ABSTRACT

Title of Dissertation: EXAMINING INTERACTIONS AMONG FUNGAL INFECTION, SLEEP, AND HOST DEFENSE IN POPULATIONS OF WORLDWIDE, SLEEP INBRED PANEL, AND MUTANT *DROSOPHILA MELANOGASTER*

Mintong Nan, Doctor of Philosophy, 2024

Dissertation directed by: Professor Raymond St. Leger, Department of Entomology

Previous studies with mutant *Drosophila melanogaster* lines and the *Drosophila* Genetic Reference Panel (DGRP) reveal substantial variation in susceptibility to infection with *Metarhizium anisopliae* strain Ma549. These differences likely arise from variations in immunity, physiology, and behavior, yet the role of selection pressures in maintaining these disparities remains understudied. Using global *Drosophila* populations, the Sleep Inbred Panel (SIP), and sleep-deprived mutants, we investigated the interactions among fungal infection, sleep, and host defense. Testing 14,957 male and 15,287 female flies from 43 lines across 28 locations, we found that resistance to Ma549 correlates with latitudinal gradients in sleep duration, temperature, and humidity. Tropical populations may exhibit stronger defenses due to fungal diversity; however, the most disease-resistant males were also more susceptible to desiccation, indicating trade-offs between abiotic stress and disease resistance. Longer-sleeping males and virgin flies survived infections longer, and increased daytime sleep post-infection was particularly protective in resistant flies. These findings suggest that sleep and disease resistance are interrelated traits possibly shaped by clinal evolution. Using 10,917 males and 11,166 females selected for extreme long or short nighttime sleep duration, we found that short-sleepers,

despite having fragmented sleep when healthy, outlived long-sleepers after Ma549 infection. Resistance differences were sex-specific: males were more resistant among short-sleepers, while females showed higher resistance among long-sleepers. Daytime sleep bout numbers correlated with dimorphic disease resistance. Additionally, Ma549 infection increased daytime sleep in both short- and long-sleepers, with short-sleeping males nearly matching long-sleepers in sleep duration post-infection. Virgins, regardless of sleep status, slept more and survived infection longer, indicating that sleep traits, sex, and mating status are closely linked to disease resistance. The study highlights that circadian rhythms influence sleep and immunity, with the sleep-deprived Sh^{mns} mutant failing to undergo sickness sleep and succumbing quickly to infection. Mutants with disrupted circadian rhythms (PER and CLK) also showed impaired sickness sleep; however, only the *per* gene offered protection against disease, while the *Clk* mutant had increased survival. Independent of mutant status, males slept more than females, and virgins slept more than mated flies, emphasizing the significance of circadian rhythms in sleep and disease resistance.

EXAMINING INTERACTIONS AMONG FUNGAL INFECTION, SLEEP, AND HOST DEFENSE IN POPULATIONS OF WORLDWIDE, SLEEP INBRED PANEL, AND MUTANT *DROSOPHILA MELANOGASTER*

By

Mintong Nan

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2024

Advisory Committee:

Professor Raymond St. Leger, Chair
Professor Kelly Hamby
Professor Meiqing Shi
Professor Louisa Wu
Professor Weiguo Fang

© Copyright by
Mintong Nan
2024

Acknowledgements

This work would not have been possible without the support of many individuals. First and foremost, I would like to express my deepest gratitude to my advisor, Dr. Raymond St. Leger, for his unwavering support, guidance, and encouragement throughout my PhD studies. It has been a true pleasure exploring both science and life with you. I would also like to thank Dr. Weiguo Fang, Dr. Kelly Hamby, Dr. Meiqing Shi, and Dr. Louisa Wu for serving on my committee and for providing invaluable guidance, advice, and feedback as I developed these projects. Special thanks to Dr. Jonathan Bohuai Wang for his initial mentoring, which laid the foundation for my PhD studies. I am grateful to Dr. Brian Richard Lovett and Huiyu Sheng for their assistance with various aspects of my research over the years, whether through helping procure supplies or offering insightful feedback on my projects. I also want to acknowledge Dr. Jeffrey Shultz, Josh Kiner, Andy Yeh, Amy Yaich, Eileen Jewison, Bill Katsereles, Pam Biery, Greg Hess, and Michael Katsereles for their significant and invaluable support in managing the administrative aspects of my research. A big thank you to Todd Waters for managing the fly kitchen and keeping our *Drosophila melanogaster* lines fed.

Additionally, I extend my appreciation to Drs. Elizabeth Brown and Alex Keene from Texas A&M University for providing the 22 worldwide fly stocks, Dr. Julie Williams from the University of Pennsylvania for providing the insomniac sleep analysis software, and Mr. Yufeng Zhu from National Oceanic and Atmospheric Administration for his assistance in extracting and verifying large temperature and precipitation datasets from the 28 collection sites.

Finally, I would like to express my heartfelt thanks to my parents, Drs. Ling Tian and Xinli Nan, for all the love, laughter, and unconditional support you have given me throughout the years. I couldn't have achieved this without you.

List of Tables

Chapter 1

Table 1.1 The localities, stock numbers, Ma549 LT_{50}s, collection years, and geographic variables including biomes of 43 *Drosophila melanogaster* populations 9
Table 1.2 Fixed effect tests on longevity effects of sex and collection date. 17
Table 1.3 REML variance component estimates on longevity effects of fly lines. 17
Table 1.4 Impact of geography and biome on resistance levels. 18
Table 1.5 Principal component loadings of environmental variables and LT_{50} for male and female flies 20
Table 1.6 Comparison of mean and variation in disease resistance between males and females 55
Table 1.7 Effects of line, sex, mating status, and infection as well as their interactions on night and daytime sleep. 64
S1.1 Table ..... (Attached separately)
S1.2 Table ..... (Attached separately)
S1.3 Table ..... (Attached separately)
S1.4 Table ..... (Attached separately)
S1.5 Table ..... (Attached separately)
S1.6 Table ..... (Attached separately)
S1.7 Table ..... (Attached separately)
S1.8 Table ..... (Attached separately)

Chapter 2

Table 2.1 The fly lines, stock numbers, female and male Ma549 LT_{50}s, standard error of the mean (SEM), and sample size (N) of 38 SIP and 10 progenitor populations. 70
Table 2.2 Correlation analyses of sleep indices and Ma549 longevity for SIPs with or without progenitors (LP/SP) and DGRPs 78
Table 2.3 Significance testing of correlation coefficient differences for SIPs with or without progenitors (LP/SP) and DGRPs 78
Table 2.4 Effects of line, sex, mating status, and infection as well as their interactions on night and daytime sleep. 88
Table 2.5 Comparison of mean and variation in disease resistance between males and females 100
S2.1 Table ..... (Attached separately)
S2.2 Table ..... (Attached separately)
S2.3 Table ..... (Attached separately)
S2.4 Table ..... (Attached separately)
S2.5 Table ..... (Attached separately)
S2.6 Table ..... (Attached separately)

Chapter 3

Table 3.1 Effects of line, sex, mating status, and infection as well as their interactions on night and daytime sleep 117
S3.1 Table (Attached separately)
S3.2 Table ..... (Attached separately)
S3.3 Table ..... (Attached separately)
S3.4 Table ..... (Attached separately)
S3.5 Table ..... (Attached separately)

List of Figures

Chapter 1

Figure 1.1 12
Figure 1.2 16
Figure 1.3 23
Figure 1.4 25
Figure 1.5 27
Figure 1.6 28
Figure 1.7 30
Figure 1.8 33
Figure 1.9 35
Figure 1.10 39

