADNAN, M., ADNANI, Q. E. S., AFZAL, M. S., AFZAL, S., AGHDAM, Z. B., AHINKORAH, B. O., AHMAD, A., AHMAD, A. R., AHMAD, R., AHMAD, S., AHMAD, S., AHMADI, S., AHMED, A., AHMED, H., AHMED, J. Q., AHMED RASHID, T., AJAMI, M., AJI, B., AKBARZADEH-KHIAVI, M., AKUNNA, C. J., AL HAMAD, H., ALAHDAB, F., AL-ALY, Z., ALDEYAB, M. A., ALEMAN, A. V., ALHALAIQA, F. A. N., ALHASSAN, R. K., ALI, B. A., ALI, L., ALI, S. S., ALIMOHAMADI, Y., ALIPOUR, V., ALIZADEH, A., ALJUNID, S. M., ALLEL, K., ALMUSTANYIR, S., AMEYAW, E. K., AMIT, A. M. L., ANANDAVELANE, N., ANCUCEANU, R., ANDREI, C. L., ANDREI, T., ANGGRAINI, D., ANSAR, A., ANYASODOR, A. E., ARABLOO, J., ARAVKIN, A. Y., AREDA, D., ARIPOV, T., ARTAMONOV, A. A., ARULAPPAN, J., ARULEBA, R. T., ASADUZZAMAN, M., ASHRAF, T., ATHARI, S. S., ATLAW, D., ATTIA, S., AUSLOOS, M., AWOKE, T., AYALA QUINTANILLA, B. P., AYANA, T. M., AZADNAJAFABAD, S., AZARI JAFARI, A., B, D. B., BADAR, M., BADIYE, A. D., BAGHCHEGHI, N., BAGHERIEH, S., BAIG, A. A., BANERJEE, I., BARAC, A., BARDHAN, M., BARONE-ADESI, F., BARQAWI, H. J., et al. 2022. Global mortality associated with 33 bacterial pathogens in 2019: a systematic analysis for the Global Burden of Disease Study 2019. The Lancet, 400, 2221-2248.

• INOUE, K., HOSHIJIMA, K., HIGUCHI, I., SAKAMOTO, H. & SHIMURA, Y. 1992. Binding of the *Drosophila* transformer and transformer-2 proteins to the regulatory elements of doublesex primary transcript for sex-specific RNA processing. Proceedings of the National Academy of Sciences, 89, 8092-8096.

• JAILLON, S., BERTHENET, K. & GARLANDA, C. 2019. Sexual Dimorphism in Innate Immunity. Clinical Reviews in Allergy & Immunology, 56, 308-321.

• JOO, H. S., FU, C. I. & OTTO, M. 2016. Bacterial strategies of resistance to antimicrobial peptides. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 371.

• KANEKO, T., GOLDMAN, W. E., MELLROTH, P., STEINER, H., FUKASE, K., KUSUMOTO, S., HARLEY, W., FOX, A., GOLENBOCK, D. & SILVERMAN, N. 2004. Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the *Drosophila* IMD pathway. Immunity, 20, 637-49.

• KEITH, S. A. 2023. Steroid hormone regulation of innate immunity in *Drosophila melanogaster*. PLOS Genetics, 19, e1010782.

• KEMP, C., MUELLER, S., GOTO, A., BARBIER, V., PARO, S., BONNAY, F., DOSTERT, C., TROXLER, L., HETRU, C., MEIGNIN, C., PFEFFER, S., HOFFMANN, J. A. & IMLER, J. L. 2013. Broad RNA interference-mediated antiviral immunity and virus-specific inducible responses in *Drosophila*. Journal of Immunology, 190, 650-8.

• KENNEDY, A. D., BUBECK WARDENBURG, J., GARDNER, D. J., LONG, D., WHITNEY, A. R., BRAUGHTON, K. R., SCHNEEWIND, O. & DELEO, F. R. 2010. Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. Journal of Infectious Diseases, 202, 1050-8.

• KITUR, K., PARKER, D., NIETO, P., AHN, D. S., COHEN, T. S., CHUNG, S., WACHTEL, S., BUENO, S. & PRINCE, A. 2015. Toxin-induced necroptosis is a major mechanism of *Staphylococcus aureus* lung damage. PLOS Pathogens, 11, e1004820.

• KLEIN, S. L. 2012. Immune Cells Have Sex and So Should Journal Articles. Endocrinology, 153, 2544-2550.

• KLEIN, S. L. & ROBERTS, C. 2010. Sex hormones and immunity to infection, Springer.

• KLINGE, I. 2008. Gender perspectives in European research. Pharmacology Research, 58, 183-9.

• KOCKS, C., CHO, J. H., NEHME, N., ULVILA, J., PEARSON, A. M., MEISTER, M., STROM,

C., CONTO, S. L., HETRU, C., STUART, L. M., STEHLE, T., HOFFMANN, J. A., REICHHART, J.-M., FERRANDON, D., RÄMET, M. & EZEKOWITZ, R. A. B. 2005. Eater, a Transmembrane Protein Mediating Phagocytosis of Bacterial Pathogens in *Drosophila*. Cell, 123, 335-346.

KONTULA, K. S. K., SKOGBERG, K., OLLGREN, J., JÄRVINEN, A. & LYYTIKÄINEN, O. 2021. Population-Based Study of Bloodstream Infection Incidence and Mortality Rates, Finland, 2004-2018. Emerging Infectious Diseases, 27, 2560-9.

• KREJČOVÁ, G., DANIELOVÁ, A., NEDBALOVÁ, P., KAZEK, M., STRYCH, L., CHAWLA, G., TENNESSEN, J. M., LIESKOVSKÁ, J., JINDRA, M., DOLEŽAL, T. & BAJGAR, A. 2019. *Drosophila* macrophages switch to aerobic glycolysis to mount effective antibacterial defense. eLife, 8, e50414.