Supplementary figure 1.1 57
Supplementary figure 1.2 58
Supplementary figure 1.3 59
Supplementary figure 1.4 60
Supplementary figure 1.5 62
Supplementary figure 1.6 63
Supplementary figure 1.7 65

Chapter 2

Figure 2.1 69
Figure 2.2 72
Figure 2.3 72
Figure 2.4 75
Figure 2.5 77
Figure 2.6 80
Figure 2.7 83
Figure 2.8 86
Figure 2.9 88
Figure 2.10 90

Chapter 3

Figure 3.1 110
Figure 3.2 113
Figure 3.3 115
Figure 3.4 118
Figure 3.5 120

Table of Contents

Acknowledgements ii

List of Tables iv

List of Figures v

Table of Contents vi

Introduction 1

Chapter 1: Latitudinal clines in climate and sleep patterns shape disease outcomes in *Drosophila melanogaster* infected by *Metarhizium anisopliae* 4

1.1 *Abstract* 4

1.2 *Introduction* 5

1.3 *Results* 9

1.3.1 The longevity of infected fruit flies varies by collection site and sex 9

1.3.2 Fly line and sex matter more than collection date 16

1.3.3 Geography and biomes impact on host susceptibility to infection 18

1.3.4 Environmental factors at the original collection sites influence host susceptibility to infection in the laboratory 18

1.3.5 Abiotic and biotic stresses link ecological pressures to variations in disease resistance. 21

1.3.6 Correlation between sleep and disease resistance 27

1.3.7 Characterization of sickness sleep triggered by Ma549 infection 28

1.3.8 The impact of host variation on fungal fitness 34

1.3.9 Potential adaptive differences between populations from Africa, France and Raleigh, North Carolina 37

1.4 *Discussion* 40

1.5 *Materials and Methods* 48

1.5.1 Fly stocks, geographic and climate data, and biomes 48

1.5.2 Fungal strains, and longevity bioassays 50

1.5.3 Desiccation assays 51

1.5.4 Fungal colonization of hosts measured by colony forming units (CFUs) 52

1.5.5 Sleep assays and data analysis 52

1.5.6 Statistical analyses on interactions among clinal cues, geographic variables, longevity, and sleep parameters 53

1.6 *Supplementary tables* 55

Chapter 2: Exploring the relationship between disease resistance and sleep in the *Drosophila melanogaster* Sleep Inbred Panel 66

2.1 *Abstract* 66

2.2 *Introduction* 66
2.3 *Results* 69
2.3.1 SIP lines preserve reliable activity patterns 69
2.3.2 The SIP and DGRP populations vary in their resistance to Ma549 infection 70
2.3.3 Correlation between sleep parameters and infection 77
2.3.4 Characterization of sickness sleep triggered by Ma549 infection 80
2.3.5 Effects of line, sex, mating status, and infection on sleep 87
2.3.6 The impact of host variation on fungal fitness 89
2.4 *Discussion* 91
2.5 *Materials and Methods* 95
2.5.1 Fly stocks and virgin fly collection 95
2.5.2 Fungal strains and longevity bioassays 96
2.5.3 Fungal colonization of hosts measured by colony forming units (CFUs) 97
2.5.4 Sleep assays and data analysis 97
2.5.5 Statistical analyses on longevity and sleep parameters 98
2.6 *Supplementary tables* 99
Chapter 3: Sleep, sex, and mating status modulate fungal (*Metarhizium anisopliae*) infections in *Drosophila melanogaster* 102
3.1 *Abstract* 102
3.2 *Introduction* 102
3.3 *Results* 106
3.3.1 Ma549 infection promotes sickness sleep in the wildtype Canton-S line 106
3.3.2 Sickness sleep is disrupted in short-sleeping *Shaker* line and clock gene mutants, with variation in survival to Ma549 infection 107
3.3.3 Sexual dimorphism in sleep and longevity in response to Ma549 infection 112
3.3.4 Impact of mating status on sleep and survival in flies following Ma549 infection 114
3.3.5 Effects of line, sex, mating status, and infection on sleep 116
3.3.6 The impact of natural host variation and sleep mutants on fungal fitness 118
3.4 *Discussion* 122
3.5 *Materials and Methods* 126
3.5.1 Fly stocks and virgin fly collection 126
3.5.2 Fungal strains and longevity bioassays 126
3.5.3 Fungal colonization of hosts measured by colony forming units (CFUs) 127
3.5.4 Sleep assays and data analysis 128

3.5.5 Statistical analyses on longevity and sleep parameters 128
3.6 *Supplementary tables* 130
Conclusion 131
Bibliography 138

Introduction

Sleep is a behavioral state marked by quiescence and a species-specific posture, easily reversed to wakefulness when an arousal threshold is breached. It is regulated by the circadian clock and homeostat. Circadian rhythms originate from an internal biological clock that drives many aspects of animal physiology[1]. The clock is synchronized in a complex manner by ecologically and physiologically relevant environmental factors, such as temperature, precipitation, relative humidity, and conspecific interactions, enabling animals to anticipate daily environmental changes and align their behavior and physiology with the time of day[2-6]. Sleep is therefore plastic, being regulated by both external agents and changes in internal state, and shows significant diversity in homeostatically-regulated behaviors, though the forces that select for, and govern, these differences are poorly understood[7, 8]. Sleep is nevertheless vital for survival[9] with continuous sleep deprivation leading to death in *Drosophila*[10, 11]. The precise mechanism(s) by which sleep enhances survival are unclear, but one hypothesis is that deprivation negatively affects immune function[12].

In mammals, both innate and adaptive arms of the immune system are regulated by circadian rhythms at molecular and cellular levels[13]. These rhythms influence organismal immunobiology in profound ways[14]. Chronic circadian disruptions, such as sleep deprivation, shift work, and jet lag, can lead to disease in healthy individuals and worsen existing diseases, particularly inflammatory conditions[15]. *Drosophila* has been widely used to study immune response mechanisms due to its relative simplicity and genetic tractability[16-18]. The humoral immune system of *Drosophila* has two major pathways, the Toll pathway and the Imd (immune deficiency) pathway. These two pathways combat different types of bacterial and fungal infections by recognizing pathogen-associated molecular patterns and activating downstream

AMPs (anti-microbial peptides) like Drosomycin (Drs) and Metchnikowin. We previously demonstrated that the Toll pathway does not substantially restrain infection with the entomopathogen *Metarhizium anisopliae* even though it is activated by this fungus and is responsible for *Drs* transcription[19].

Similar to mammals, the *Drosophila* immune response is rhythmic, with immune genes showing rhythmic expression, and flies exhibiting time-of-day-dependent resistance to bacterial infections[20, 21]. However, it is unclear how this might relate to fly populations and their ecology. Conversely, despite conserved immune responses, individual fruit flies and humans vary extensively in resistance mechanisms that activate immune pathways and tolerance mechanisms that minimize tissue damage[22].

Using fruit flies for these studies offers the advantage of accessing numerous global laboratory cultures. Several reports indicate that prolonged domestication of inbred *Drosophila* lines does not alter their physiological phenotypes, allowing the possibility of population studies with laboratory cultures (Chapter I Introduction). For example, high-latitude populations retain their original starvation, cold, and heat tolerance after several years being cultured in the laboratory[23].

Existing research on disease resistance and circadian rhythms predominantly focuses on bacteria and viruses. The sole fungal exception seems to be the demonstration of time-to-day variation in the clearance of *Aspergillus fumigatus* from mice lungs[24]. However, around 300 fungi are confirmed human pathogens[25] affecting over 300 million people and causing an estimated 1.7 million deaths annually[26], outpacing malaria and rivaling tuberculosis. Fungi also destroy a third of all food crops annually, sufficient to feed 600 million people[27]. Of particular relevance to our study, fungi cause the majority of insect diseases, infecting through cuticle

penetration via cuticle-degrading enzymes and mechanical pressure[28-30]. Once in the hemocoel, fungi proliferate by competing for nutrients and evading antimicrobial proteins and hemocytes capable of phagocytosis and encapsulation of invading microorganisms[31]. After the host is dead, the fungus breaches the cuticle from within, allowing the formation of conidial spores that initiate new infections.

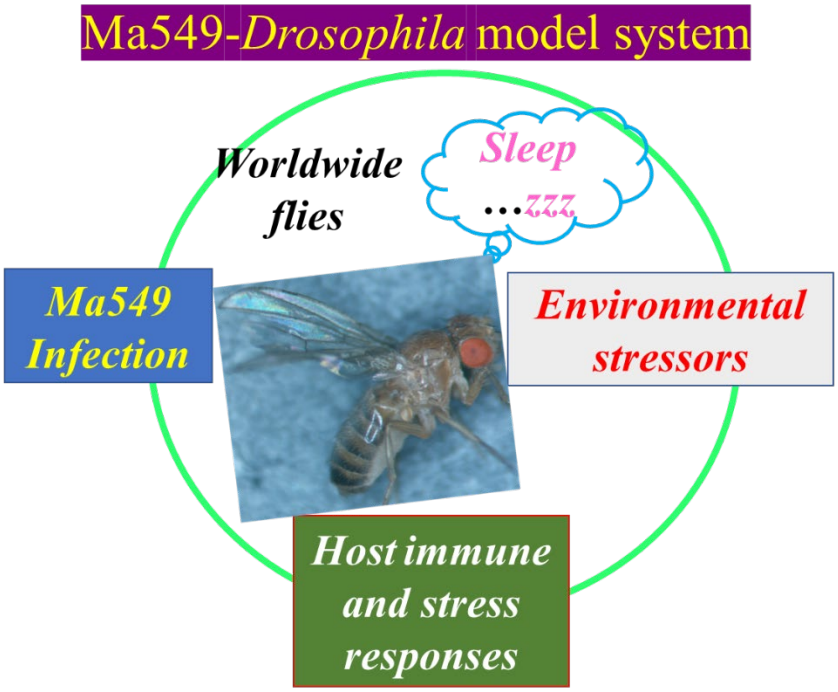
Metarhizium spp are probably the best studied insect pathogenic fungi and have been used to explore immune system evolution and provide insights into emerging human pathogens[31, 32]. Aside playing a critical role in natural ecosystems, they are also being developed as eco-friendly alternatives to chemical insecticides for pest control[33, 34]. Progress in understanding immune defense against fungal infections in insects has implications for the biological control of vector-borne human diseases[35]. The strain used in this study, *M. anisopliae* ARSEF549 (Ma549), is naturally pathogenic to *Drosophila*[19] and unlike other species, does not produce toxins that inhibit the innate immune responses[36]. By establishing conditions where individuals succumbed slowly to the infection, we could better evaluate whether sleep modulates the ability of flies to successfully combat pathogenic infection.

Chapter 1: Latitudinal clines in climate and sleep patterns shape disease outcomes in *Drosophila melanogaster* infected by *Metarhizium anisopliae*

(Prepared for submission)

1.1 *Abstract*

Major latitudinal clines have been observed in *Drosophila melanogaster*, a human commensal that originated in tropical Africa and has spread world-wide to colonize temperate habitats. However, despite the significant impact of pathogens on species distribution, the influence of geographical factors on disease susceptibility remains poorly understood. This study investigated the effects of latitudinal clines and biomes on disease resistance using the common fly pathogen *Metarhizium anisopliae* and 43 global *Drosophila melanogaster* populations. The results showed that disease resistance was correlated with latitudinal gradients of sleep duration, temperature and humidity. While fungal diversity at tropical latitudes may drive enhanced defenses, the most disease-resistant males were also the most susceptible to desiccation, indicating potential trade-offs between abiotic stress resistance, necessary for survival in temperate habitats, and disease resistance. The study also found that sex, mating status, and sleep interacted with abiotic stresses to impact disease resistance, with longer-sleeping males and virgin flies surviving infections longer, and extra daytime sleep post-infection being protective, especially in resistant flies. These findings promote the idea that sleep and defense are intertwined traits related to organismal fitness and subject to joint clinal evolution. However, it remains unclear to what extent selection acts on disease resistance through changes in stress responses and sleep, or if disease resistance is often a byproduct of selection on sleep and multi-stress resistance.



1.2 *Introduction*

Understanding how organisms adapt to their environment, especially which biological processes are most relevant, is still an open question in biology[37] and one that has become increasingly critical with growing threats to biodiversity from human activities. The fruit fly *Drosophila melanogaster*, a key evolutionary model, offers unique opportunities to investigate historical processes experienced by human commensals[38]. Originating in sub-Saharan Africa and initially specializing to marula fruit (*Sclerocarya birrea*), this species first associated with people when marula was stored by cave-dwellers[39]. Flies maintained under laboratory conditions since 1916 still prefer marula over other fruits[39]. *D. melanogaster* expanded its range to the Middle East around 13,000 years ago and to Europe and Asia about 1,800 years ago, coinciding with citrus and grape cultivation[40]. Further colonization events, driven by human dispersal, have spread fly populations globally, including North America and Australia about 200 years ago[41, 42].

Colonizing natural habitats, environmental clines, and geographic variables across continents suggests the capacity to rapidly adapt to local conditions[37, 43], making *D. melanogaster* a robust model for studying evolutionary processes of range expansion and adaptation[38] with promise for understanding evolutionary change in response to future environmental challenges. Thus, variations in *D. melanogaster* lines from diverse locales have identified sleep differences[44] linked to metabolic and foraging needs[45] and associated with latitudinal clines and elevated temperatures[44]. Other clines such body size and chill resistance that vary predictably in abiotic factors have been the subject of much theoretical and experimental work but relatively little is known about clines whose evolution may be governed by interspecific interactions.

Flies potentially encounter distinct parasitic and pathogenic pressures in different environments, and according to the Red Queen hypothesis, hosts continually evolve to minimize the effects of pathogen adaptation[43, 46, 47]. Disease resistance varies across *Drosophila* species; those with a broad diet (e.g., *D. melanogaster, D. simulans, D. repleta, D. arizonae*) tend to be more resistant to *Metarhizium anisopliae* strain Ma549 than dietary specialists (e.g., *D. erecta, D. sechellei*)[48], possibly because insects with a broad diet have more opportunities to encounter and develop resistance to a variety of pathogens[48, 49]. There is also a great deal of intraspecific variation in disease resistance in several species[48, 49], but whether any of this variation is predictable across environments is poorly understood. However, four African *D. melanogaster* lines were found to be more resistant to the fungal pathogen *Beauveria bassiana* than two non-African populations[50].

Overall, the ecological and evolutionary processes contributing to disease resistance are understudied. In contrast, there is a voluminous literature on the molecular mechanisms of host–pathogen interactions using *D. melanogaster*[16, 17] and these have shown a notable conservation of

pathogenesis and host defense mechanisms between higher host organisms and *Drosophila*[51, 52]. Nonetheless, both individual fruit flies and humans exhibit extensive variability in their responses to infectious agents[31, 53], as demonstrated by human variability to COVID-19. Understanding why two individuals exposed to identical infections have different outcomes is a central issue in infection biology[54], but presumably is a consequence of their different evolutionary histories.