• KUBICA, M., GUZIK, K., KOZIEL, J., ZAREBSKI, M., RICHTER, W., GAJKOWSKA, B., GOLDA, A., MACIAG-GUDOWSKA, A., BRIX, K., SHAW, L., FOSTER, T. & POTEMPA, J. 2008. A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. PLOS One, 3, e1409.

• KURUCZ, É., MÁRKUS, R., ZSÁMBOKI, J., FOLKL-MEDZIHRADSZKY, K., DARULA, Z., VILMOS, P., UDVARDY, A., KRAUSZ, I., LUKACSOVICH, T., GATEFF, E., ZETTERVALL, C.-J.,
HULTMARK, D. & ANDÓ, I. 2007. Nimrod, a Putative Phagocytosis Receptor with EGF Repeats in *Drosophila* Plasmatocytes. Current Biology, 17, 649-654.

• KUTCH, I. & FEDORKA, K. 2017. A test for Y-linked additive and epistatic effects on surviving bacterial infections in *Drosophila melanogaster*. Journal of Evolutionary Biology, 30, 1400-1408.

• KWIECINSKI, J. M. & HORSWILL, A. R. 2020. *Staphylococcus aureus* bloodstream infections: pathogenesis and regulatory mechanisms. Current Opinion in Microbiology, 53, 51-60.

• LANOT, R., ZACHARY, D., HOLDER, F. & MEISTER, M. 2001. Postembryonic hematopoiesis in *Drosophila*. Developmental Biology, 230, 243-57.

• LAWRENCE, P. A. & JOHNSTON, P. 1986. The muscle pattern of a segment of *Drosophila* may be determined by neurons and not by contributing myoblasts. Cell, 45, 505-513.

• LEE, A. S., DE LENCASTRE, H., GARAU, J., KLUYTMANS, J., MALHOTRA-KUMAR, S., PESCHEL, A. & HARBARTH, S. 2018a. Methicillin-resistant *Staphylococcus aureus*. Nature Reviews Disease Primers, 4, 18033.
• LEE, Y.-J., JANG, H.-J., CHUNG, I.-Y. & CHO, Y.-H. 2018b. *Drosophila melanogaster* as a polymicrobial infection model for *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Journal of Microbiology, 56, 534-541.

• LEECH, T., EVISON, S. E. F., ARMITAGE, S. A. O., SAIT, S. M. & BRETMAN, A. 2019b. Interactive effects of social environment, age and sex on immune responses in *Drosophila melanogaster*. Journal of Evolutionary Biology, 32, 1082-1092.

• LEITÃO, A. B., ARUNKUMAR, R., DAY, J. P., GELDMAN, E. M., MORIN-POULARD, I., CROZATIER, M. & JIGGINS, F. M. 2020. Constitutive activation of cellular immunity underlies the evolution of resistance to infection in *Drosophila*. eLife, 9, e59095.

• LEMAITRE, B. & HOFFMANN, J. 2007. The host defense of *Drosophila melanogaster*.
Annual Review of Immunology, 25, 697-743.

• LEULIER, F., PARQUET, C., PILI-FLOURY, S., RYU, J. H., CAROFF, M., LEE, W. J., MENGINLECREULX, D. & LEMAITRE, B. 2003. The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. Nature Immunology, 4, 478-84.

• LEVASHINA, E. A., OHRESSER, S., BULET, P., REICHHART, J. M., HETRU, C. & HOFFMANN, J. A. 1995. Metchnikowin, a Novel Immune-Inducible Proline-Rich Peptide from *Drosophila* with Antibacterial and Antifungal Properties. European Journal of Biochemistry, 233, 694-700.

• LEVIN, T. C. & MALIK, H. S. 2017. Rapidly evolving Toll-3/4 genes encode male-specific Toll-like receptors in *Drosophila*. Molecular Biology and Evolution, 34, 2307-2323.

• LI, K., XU, W., GUO, Q., JIANG, Z., WANG, P., YUE, Y. & XIONG, S. 2009. Differential
Macrophage Polarization in Male and Female BALB/c Mice Infected with Coxsackievirus B3 Defines Susceptibility to Viral Myocarditis. Circulation Research, 105, 353-364.

• LIBERT, C., DEJAGER, L. & PINHEIRO, I. 2010. The X chromosome in immune functions: when a chromosome makes the difference. Nature Reviews Immunology, 10, 594-604.

• LIU, G. Y., ESSEX, A., BUCHANAN, J. T., DATTA, V., HOFFMAN, H. M., BASTIAN, J. F., FIERER, J. & NIZET, V. 2005. *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. Journal of Experimental Medicine, 202, 209-15.

• LIU, K. A. & MAGER, N. A. 2016. Women's involvement in clinical trials: historical perspective and future implications. Pharmacy Practice (Granada), 14, 708.

LIVA, S. M. & VOSKUHL, R. R. 2001. Testosterone acts directly on CD4+ T lymphocytes to increase IL-10 production. Journal of Immunology, 167, 2060-7.

• LÜ, F. X., ABEL, K., MA, Z., ROURKE, T., LU, D., TORTEN, J., MCCHESNEY, M. & MILLER, C. J. 2002. The strength of B cell immunity in female rhesus macaques is controlled by CD8+ T cells under the influence of ovarian steroid hormones. Clinical and Experimental Immunology, 128, 10-20.

• MANOLAKOU, P., LAVRANOS, G. & ANGELOPOULOU, R. 2006. Molecular patterns of sex determination in the animal kingdom: a comparative study of the biology of reproduction. Reproductive Biology and Endocrinology, 4, 59.