Fungi cause the majority of insect diseases, infecting insects by directly penetrating the cuticle with a combination of cuticle-degrading enzymes and mechanical pressure[55, 56]. The entomopathogenic fungus *M. anisopliae* is widespread globally, and strain ARSEF549 (Ma549) is naturally pathogenic to *Drosophila*[19]. Unlike many other entomopathogens, it does not produce toxins that inhibit innate immune responses[36]. Previous studies showed that about 9% of mutant *D. melanogaster* lines exhibit altered disease resistance to Ma549[19] and that *Drosophila* Genetic Reference Panel (DGRP) flies from Raleigh, North Carolina, vary significantly in longevity after Ma549 infection[49]. Our GWAS analysis of DGRP flies identified a complex network of immune and physiological genes, particularly those related to sleep, involved in *D. melanogaster* interactions with Ma549[49]. However, DGRP data only reflect genetic variation from a single location (North-Eastern USA), while a major portion of the genetic diversity of the species resides elsewhere, notably in Africa its ancestral home[57]. Thus, DGRP data are inadequate for studying global pathogen susceptibility.

Understanding the relationship between organisms and their habitats is central to ecological and environmental research. Diverse biomes, each with unique environmental conditions, exemplify this complexity[58]. Examples of biomes with aseasonal climates include Tropical and Subtropical Moist Broadleaf Forests (TSMF), Tropical and Subtropical Grasslands,

Savannas, and Shrublands (TSGSS)[59], and Tropical and Subtropical Dry Broadleaf Forests (TSDF). In contrast, seasonal biomes like Temperate Broadleaf and Mixed Forests (TBMF), Mediterranean Forests, Woodlands, and Scrub (MFWS), and Desert Xeric Shrublands (DXS) experience greater climatic variability. Each biome's unique climatic, topographic, and ecological variations significantly influence the resident organisms[58, 60], including the distribution of *D. melanogaster* populations and fungal species[60-62].

We used the Ma549-*Drosophila* model to investigate how environmental factors, genetic diversity, and sleep behavior affect disease resistance in global fly populations. The study focuses on temperature and humidity due to their significant impact on both fungal infection prevalence and insect resistance[63]. Increasing temperatures, changes in precipitation and extreme events under climate change threaten the persistence of many insects and their pathogens[64] so understanding adaptive responses to these stresses is crucial for predicting climate change impacts[65]. We analyzed flies from the two ends of the resistance and biome spectrum by choosing Monkey Hills (MH), St. Kitts, characterized by a humid and stable TSMF biome[66], and Ica, Peru (IP), a hyper-arid landscape with significant climatic variability[67], for particular study to check the relationship between abiotic and biotic stress.

Our findings show significant differences in Ma549 resistance among *Drosophila* populations from various global sites. This variation correlates with geographic origins and ecoregional conditions, suggesting persistent macro-physiological relationships between disease resistance and other traits. Sexual dimorphism also affects host-pathogen interactions regardless of geographic origin, with infection impacting sleep patterns more in males. Moreover, sexual activity influences disease resistance and sleep patterns, highlighting the complex interplay of genetic, environmental, and behavioral factors in shaping host responses to pathogens.

1.3 *Results*

1.3.1 The longevity of infected fruit flies varies by collection site and sex.

We assessed the ability of 43 age-matched fly lines/populations from diverse geographic regions to survive infection by Ma549. Infection was performed via the natural route of applying spores to the body surface. Median lethal times (LT_{50}) were monitored using five replicates (~35 flies each) per sex per fly line, and the experiments were conducted at least twice. Lines with an unusually large disparity between males and females were screened three times to validate differences. A total of 14,957 male flies and 15,287 female flies were assayed, and the collection locations for the fly populations are listed in Table 1.1 and S1.1 Table.

Table 1.1 The localities, stock numbers, Ma549 LT_{50}s, collection years, and geographic variables including biomes of 43 *Drosophila melanogaster* populations

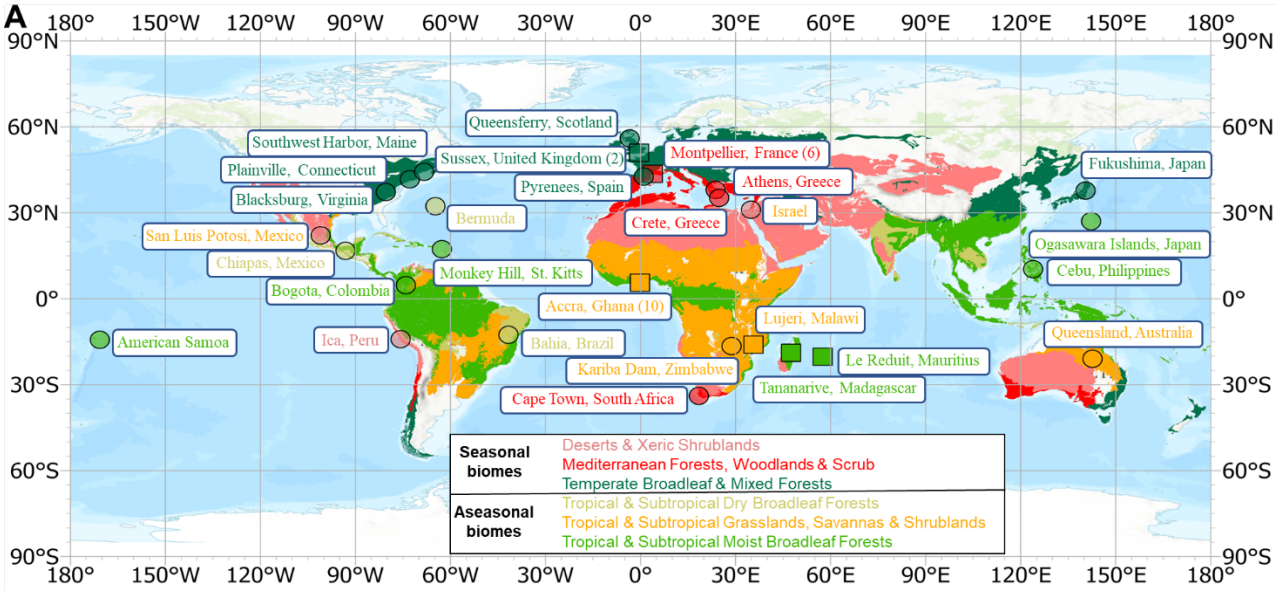
Fly lines	Stock	Female			Male			Collection date	Lat.	Lon.	Alt.	Biomes
		LT_{50}	±SEM	N	LT_{50}	±SEM	N					
American Samoa	134	5.28	0.12	326	5.48	0.04	359	2009	-14.30	-170.70	157	TSMF
Bogota, Colombia	59	5.44	0.10	345	5.27	0.10	390	1962	4.71	-74.07	2258	TSMF
Cebu, Philippines	129	4.73	0.04	433	4.49	0.03	394	2008	10.32	123.89	35	TSMF
Le Reduit, Mauritius	53	5.44	0.09	365	6.24	0.09	376	2006	-20.23	57.49	282	TSMF
Monkey Hill, St. Kitts	35	5.28	0.18	501	8.09	0.13	373	2005	17.32	-62.73	121	TSMF
Ogasawara Islands, Japan	137	5.83	0.09	356	6.03	0.15	322	2009	27.08	142.21	152	TSMF
Tananarive, Madagascar	125	4.53	0.08	357	5.47	0.11	398	1982	-18.87	47.51	1250	TSMF
Bahia, Brazil	15	4.00	0.07	295	4.10	0.03	365	Not Listed	-12.58	-41.70	907	TSDF
Bermuda	58	4.57	0.13	446	4.40	0.08	497	1954	32.31	-64.75	14	TSDF
Chiapas, Mexico	22	4.72	0.05	321	4.94	0.15	280	2002	16.76	-93.13	536	TSDF
Accra, Ghana.163	163	5.84	0.08	396	6.86	0.13	354	2010	5.60	-0.19	54	TSGSS
Accra, Ghana.165	165	5.24	0.05	387	6.29	0.07	395	2010	5.60	-0.19	54	TSGSS
Accra, Ghana.167	167	5.25	0.04	360	6.10	0.06	305	2010	5.60	-0.19	54	TSGSS
Accra, Ghana.169	169	6.25	0.08	404	7.16	0.05	279	2010	5.60	-0.19	54	TSGSS
Accra, Ghana.171	171	5.68	0.06	359	7.22	0.11	312	2010	5.60	-0.19	54	TSGSS

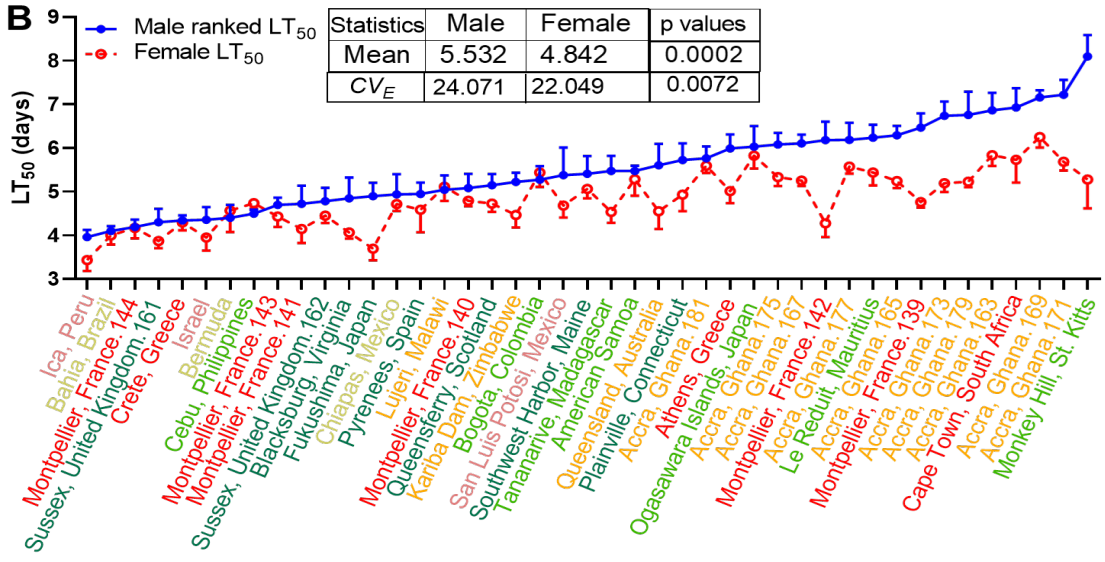
Accra, Ghana.173	173	5.20	0.06	332	6.74	0.10	337	2010	5.60	-0.19	54	TSGSS
Accra, Ghana.175	175	5.34	0.07	281	6.08	0.08	292	2010	5.60	-0.19	54	TSGSS
Accra, Ghana.177	177	5.57	0.05	375	6.18	0.12	350	2010	5.60	-0.19	54	TSGSS
Accra, Ghana.179	179	5.23	0.04	338	6.76	0.17	311	2010	5.60	-0.19	54	TSGSS
Accra, Ghana.181	181	5.59	0.05	350	5.77	0.08	346	2010	5.60	-0.19	54	TSGSS
Kariba Dam, Zimbabwe	64	4.46	0.09	304	5.23	0.07	337	1963	-16.52	28.76	485	TSGSS
Lujeri, Malawi	76	5.11	0.10	369	5.04	0.10	329	2009	-16.04	35.65	717	TSGSS
Queensland, Australia	03	4.55	0.13	327	5.60	0.16	330	Not Listed	-20.92	142.70	185	TSGSS
Ica, Peru	01	3.43	0.08	281	3.96	0.05	344	1956	-14.08	-75.73	403	DXS
Israel	68	3.95	0.09	323	4.35	0.09	336	1954	31.05	34.85	485	DXS
San Luis Potosi, Mexico	43	4.68	0.09	330	5.38	0.20	314	2005	22.16	-100.99	1878	DXS
Athens, Greece	69	5.01	0.09	340	5.99	0.10	310	1965	37.98	23.73	69	MFWS
Cape Town, South Africa	62	5.73	0.17	324	6.93	0.14	327	1954	-33.92	18.42	7	MFWS
Crete, Greece	23	4.30	0.06	422	4.34	0.04	352	2002	35.24	24.81	1667	MFWS
Montpellier, France.139	139	4.76	0.04	375	6.47	0.10	377	2010	43.62	3.86	47	MFWS
Montpellier, France.140	140	4.79	0.04	392	5.08	0.10	382	2010	43.62	3.86	47	MFWS
Montpellier, France.141	141	4.14	0.10	353	4.72	0.13	377	2010	43.62	3.86	47	MFWS
Montpellier, France.142	142	4.28	0.10	337	6.18	0.13	333	2010	43.62	3.86	47	MFWS
Montpellier, France.143	143	4.43	0.08	376	4.70	0.05	365	2010	43.62	3.86	47	MFWS
Montpellier, France.144	144	4.17	0.08	336	4.19	0.05	302	2010	43.62	3.86	47	MFWS
Blacksburg, Virginia	61	4.06	0.04	328	4.85	0.15	346	1954	37.23	-80.41	641	TBMF
Fukushima, Japan	136	3.70	0.08	335	4.90	0.10	290	2009	37.76	140.47	65	TBMF
Plainville, Connecticut	56	4.93	0.10	370	5.72	0.10	437	2007	41.68	-72.86	54	TBMF
Pyrenees, Spain	67	4.59	0.16	282	4.95	0.08	319	1965	42.67	1.00	2311	TBMF
Queensferry, Scotland	130	4.72	0.06	325	5.15	0.08	358	2009	55.99	-3.40	25	TBMF
Southwest Harbor, Maine	132	5.06	0.07	321	5.41	0.13	331	2009	44.28	-68.33	13	TBMF
Sussex, United Kingdom.161	161	3.87	0.05	377	4.30	0.10	364	2011	50.97	-0.47	53	TBMF
Sussex, United Kingdom.162	162	4.45	0.05	433	4.78	0.10	362	2011	50.97	-0.47	53	TBMF

Stocks all contain 14021-0231. prefixes. Abbreviations: Lat. (Latitude), Lon. (Longitude), Alt. (Altitude), TSMF (Tropical and Subtropical Moist broadleaf Forests), TSDF (Tropical and Subtropical Dry broadleaf Forests), TSGSS (Tropical and Subtropical Grasslands, Savannas, and Shrublands), DXS (Deserts and Xeric Shrublands), MFWS (Mediterranean Forests, Woodlands, and Scrub), and TBMF (Temperate Broadleaf and Mixed Forests).

The 43 fly populations were sorted into six biomes based on the latitude and longitude of each collection site using ArcGIS Pro, and the 14 biomes defined in Ecoregions 2017[59] as follows: TSMF (Tropical and Subtropical Moist broadleaf Forests), TSDF (Tropical and Subtropical Dry broadleaf Forests), TSGSS (Tropical and Subtropical Grasslands, Savannas, and Shrublands), DXS (Deserts and Xeric Shrublands), MFWS (Mediterranean Forests, Woodlands, and Scrub), and TBMF (Temperate Broadleaf and Mixed Forests).