• MARTIN, F. J., PARKER, D., HARFENIST, B. S., SOONG, G. & PRINCE, A. 2011. Participation of CD11c(+) leukocytes in methicillin-resistant *Staphylococcus aureus* clearance from the lung. Infection and Immunity, 79, 1898-904.

• MARTIN, R. A. & TATE, A. T. 2023. Pleiotropy promotes the evolution of inducible immune responses in a model of host-pathogen coevolution. PLOS Computational Biology, 19, e1010445.

• MASE, A., AUGSBURGER, J. & BRÜCKNER, K. 2021. Macrophages and Their Organ
Locations Shape Each Other in Development and Homeostasis - A *Drosophila* Perspective. Frontiers in Cell and Developmental Biology, 9, 630272.

• MCKEOWN, M., BELOTE, J. M. & BAKER, B. S. 1987. A molecular analysis of transformer, a gene in *Drosophila melanogaster* that controls female sexual differentiation. Cell, 48, 489-499.

• MEDITZ, A. L., MAWHINNEY, S., ALLSHOUSE, A., FESER, W., MARKOWITZ, M., LITTLE, S., HECHT, R., DAAR, E. S., COLLIER, A. C., MARGOLICK, J., KILBY, J. M., ROUTY, J. P., CONWAY, B., KALDOR, J., LEVY, J., SCHOOLEY, R., COOPER, D. A., ALTFELD, M., RICHMAN, D. & CONNICK, E. 2011. Sex, race, and geographic

region influence clinical outcomes following primary HIV-1 infection. Journal of Infectious Diseases, 203, 442-51.

• MEISTER, M., BRAUN, A., KAPPLER, C., REICHHART, J.-M. & HOFFMANN, J. A. 1994. Insect immunity. A transgenic analysis in *Drosophila* defines several functional domains in the diptericin promoter. The EMBO Journal, 13, 5958-5966.

• MEISTER, M., LEMAITRE, B. & HOFFMANN, J. A. 1997. Antimicrobial peptide defense in *Drosophila*. BioEssays, 19, 1019-26.

MELCARNE, C., RAMOND, E., DUDZIC, J., BRETSCHER, A. J., KURUCZ, É., ANDÓ, I. & LEMAITRE, B. 2019. Two Nimrod receptors, NimC1 and Eater, synergistically contribute to bacterial phagocytosis in *Drosophila melanogaster*. FEBS Journal, 286, 2670-2691.
• MIGEON, B. R. 2007. Why females are mosaics, X-chromosome inactivation, and sex differences in disease. Gender Medicine, 4, 97-105.

• MILLER, L. & HUNT, J. S. 1996. Sex steroid hormones and macrophage function. Life Sciences, 59, 1-14.

• MOHUS, R. M., GUSTAD, L. T., FURBERG, A.-S., MOEN, M. K., LIYANARACHI, K. V., ASKIM, Å., ÅSBERG, S. E., DEWAN, A. T., ROGNE, T., SIMONSEN, G. S., NILSEN, T. I. L., ÅSVOLD, B. O., DAMÅS, J. K. & SOLLIGÅRD, E. 2022. Explaining sex differences in risk of bloodstream infections using mediation analysis in the population-based HUNT study in Norway. Scientific Reports, 12, 8436.

• MONTEITH, K. 2018. Recipe for apple-agar embryo collection plates [Online]. University of Edinburgh. Available: https://www.protocols.io/view/recipefor-apple-agar-embryo-collection-plates-36wgqw9xgk57/v1 [Accessed].

• MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. Nature Reviews Immunology, 8, 958-69.

• MURRAY, P. J., ALLEN, J. E., BISWAS, S. K., FISHER, E. A., GILROY, D. W., GOERDT, S., GORDON, S., HAMILTON, J. A., IVASHKIV, L. B. & LAWRENCE, T. 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity, 41, 14-20.

• MYLLYMÄKI, H. & RÄMET, M. 2014. JAK/STAT pathway in *Drosophila* immunity. Scandinavian Journal of Immunology, 79, 377-385.

• NABER, C. K. 2009. *Staphylococcus aureus* Bacteremia: Epidemiology, Pathophysiology, and Management Strategies. Clinical Infectious Diseases, 48, S231-S237.
• NAPPI, A., POIRIÉ, M. & CARTON, Y. 2009. The role of melanization and cytotoxic byproducts in the cellular immune responses of *Drosophila* against parasitic wasps. Advances in Parasitology, 70, 99-121.

• NEEDHAM, A. J., KIBART, M., CROSSLEY, H., INGHAM, P. W. & FOSTER, S. J. 2004. *Drosophila melanogaster* as a model host for *Staphylococcus aureus* infection. Microbiology, 150, 2347-2355.

• NEHME, N. T., QUINTIN, J., CHO, J. H., LEE, J., LAFARGE, M.-C., KOCKS, C. & FERRANDON, D. 2011. Relative Roles of the Cellular and Humoral Responses in the *Drosophila* Host Defense against Three Gram-Positive Bacterial Infections. PLOS One, 6, e14743.

• NELLIOT, A., BOND, N. & HOSHIZAKI, D. K. 2006. Fat-body remodeling in *Drosophila melanogaster*. Genesis, 44, 396-400.

• NELSON, R. E., FESSLER, L. I., TAKAGI, Y., BLUMBERG, B., KEENE, D. R., OLSON, P. F., PARKER, C. G. & FESSLER, J. H. 1994. Peroxidasin: a novel enzyme-matrix protein of *Drosophila* development. EMBO Journal, 13, 3438-47.

• NONAKA, S., NAGAOSA, K., MORI, T., SHIRATSUCHI, A. & NAKANISHI, Y. 2013. Integrin-mediated Phagocytosis of Apoptotic Cells and Bacteria in *Drosophila*. Journal of Biological Chemistry, 288, 10374-10380.