We also subdivided the 43 lines into four distinct geographical regions based on their collection sites: African (15), European/Middle East (13), Asian/Pacific (4), and American (11). The African lines were from Ghana (10), Madagascar (1), Malawi (1), Mauritius (1), South Africa (1), and Zimbabwe (1), spanning the TSMF, TSGSS, and MFWS biomes. The European/Middle East lines were from England (2), France (6), Greece (2), Israel (1), Scotland (1), and Spain (1), covering the DXS, MFWS, and TBMF biomes. The Asian/Pacific lines were from Australia (1), Japan (2), and the Philippines (1), representing the TSMF, TSGSS, and TBMF biomes. The American lines were from Bermuda (1), Brazil (1), Colombia (1), Mexico (2), Peru (1), and various locations in the United States (5), encompassing the TSMF, TSDF, DXS, and TBMF biomes (Table 1.1, Figure 1.1A).


A
Queensferry, Scotland
Southwest Harbor, Maine
Sussex, United Kingdom (2)
Montpellier, France (6)
Plainville, Connecticut
Pyrenees, Spain
Athens, Greece
Fukushima, Japan
Blacksburg, Virginia
Bermuda
Israel
Crete, Greece
San Luis Potosi, Mexico
Ogasawara Islands, Japan
Chiapas, Mexico
Cebu, Philippines
Monkey Hill, St. Kitts
Bogota, Colombia
Accra, Ghana (10)
Lujeri, Malawi
American Samoa
Ica, Peru
Bahia, Brazil
Queensland, Australia
Le Reduit, Mauritius
Kariba Dam, Zimbabwe
Tananarive, Madagascar
Cape Town, South Africa
Seasonal biomes
Deserts & Xeric Shrublands
Mediterranean Forests, Woodlands & Scrub
Temperate Broadleaf & Mixed Forests
Aseasonal biomes
Tropical & Subtropical Dry Broadleaf Forests
Tropical & Subtropical Grasslands, Savannas & Shrublands
Tropical & Subtropical Moist Broadleaf Forests


B
Male ranked LT50
Female LT50
LT50 (days)
Statistics | Male | Female | p values
Mean | 5.532 | 4.842 | 0.0002
CVE | 24.071 | 22.049 | 0.0072

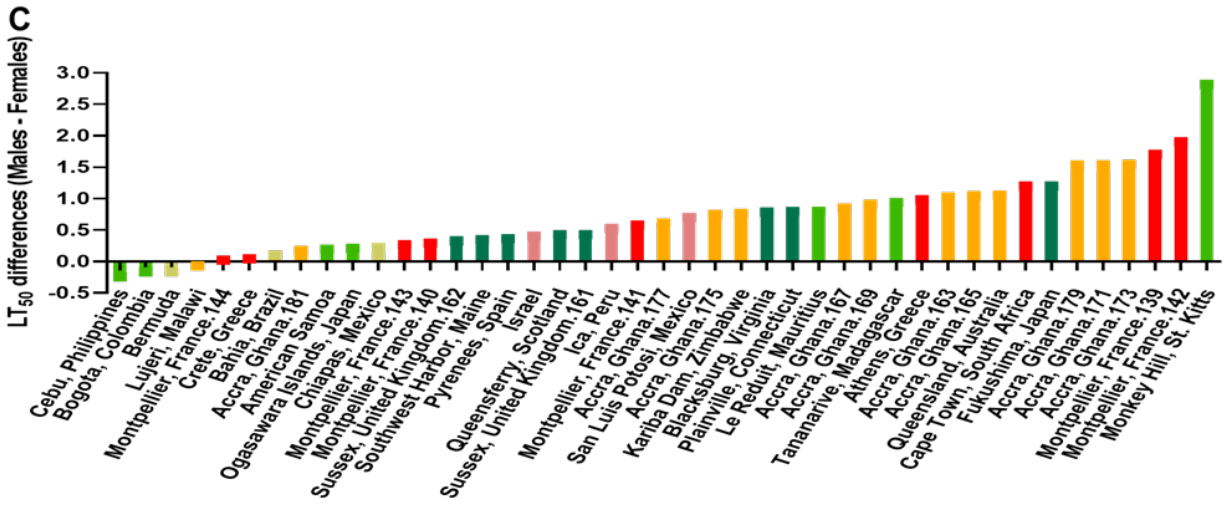

C
LT50 differences (Males - Females)

Figure 1.1 **(A)** Collection locations and biomes of *Drosophila melanogaster* populations. The geographic and biomes map[59] generated by ArcGIS Pro displays the collection locations and biomes of 43 fly populations. The biomes are color coded for this and subsequent figures: Bright Green for Tropical & Subtropical Moist Broadleaf Forests (TSMF), Light Olive for Tropical & Subtropical Dry Broadleaf Forests (TSDF), Gold for Tropical & Subtropical Grasslands, Savannas & Shrublands (TSGSS), Pink for Deserts & Xeric Shrublands (DXS), Red for Mediterranean Forests, Woodlands & Scrub (MFWS), and Forest Green for Temperate Broadleaf & Mixed Forests (TBMF). The numbers in parentheses represent the number of fly lines collected at each site. The 22 circles mark the collection sites of previously deployed fly stocks[44] (gifted by Dr. Alex Keene), while six squares denote collection sites of 21 fly stocks from the *Drosophila* Species Stock Center. **(B)** The graph shows the mean LT_{50} values for females (red circles) and males (blue dots) across all 43 global fly lines, ranked by males from least to most resistant to Ma549 infection. Welch's t test and unpaired t-test were used to obtain p-values for mean and coefficient of variation (CV_E), respectively. **(C)** The graph illustrates sex-based disparities in Ma549 LT_{50} values, calculated by subtracting female LT_{50} values from male LT_{50} values for each of the 43 lines, averaged from 10-15 replicates (each containing an average of 35 flies), per sex per line in 2-3 experiments. Positive values indicate greater resistance in males, while negative values indicate greater resistance in females.

To identify sexual dimorphism, we measured resistance to Ma549 for males and females separately. Two-way ANOVA revealed significant interaction effects between line/genetic factor and sex ($F_{(42, 800)} = 17.49$, $p < 0.0001$), accounting for 9.87% of the total variation. Genetic factors were the most impactful ($F_{(42, 800)} = 117.3$, $p < 0.0001$), explaining 66.17% of the total variation, while sex contributed 12.81% ($F_{(1, 800)} = 953.5$, $p < 0.0001$) (Figure 1.2A, S1.1A and S1.8A Tables), showing that although males generally have longer lifespans, the extent of the differences between the sexes varies significantly across strains. Mean survival time (MST) and LT_{50} values showed strong correlations for both males ($r = 0.9963$, $p < 0.0001$) and females ($r = 0.9992$, $p < 0.0001$) (Figure 1.2B), indicating that both metrics reliably measure disease resistance. We mostly used LT_{50} values to compare resistance across lines and MST values for analyses related to plasticity.

Kaplan-Meier survival plots confirmed a sex-based disparity in fly longevity, with males outliving females in 39 of 43 lines (S1.1A Table, Figure 1.2A and Figure 1.1B, C). The LT_{50} values for both female and male flies across diverse geographical regions were normally

distributed (Figure 1.2C). The average LT_{50} for worldwide males (females) was 5.53 (4.84) days so males were, on average, 1.14-fold more resistant ($t = 3.871$, $p = 0.0002$, Figure 1.1B). The divergence between the most resistant and the most susceptible males (females) was 4.13 (2.82) days, ranging from 3.96 (3.43) to 8.09 (6.25) days (Figure 1.1B, C and Figure 1.2C, Table 1.1), showing that male flies displayed a wider range of LT_{50} values than females. However, cross-sex LT_{50} correlations were strong ($r = 0.7778$, $p < 0.0001$, Figure 1.2D), closely mirroring the DGRP lines ($r = 0.7374$, $p < 0.0001$)[49], indicating that many of the same genetic and environmental factors identified in the DGRP lines affect infection resistance in males and females worldwide.

The most susceptible line was from Ica, Peru (DXS biome), with an LT_{50} of 3.96 (3.43) days for males (females). The most resistant male flies were obtained from Monkey Hill (MH), St. Kitts (TMSF biome) with an LT_{50} of 8.09 days (Figure 1.1B, Table 1.1). Males from the MH populations exhibited 1.53-fold higher resistance than MH females, resulting in them having the largest sexual disparity of 2.81 days (Figure 1.1C, Table 1.1). Four lines (Cebu/Philippines, Bogota/Colombia, Bermuda, and Lujeri/Malawi) with intermediate LT_{50} values showed longer female lifespans (Figure 1.1C, Table 1.1), although these longevity differences were only significant in the Cebu/Philippines line ($t = 3.68$, $p = 0.0003$, Table 1.6). Notably, three of these four lines, excluding Lujeri/Malawi, originated from aseasonal regions characterized by high humidity and uniform temperatures. This suggests that females in these lines might have locally adapted to their specific environmental conditions differently than males did.

Ectothermic organisms commonly exhibit phenotypic plasticity, allowing a single genotype to produce different phenotypes to adapt to environmental stressors like temperature[68]. This plasticity is typically measured through variability among individuals[68]. Although the global lines are highly inbred they are not necessarily clones, so following common

practice we estimated phenotypic plasticity by within-line variability expressed as a coefficient of environmental variation (CV_E), which removes the association between mean and variance[69]. The within-line standard deviation (σ_E) has been used to measure variability among individuals in clonal lines of the DGRP[68], and we used σ_E to demonstrate that plasticity in disease resistance is highly heritable[49]. σ_E positively ($p < 0.0001$) correlates with CV_E (males $r = 0.7605$; females $r = 0.7157$) and MST values (males $r = 0.8300$; females $r = 0.5652$), indicating that the most resistant lines also have the greatest variance (Supplementary figure 1.1A, B).

However, CV_E is not significantly correlated with MST values in males ($r = 0.2786$, $p = 0.0705$) or females ($r = -0.1634$, $p = 0.2952$) (Supplementary figure 1.1C), which is generally true for mean and CV_E plasticity estimates in *D. melanogaster*[69]. This suggests that disease resistance plasticity is independent of dimension, consistent with specific disease plasticity genes distinct from genes determining MST values as previously found in the DGRP[49]. The CV_E ranged from 17.68 to 32.56 in males and 14.35 to 29.36 in females. Levene's test was used to compare sex-based variations in disease resistance (Table 1.6). Of the 43 strains, 27 had higher CV_E in males, with 23 showing significant differences per Levene's test. Variation between sexes across the 43 lines was assessed by comparing CV_E using a paired t-test, revealing significantly larger male variance across lines ($t = 2.755$, $p = 0.0072$, Table 1.6), confirming sex- and line-specific differences in variance. MH males showed the third highest plasticity and IP males the second lowest CV_E (S1.1 Table, $p \leq 0.0024$, Supplementary figure 1.1D, E), while for σ_E, MH males were the most plastic and IP males the least (S1.1 Table, $p < 0.0001$, Supplementary figure 1.1F, G).

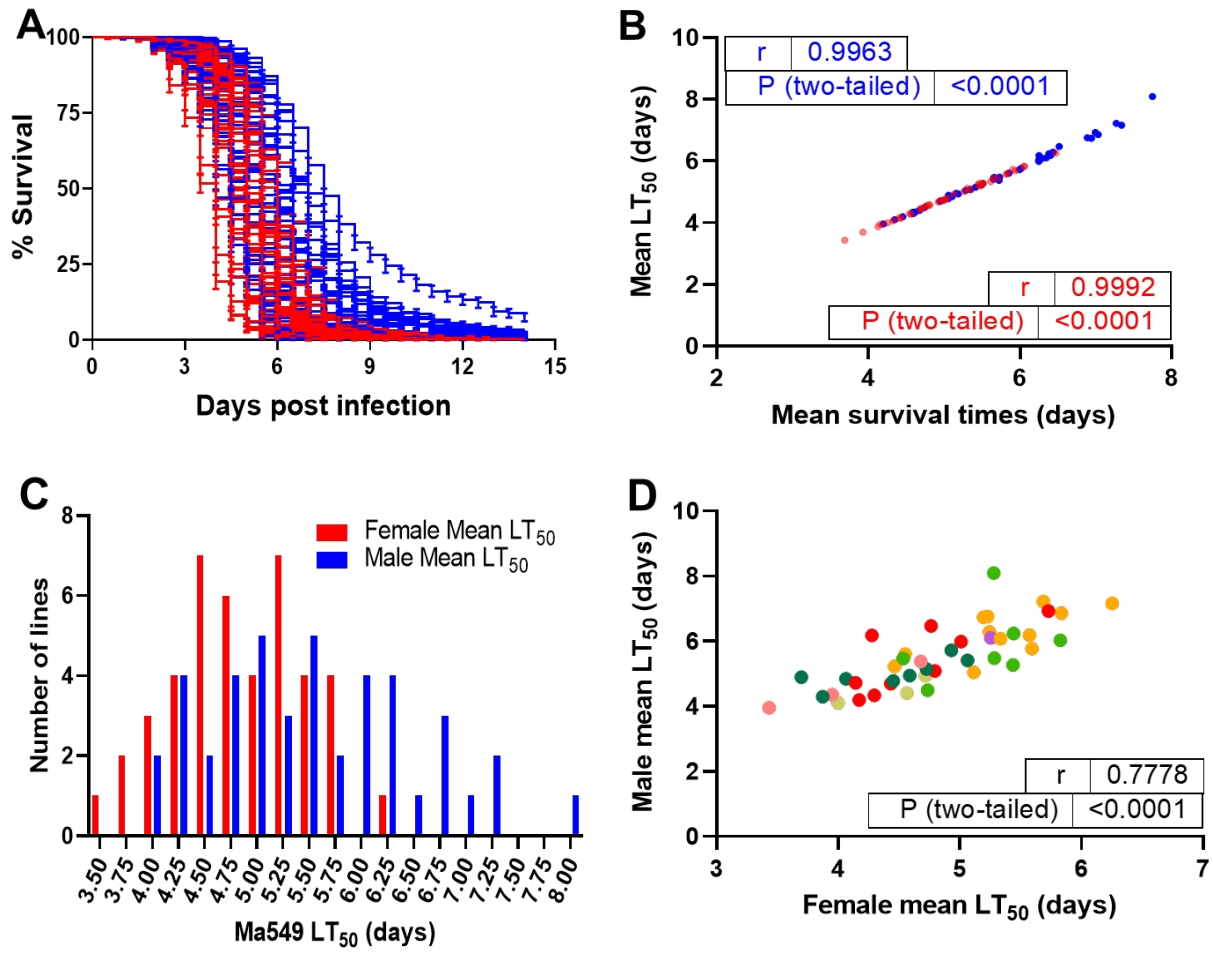


Figure 1.2 **(A)** The survival data of 43 *Drosophila* populations infected with Ma549. The mean percentage survival ± SEM for females (red, n = 281-501) and males (blue, n = 279-497) across 10-15 replicates per sex per line in 2-3 experiments. **(B)** The correlation between mean survival times and LT_{50} values in male (blue dots) and female (red dots) lines infected with Ma549, with Pearson correlation coefficient (r) and p-values indicated. **(C)** The distribution of longevity in females (red bars) and males (blue bars) confirming the normal distribution of mean LT_{50} values from the 43 lines using D'Agostino & Pearson and Shapiro-Wilk tests ($p > 0.05$). **(D)** Correlation analysis of LT_{50} values between the sexes, including the Pearson correlation coefficient (r) and p-values.