• NUNES, C., SUCENA, É. & KOYAMA, T. 2021. Endocrine regulation of immunity in insects. The FEBS Journal, 288, 3928-3947.

• OBIANDU, C., OKERENGWO, A. A. & DAPPER, D. V. 2013. Levels of serum immunoglobulins in apparently healthy children and adults in Port Harcourt, Nigeria. Nigerian Journal of Physiological Sciences, 28, 23-7.

• OSTERWALDER, T., YOON, K. S., WHITE, B. H. & KESHISHIAN, H. 2001. A conditional tissue-specific transgene expression system using inducible GAL4. Proceedings of the National Academy of Sciences USA, 98, 12596-601.

• PEACOCK, S. J., JUSTICE, A., GRIFFITHS, D., DE SILVA, G. D., KANTZANOU, M. N., CROOK, D., SLEEMAN, K. & DAY, N. P. 2003. Determinants of acquisition and carriage of *Staphylococcus aureus* in infancy. Journal of Clinical Microbiology, 41, 5718-25.

• PEARSON, A., LUX, A. & KRIEGER, M. 1995. Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*. Proceedings of the National Academy of Sciences, 92, 4056-4060.

• PEARSON, A. M., BAKSA, K., RÄMET, M., PROTAS, M., MCKEE, M., BROWN, D. & EZEKOWITZ, R. A. B. 2003. Identification of cytoskeletal regulatory proteins required for efficient phagocytosis in *Drosophila*. Microbes and Infection, 5, 815-824.

• PEPE, G., BRAGA, D., RENZI, T. A., VILLA, A., BOLEGO, C., D'AVILA, F., BARLASSINA, C., MAGGI, A., LOCATI, M. & VEGETO, E. 2017. Self-renewal and phenotypic conversion are the main physiological responses of macrophages to the endogenous estrogen surge. Scientific reports, 7, 44270.

• PERRIMON, N. & MAHOWALD, A. P. 1986. l (1) hopscotch, a larval-pupal zygotic lethal with a specific maternal effect on segmentation in *Drosophila*. Developmental biology, 118, 28-41.

• PHE 2019. Laboratory surveillance of *Staphylococcus aureus* bloodstream infections in England, Wales and Northern Ireland: 2018.

• PHILIPS, J. A., RUBIN, E. J. & PERRIMON, N. 2005. *Drosophila* RNAi Screen Reveals CD36 Family Member Required for Mycobacterial Infection. Science, 309, 1251-1253.

• PIASECKA, B., DUFFY, D., URRUTIA, A., QUACH, H., PATIN, E., POSSEME, C., BERGSTEDT, J., CHARBIT, B., ROUILLY, V., MACPHERSON, C. R., HASAN, M., ALBAUD, B., GENTIEN, D., FELLAY, J., ALBERT, M. L. & QUINTANA-MURCI, L. 2018. Distinctive roles of age, sex, and genetics in shaping transcriptional variation of human immune responses to microbial challenges. Proceeding of the National Academy of Sciences USA, 115, E488-e497.

• PIDWILL, G. R., GIBSON, J. F., COLE, J., RENSHAW, S. A. & FOSTER, S. J. 2020. The Role of Macrophages in *Staphylococcus aureus* Infection. Frontiers in Immunology, 11, 620339.

• PISITKUN, P., DEANE, J. A., DIFILIPPANTONIO, M. J., TARASENKO, T., SATTERTHWAITE, A. B. & BOLLAND, S. 2006. Autoreactive B cell responses to RNA-related antigens due to TLR7 gene duplication. Science, 312, 1669-72.

• POKHREL, S., TRIPLETT, K. D., DALY, S. M., JOYNER, J. A., SHARMA, G., HATHAWAY, H. J., PROSSNITZ, E. R. & HALL, P. R. 2020. Complement Receptor 3

Contributes to the Sexual Dimorphism in Neutrophil Killing of *Staphylococcus aureus*. Journal of Immunology, 205, 15931600.

• POLAND, G. A., OVSYANNIKOVA, I. G. & JACOBSON, R. M. 2008. Personalized vaccines: the emerging field of vaccinomics. Expert Opinion on Biological Therapy, 8, 1659-67.

• POLLITT, E. J. G., SZKUTA, P. T., BURNS, N. & FOSTER, S. J. 2018. *Staphylococcus aureus* infection dynamics. PLoS Pathogens, 14, e1007112.

• PRAJSNAR, T. K., CUNLIFFE, V. T., FOSTER, S. J. & RENSHAW, S. A. 2008. A novel vertebrate model of *Staphylococcus aureus* infection reveals phagocyte-dependent resistance of zebrafish to non-host specialized pathogens. Cellular Microbiology, 10, 2312-25.

• PRAJSNAR, T. K., SERBA, J. J., DEKKER, B. M., GIBSON, J. F., MASUD, S., FLEMING, A., JOHNSTON, S. A., RENSHAW, S. A. & MEIJER, A. H. 2021. The autophagic response to *Staphylococcus aureus* provides an intracellular niche in neutrophils. Autophagy, 17, 888-902.

• PRESTINACI, F., PEZZOTTI, P. & PANTOSTI, A. 2015. Antimicrobial resistance: a global multifaceted phenomenon. Pathogens and Global Health, 109, 309-18.

• RÄMET, M., MANFRUELLI, P., PEARSON, A., MATHEY-PREVOT, B. & EZEKOWITZ, R. A. 2002. Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for *E. coli*. Nature, 416, 644-8.

• RÄMET, M., PEARSON, A., MANFRUELLI, P., LI, X., KOZIEL, H., GÖBEL, V., CHUNG, E., KRIEGER, M. & EZEKOWITZ, R. A. B. 2001. *Drosophila* Scavenger Receptor CI Is a Pattern Recognition Receptor for Bacteria. Immunity, 15, 1027-1038.

• RAMOND, E., DUDZIC, J. P. & LEMAITRE, B. 2020. Comparative RNA-Seq analyses of *Drosophila* plasmatocytes reveal gene-specific signatures in response to clean injury and septic injury. PLOS One, 15, e0235294.

• RAMOND, E., JAMET, A., DING, X., EUPHRASIE, D., BOUVIER, C., LALLEMANT, L., HE, X., ARBIBE, L., COUREUIL, M. & CHARBIT, A. 2021. Reactive Oxygen Species-Dependent Innate Immune Mechanisms Control Methicillin-Resistant *Staphylococcus aureus* Virulence in the *Drosophila* Larval Model. mBio, 12, 10.1128/mbio.00276-21.

• REGAN, J. C., BRANDÃO, A. S., LEITÃO, A. B., MANTAS DIAS, Â. R., SUCENA, É., JACINTO, A. & ZAIDMAN-RÉMY, A. 2013. Steroid Hormone Signaling Is Essential to Regulate Innate Immune Cells and Fight Bacterial Infection in *Drosophila*. PLOS Pathogens, 9, e1003720.

• REGAN, J. C., KHERICHA, M., DOBSON, A. J., BOLUKBASI, E., RATTANAVIROTKUL, N. & PARTRIDGE, L. 2016. Sex difference in pathology of the ageing gut mediates the greater response of female lifespan to dietary restriction. eLife, 5, e10956.

• REGAN, J. C., LU, Y. X., UREÑA, E., MEILENBROCK, R. L., CATTERSON, J. H., KISSLE, D., FRÖHLICH, J., FUNK, E. & PARTRIDGE, L. 2022. Sexual identity of enterocytes regulates autophagy to determine intestinal health, lifespan and responses to rapamycin. Nature Aging, 2, 1145-1158.

• REHORN, K.-P., THELEN, H., MICHELSON, A. M. & REUTER, R. 1996. A molecular aspect of hematopoiesis and endoderm development common to vertebrates and *Drosophila*. Development, 122, 4023-4031.

• RETTEW, J. A., HUET, Y. M. & MARRIOTT, I. 2009. Estrogens augment cell surface TLR4 expression on murine macrophages and regulate sepsis susceptibility in vivo. Endocrinology, 150, 3877-84.

• RETTEW, J. A., HUET-HUDSON, Y. M. & MARRIOTT, I. 2008. Testosterone reduces macrophage expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. Biology of Reproduction, 78, 432-7.

• RIBAS, V., DREW, B. G., LE, J. A., SOLEYMANI, T., DARAEI, P., SITZ, D., MOHAMMAD, L., HENSTRIDGE, D. C., FEBBRAIO, M. A. & HEWITT, S. C. 2011. Myeloid-specific estrogen receptor α deficiency impairs metabolic homeostasis and accelerates atherosclerotic lesion development. Proceedings of the National Academy of Sciences, 108, 1645716462.

• RIDKER, P. M., COOK, N. R., LEE, I.-M., GORDON, D., GAZIANO, J. M., MANSON, J. E., HENNEKENS, C. H. & BURING, J. E. 2005. A randomized trial of low-dose aspirin in the primary prevention of cardiovascular disease in women. New England Journal of Medicine, 352, 1293-1304.

• ROBERTS, C. W., WALKER, W. & ALEXANDER, J. 2001. Sex-associated hormones and immunity to protozoan parasites. Clinical Microbiology Reviews, 14, 476-88.

• ROBINETT, C. C., VAUGHAN, A. G., KNAPP, J.-M. & BAKER, B. S. 2010. Sex and the Single Cell. II. There Is a Time and Place for Sex. PLOS Biology, 8, e1000365.

• ROBINSON, D. P., HUBER, S. A., MOUSSAWI, M., ROBERTS, B., TEUSCHER, C., WATKINS, R., ARNOLD, A. P. & KLEIN, S. L. 2011. Sex chromosome complement contributes to sex differences in coxsackievirus B3 but not influenza A virus pathogenesis. Biology of Sex Differences, 2, 8.

• ROGERS, I. Development, characterisation and application of an inducible model of Alzheimer's Disease in the fruit fly, *Drosophila melanogaster*. Doctoral Thesis, University College London.

• ROLFF, J. 2002. Bateman's principle and immunity. Proceedings of the Royal Society of London. Series B: Biological Sciences, 269, 867-872.

• ROUSSET, F. & FERDY, J.-B. 2014. Testing environmental and genetic effects in the presence of spatial autocorrelation. Ecography, 37, 781-790.

• RYNER, L. C., GOODWIN, S. F., CASTRILLON, D. H., ANAND, A., VILLELLA, A., BAKER, B. S., HALL, J. C., TAYLOR, B. J. & WASSERMAN, S. A. 1996. Control of male sexual behavior and sexual orientation in *Drosophila* by the fruitless gene. Cell, 87, 1079-1089.

• SABIKUNNAHAR, B., LAHUE, K. G., ASARIAN, L., FANG, Q., MCGILL, M. M., HAYNES, L., TEUSCHER, C. & KREMENTSOV, D. N. 2022. Sex differences in susceptibility to influenza A virus infection depend on host genotype. PLOS One, 17, e0273050.

• SAIA, R. S., GARCIA, F. M. & CÁRNIO, E. C. 2015. Estradiol protects female rats against sepsis induced by Enterococcus faecalis improving leukocyte bactericidal activity. Steroids, 102, 17-26.

• SAVITA & RAI, U. 1998. Sex steroid hormones modulate the activation of murine peritoneal macrophages: receptor mediated modulation. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 119, 199-204.

• SCHLENKE, T. A., MORALES, J., GOVIND, S. & CLARK, A. G. 2007. Contrasting Infection Strategies in Generalist and Specialist Wasp Parasitoids of *Drosophila melanogaster*. PLOS Pathogens, 3, e158.

• SCHWENKE, R. A. & LAZZARO, B. P. 2017. Juvenile Hormone Suppresses Resistance to Infection in Mated Female *Drosophila melanogaster*. Current Biology, 27, 596-601.

• SCOTLAND, R. S., STABLES, M. J., MADALLI, S., WATSON, P. & GILROY, D. W. 2011. Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. Blood, 118, 5918-27.

• SERIZIER, S. B. & MCCALL, K. 2017. Scrambled Eggs: Apoptotic Cell Clearance by NonProfessional Phagocytes in the *Drosophila* Ovary. Frontiers in Immunology, 8, 1642.

• SHAH, K., MCCORMACK, C. E. & BRADBURY, N. A. 2014. Do you know the sex of your cells? American Journal of Physiology and Cell Physiology, 306, C3-18.

• SHARMA, S. & EGHBALI, M. 2014. Influence of sex differences on microRNA gene regulation in disease. Biology of Sex Differences, 5, 3.

• SHIA, A. K., GLITTENBERG, M., THOMPSON, G., WEBER, A. N., REICHHART, J. M. & LIGOXYGAKIS, P. 2009. Toll-dependent antimicrobial responses in *Drosophila* larval fat body require Spätzle secreted by haemocytes. Journal of Cell Science, 122, 4505-15.

• SHIN, M., CHA, N., KORANTENG, F., CHO, B. & SHIM, J. 2020. Subpopulation of Macrophage-Like Plasmatocytes Attenuates Systemic Growth via JAK/STAT in the *Drosophila* Fat Body. Frontiers in Immunology, 11, 63.

• SHIRATSUCHI, A., MORI, T., SAKURAI, K., NAGAOSA, K., SEKIMIZU, K., LEE, B. L. & NAKANISHI, Y. 2012. Independent Recognition of *Staphylococcus aureus* by Two Receptors for Phagocytosis in *Drosophila*. Journal of Biological Chemistry, 287, 2166321672.

• SHKLOVER, J., LEVY-ADAM, F. & KURANT, E. 2015. Apoptotic cell clearance in development. Current Topics in Developmental Biology, 114, 297-334.

• SHORT, S. M. & LAZZARO, B. P. 2010. Female and male genetic contributions to postmating immune defence in female *Drosophila melanogaster*. Proceedings of the Royal Society B: Biological Sciences, 277, 3649-3657.

• SICA, A. & MANTOVANI, A. 2012. Macrophage plasticity and polarization: in vivo veritas. Journal of Clinical Investigation, 122, 787-95.

• SINENKO, S. A. & MATHEY-PREVOT, B. 2004. Increased expression of *Drosophila* tetraspanin, Tsp68C, suppresses the abnormal proliferation of ytr-deficient and Ras/Raf-activated hemocytes. Oncogene, 23, 9120-8.

• SIWCZAK, F., CSERESNYES, Z., HASSAN, M. I. A., AINA, K. O., CARLSTEDT, S., SIGMUND, A., GROGER, M., SUREWAARD, B. G. J., WERZ, O., FIGGE, M. T., TUCHSCHERR, L., LOFFLER, B. & MOSIG, A. S. 2022. Human macrophage polarization determines bacterial persistence of *Staphylococcus aureus* in a liver-on-chip-based infection model. Biomaterials, 287, 121632.

• SORRENTINO, R. P., CARTON, Y. & GOVIND, S. 2002. Cellular immune response to parasite infection in the *Drosophila* lymph gland is developmentally regulated. Developmental Biology, 243, 65-80.

• SOUYRIS, M., CENAC, C., AZAR, P., DAVIAUD, D., CANIVET, A., GRUNENWALD, S., PIENKOWSKI, C., CHAUMEIL, J., MEJÍA, J. E. & GUÉRY, J. C. 2018. TLR7 escapes X chromosome inactivation in immune cells. Science Immunology, 3.

• SPACH, K. M., BLAKE, M., BUNN, J. Y., MCELVANY, B., NOUBADE, R., BLANKENHORN, E. P. & TEUSCHER, C. 2009. Cutting edge: the Y chromosome controls the age-dependent experimental allergic encephalomyelitis sexual dimorphism in SJL/J mice. Journal of Immunology, 182, 1789-93.

STEPHENSON, H. N., STREECK, R., GRÜBLINGER, F., GOOSMANN, C. & HERZIG, A. 2022. Hemocytes are essential for *Drosophila melanogaster* post-embryonic development, independent of control of the microbiota. Development, 149.

• STRAFACE, E., GAMBARDELLA, L., BRANDANI, M. & MALORNI, W. 2012. Sex Differences at Cellular Level: “Cells Have a Sex”. In: REGITZ-ZAGROSEK, V. (ed.) Sex and Gender Differences in Pharmacology. Berlin, Heidelberg: Springer Berlin Heidelberg.

• STRAUB, R. H. 2007. The complex role of estrogens in inflammation. Endocrine Reviews, 28, 521-74.

• STROSCHEIN-STEVENSON, S. L., FOLEY, E., O'FARRELL, P. H. & JOHNSON, A. D. 2005. Identification of *Drosophila* Gene Products Required for Phagocytosis of Candida albicans. PLOS Biology, 4, e4.

• STUART, L. M., BOULAIS, J., CHARRIERE, G. M., HENNESSY, E. J., BRUNET, S., JUTRAS, I., GOYETTE, G., RONDEAU, C., LETARTE, S., HUANG, H., YE, P., MORALES, F., KOCKS, C., BADER, J. S., DESJARDINS, M. & EZEKOWITZ, R. A.

B. 2007. A systems biology analysis of the *Drosophila* phagosome. Nature, 445, 95-101.

• STUART , L. M., DENG , J., SILVER , J. M., TAKAHASHI , K., TSENG , A. A., HENNESSY , E. J., EZEKOWITZ , R. A. B. & MOORE , K. J. 2005. Response to *Staphylococcus aureus* requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. Journal of Cell Biology, 170, 477-485.

• STUART, L. M. & EZEKOWITZ, R. A. B. 2005. Phagocytosis: Elegant Complexity. Immunity, 22, 539-550.

• SU, L., SUN, Y., MA, F., LÜ, P., HUANG, H. & ZHOU, J. 2009. Progesterone inhibits Toll like receptor 4-mediated innate immune response in macrophages by suppressing NFkappaB activation and enhancing SOCS1 expression. Immunology Letters, 125, 151-5.

• SUREWAARD, B. G., DENISET, J. F., ZEMP, F. J., AMREIN, M., OTTO, M., CONLY, J., OMRI, A., YATES, R. M. & KUBES, P. 2016. Identification and treatment of the *Staphylococcus aureus* reservoir in vivo. Journal of Experimental Medicine, 213, 1141-51.

• TABUCHI, Y., SHIRATSUCHI, A., KUROKAWA, K., GONG, J. H., SEKIMIZU, K., LEE, B. L. & NAKANISHI, Y. 2010. Inhibitory role for D-alanylation of wall teichoic acid in activation of insect Toll pathway by peptidoglycan of *Staphylococcus aureus*. Journal of Immunology, 185, 2424-31.

• TAKEUCHI, O. & AKIRA, S. 2010. Pattern recognition receptors and inflammation. Cell, 140, 805-20.

• TATTIKOTA, S. G., CHO, B., LIU, Y., HU, Y., BARRERA, V., STEINBAUGH, M. J., YOON, S.-H., COMJEAN, A., LI, F., DERVIS, F., HUNG, R.-J., NAM, J.-W., HO SUI, S., SHIM, J. & PERRIMON, N. 2020. A single-cell survey of *Drosophila* blood. eLife, 9, e54818.

• TER HORST, R., JAEGER, M., SMEEKENS, S. P., OOSTING, M., SWERTZ, M. A., LI, Y.,
KUMAR, V., DIAVATOPOULOS, D. A., JANSEN, A. F. M., LEMMERS, H., TOENHAKEDIJKSTRA, H., VAN HERWAARDEN, A. E., JANSSEN, M., VAN DER MOLEN, R. G., JOOSTEN, I., SWEEP, F., SMIT, J. W., NETEA-MAIER, R. T., KOENDERS, M., XAVIER, R. J., VAN DER MEER, J. W. M., DINARELLO, C. A., PAVELKA, N., WIJMENGA, C., NOTEBAART, R. A., JOOSTEN, L. A. B. & NETEA,

M. G. 2016. Host and Environmental Factors Influencing Individual Human Cytokine Responses. Cell, 167, 1111-1124.e13.

• THERNEAU, T. M. 2023. A Package for Survival Analysis in R. R package version 3.5-7 ed.

• TONG, S. Y., DAVIS, J. S., EICHENBERGER, E., HOLLAND, T. L. & FOWLER, V. G., JR. 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. Clinical Microbiology Reviews, 28, 603-61.

• TUKIAINEN, T., VILLANI, A. C., YEN, A., RIVAS, M. A., MARSHALL, J. L., SATIJA, R., AGUIRRE, M., GAUTHIER, L., FLEHARTY, M., KIRBY, A., CUMMINGS, B. B., CASTEL, S. E., KARCZEWSKI, K. J., AGUET, F., BYRNES, A., LAPPALAINEN, T., REGEV, A., ARDLIE, K. G., HACOHEN, N. & MACARTHUR, D. G. 2017. Landscape of X chromosome inactivation across human tissues. Nature, 550, 244-248.

• TZOU, P., OHRESSER, S., FERRANDON, D., CAPOVILLA, M., REICHHART, J.-M., LEMAITRE, B., HOFFMANN, J. A. & IMLER, J.-L. 2000. Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. Immunity, 13, 737-748.

• TZOU, P., REICHHART, J.-M. & LEMAITRE, B. 2002. Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient
Drosophila mutants. Proceedings of the National Academy of Sciences, 99, 2152-2157.

• UNDERHILL, D. M. & GOODRIDGE, H. S. 2012. Information processing during phagocytosis. Nature Reviews Immunology, 12, 492-502.

• UPPAL, S. S., VERMA, S. & DHOT, P. S. 2003. Normal values of CD4 and CD8 lymphocyte subsets in healthy indian adults and the effects of sex, age, ethnicity, and smoking. Cytometry Part B: Clinical Cytometry, 52, 32-6.

• VAZ, F., KOUNATIDIS, I., COVAS, G., PARTON, R. M., HARKIOLAKI, M., DAVIS, I., FILIPE, S. R. & LIGOXYGAKIS, P. 2019. Accessibility to Peptidoglycan Is Important for the Recognition of Gram-Positive Bacteria in *Drosophila*. Cell Reports, 27, 2480-2492.e6.

• VÁZQUEZ-MARTÍNEZ, E. R., GARCÍA-GÓMEZ, E., CAMACHO-ARROYO, I. & GONZÁLEZPEDRAJO, B. 2018. Sexual dimorphism in bacterial infections. Biology of Sex Differences, 9, 27.

• VIERSTRAETE, E., VERLEYEN, P., BAGGERMAN, G., D'HERTOG, W., VAN DEN BERGH, G., ARCKENS, L., DE LOOF, A. & SCHOOFS, L. 2004. A proteomic approach for the analysis of instantly released wound and immune proteins in *Drosophila melanogaster* hemolymph. Proceedings of the National Academy of Sciences USA, 101, 470-5.

• VINCENT, C. M. & SHARP, N. P. 2014. Sexual antagonism for resistance and tolerance to infection in *Drosophila melanogaster*. Proceedings of the Royal Society B: Biological Sciences, 281, 20140987.

• VLISIDOU, I. & WOOD, W. 2015. *Drosophila* blood cells and their role in immune responses. The FEBS Journal, 282, 1368-1382.

• VOSKUHL, R. 2011. Sex differences in autoimmune diseases. Biology of Sex Differences, 2, 1.

• WALD, C. & WU, C. 2010. Of Mice and Women: The Bias in Animal Models. Science, 327, 1571-1572.

• WALTER, F., OTT, J. J., CLAUS, H. & KRAUSE, G. 2018. Sex- and age patterns in incidence of infectious diseases in Germany: analyses of surveillance records over a 13-year period (2001–2013). Epidemiology & Infection, 146, 372-378.

• WATSON, F. L., PÜTTMANN-HOLGADO, R., THOMAS, F., LAMAR, D. L., HUGHES, M., KONDO, M., REBEL, V. I. & SCHMUCKER, D. 2005. Extensive diversity of Ig-superfamily proteins in the immune system of insects. Science, 309, 1874-1878.

• WEBER, A. N., TAUSZIG-DELAMASURE, S., HOFFMANN, J. A., LELIÈVRE, E., GASCAN, H., RAY, K. P., MORSE, M. A., IMLER, J. L. & GAY, N. J. 2003. Binding of the *Drosophila* cytokine Spätzle to Toll is direct and establishes signaling. Nature Immunology, 4, 794-800.

• WEINSTEIN, Y., RAN, S. & SEGAL, S. 1984. Sex-associated differences in the regulation of immune responses controlled by the MHC of the mouse. J Immunol, 132, 656-61.

• WERNER, T., LIU, G., KANG, D., EKENGREN, S., STEINER, H. & HULTMARK, D. 2000. A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*. Proceedings of the National Academy of Sciences USA, 97, 13772-7.

• WERTHEIM, B., KRAAIJEVELD, A. R., SCHUSTER, E., BLANC, E., HOPKINS, M., PLETCHER, S. D., STRAND, M. R., PARTRIDGE, L. & GODFRAY, H. C. 2005a. Genome-wide gene expression in response to parasitoid attack in *Drosophila*. Genome Biology, 6, R94.

• WERTHEIM, H. F., MELLES, D. C., VOS, M. C., VAN LEEUWEN, W., VAN BELKUM, A., VERBRUGH, H. A. & NOUWEN, J. L. 2005b. The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infectious Diseases, 5, 751-62.

• WERZ, O., GERSTMEIER, J., LIBREROS, S., DE LA ROSA, X., WERNER, M., NORRIS, P. C., CHIANG, N. & SERHAN, C. N. 2018. Human macrophages differentially produce specific resolvin or leukotriene signals that depend on bacterial pathogenicity. Nature Communications, 9, 59.

• WESTERGAARD, D., MOSELEY, P., SØRUP, F. K. H., BALDI, P. & BRUNAK, S. 2019. Population-wide analysis of differences in disease progression patterns in men and women. Nature Communications, 10, 666.

• WOODCOCK, KATIE J., KIERDORF, K., POUCHELON, CLARA A., VIVANCOS, V., DIONNE, MARC S. & GEISSMANN, F. 2015. Macrophage-Derived upd3 Cytokine Causes Impaired Glucose Homeostasis and Reduced Lifespan in *Drosophila* Fed a Lipid-Rich Diet. Immunity, 42, 133-144.

• YAJJALA, V. K., THOMAS, V. C., BAUER, C., SCHERR, T. D., FISCHER, K. J., FEY, P. D., BAYLES, K. W., KIELIAN, T. & SUN, K. 2016. Resistance to Acute Macrophage Killing Promotes Airway Fitness of Prevalent Community-Acquired *Staphylococcus aureus* Strains. Journal of Immunology, 196, 4196-203.

• YANG, H. & HULTMARK, D. 2016. Tissue communication in a systemic immune response of *Drosophila*. Fly, 10, 115-122.

• YAW, L. K., ROBINSON, J. O. & HO, K. M. 2014. A comparison of long-term outcomes after meticillin-resistant and meticillin-sensitive *Staphylococcus aureus* bacteraemia: an observational cohort study. Lancet Infectious Diseases, 14, 967-975.

• YOUNES, S., AL-SULAITI, A., NASSER, E. A. A., NAJJAR, H. & KAMAREDDINE, L. 2020. *Drosophila* as a Model Organism in Host-Pathogen Interaction Studies. Frontiers in Cellular and Infection Microbiology, 10, 214.

• YOUNGER-SHEPHERD, S., VAESSIN, H., BIER, E., JAN, L. Y. & JAN, Y. N. 1992. deadpan, an essential pan-neural gene encoding an HLH protein, acts as a denominator in *Drosophila* sex determination. Cell, 70, 911-22.

• YOUSEFIAN, J., TROOST, T., GRAWE, F., SASAMURA, T., FORTINI, M. & KLEIN, T. 2013. Dmon1 controls recruitment of Rab7 to maturing endosomes in *Drosophila*. Journal of Cell Science, 126, 1583-1594.

• ZHU, F., ZHOU, Y., JIANG, C. & ZHANG, X. 2015. Role of JAK-STAT signaling in maturation of phagosomes containing *Staphylococcus aureus*. Scientific Reports, 5, 14854.

• ZUK, M. 2009. The sicker sex. PLOS Pathog, 5, e1000267.