1.3.2 Fly line and sex matter more than collection date

As the 43 fly lines had been inbred in laboratories for 13-70 years (Table 1.1), we examined whether domestication contributed to their variable longevity following infection. Excluding two lines with unspecified collection dates (Bahia/Brazil and Queensland/Australia), we analyzed the remaining 41 lines.

To assess the effect of fly line, sex, and collection date on fly longevity (LT_{50}) following Ma549 infection, we used a linear mixed model formulated as: $Y_{LT50} = \mu+S+C+S\times C+L+\varepsilon$, where Y_{LT50} is the response variable representing LT_{50} values, μ is the overall mean, S denotes the fixed effect of sex, C represents the fixed effect of collection date, S×C indicates the interaction term between sex and collection date, L is the random effect of the fly lines, and ε is the residual error (Tables 1.2-1.3).

The fixed effects model revealed significant main effects of sex ($F_{(1, 41.79)} = 20.90$, $p < 0.0001$) but not collection date ($F_{(14, 27.47)} = 1.26$, $p = 0.2935$) on LT_{50} values (Table 1.2). There was also no significant interaction between sex and collection date ($F_{(14, 28.16)} = 1.31$, $p = 0.2618$). Thus, the significant effect of sex on LT_{50} values is independent of the collection date.

Table 1.2 Fixed effect tests on longevity effects of sex and collection date.

Source	Nparm	DF	DFDen	F Ratio	p value
Sex	1	1	41.79	20.9029	<0.0001
Collection date	14	14	27.47	1.2574	0.2935
Sex*Collection date	14	14	28.16	1.3107	0.2618

Source: This is a list of the tested factors. Nparm: Number of parameters associated with the effect. DF: degrees of freedom for each source of variation. DFDen: Denominator degrees of freedom associated with the error term or residuals. F Ratio: the mean square of the factor divided by the mean square of the error. A p-value less than 0.05 means that the factor has a statistically significant effect.

Table 1.3 REML variance component estimates on longevity effects of fly lines.

Random Effect	Variance Ratio	Variance Component	Std Error	95% Lower	95% Upper	p value	% of Total
Fly lines	2.4757	0.4393	0.1457	0.1537	0.7245	0.0026	71.23
Residual		0.1775	0.0483	0.1109	0.3288		28.77
Total		0.6168	0.1457	0.4075	1.0423		100.00

REML, restricted maximum likelihood, is a statistical method used to estimate variance components in linear mixed models, providing unbiased estimates by accounting for the loss of degrees of freedom when estimating fixed effects.

1.3.3 Geography and biomes impact on host susceptibility to infection

Dividing the 43 global fly populations into four geographic regions (Africa, Europe/Middle East, Asia/Pacific, and America) and six biomes allowed us to determine the relative contributions of geographic location and biome characteristics in disease resistance. Stepwise regression analysis showed significant effects of both factors ($p < 0.0001$, Tables 1.1 and 1.4), with biome effects ($\eta^2 = 0.1291$ or 12.91%) being about double those of geography ($\eta^2 = 0.0624$ or 6.24%).

African populations, comprising 35% of the 43 lines, had mean LT_{50} values of 5.98 days (males) and 5.65 days (females), showing 1.12 to 1.24-fold greater disease resistance than other regions, including the N. Carolina DGRPs[49] ($p \leq 0.0484$, Supplementary figure 1.2, and S1.8B Table). Males outlived females by 1.06 to 1.07-fold within each region ($p < 0.0001$), but African females outlived European/Middle Eastern males by 1.16-fold ($p = 0.0021$).

Table 1.4 Impact of geography and biome on resistance levels.

Source	Nparm	DF	Sum of Squares	F Ratio	p value	η² Effect Size
Geography	3	3	49.79355	28.7166	<0.0001	0.0624
Biome	5	5	103.07443	35.6666	<0.0001	0.1291

Source: This is a list of the tested factors. Nparm: Number of parameters associated with the effect. DF: degrees of freedom for each source of variation. Sum of Squares: sum of squares (SS) for each source of variation, along with the total from all sources. F Ratio: the mean square of the factor divided by the mean square of the error. η² (eta squared) is a measure of effect size for analysis of variance (ANOVA) models. A p-value less than 0.05 means that the factor has a statistically significant effect.

1.3.4 Environmental factors at the original collection sites influence host susceptibility to infection in the laboratory

We focused on temperature and humidity, key abiotic factors[70] that act as stressors for many organisms, especially ectotherms when outside their preferred ranges[71]. Latitude strongly

correlates with annual temperature minimums and ranges, while altitude moderately correlates with annual relative humidity minimums and ranges (Supplementary figure 1.3A-F). Relative humidity decreases linearly with altitude[72], indicating altitude's indirect influence on latitudinal humidity differences.

The clinal patterns in disease resistance for the 43 fly populations are shown in Supplementary figure 1.4A-G. Both females and males exhibit a marked and linear decrease in disease resistance (Ma549 LT_{50} values) with latitude (females: $R^2 = 0.3342$, $p < 0.0001$; males: $R^2 = 0.2102$, $p = 0.0020$). There was a smaller association between disease resistance and altitude in males ($R^2 = 0.09931$, $p = 0.0396$), that fell short of significance in females ($R^2 = 0.07018$, $p = 0.0860$). One potential explanation for a diversity of patterns in intraspecific analysis is that latitude or climatic variables *per se* may be less important in disease resistance than a population's position relative to the species range[73]. We found that disease resistance was greater towards the range center (Ghana) but the association was less than latitude, and populations from low latitudes, such as the Monkey Hill (MH) population were persistently more resistant despite distance.

Both sexes exhibit increased Ma549 LT_{50} values with annual average, high, and low temperatures at collection sites (S1.2 Table, Supplementary figure 1.4C-E). Annual mean precipitation (M) and precipitation in the wettest month (W) also correlate with LT_{50} values (S1.2 Table, Supplementary figure 1.4F, G, and Materials and Methods). Despite differences in intercepts caused by males being more resistant, regression slopes for males were slightly, but not significantly, steeper, suggesting the sexes were similar in how LT_{50} values trended with changes in temperature and precipitation.

The principal component analysis (PCA) summarized in Table 1.5 shows that the first principal component, capturing 57.5% of variance in disease resistance, reflects a general gradient of environmental temperature, latitude, and precipitation as well as relative humidity. The second component, explaining 15.8% of the variance, does not appear to be related to male and female LT_{50} values as indicated by the low correlation coefficients (0.141419 for male LT_{50} and 0.214348 for female LT_{50}). The third component, accounting for 10.9% of the variance, defining the impact of dispersal distance on male disease resistance. According to the eigenvalue greater than 1.0 Kaiser-Guttman rule, only the first three principal components (Prin 1-3) with eigenvalues greater than 1.0 are retained, as they explain more variance than any individual original variable. Thus, while temperature and latitude are the primary factors, humidity and precipitation also play secondary roles.

Table 1.5 Principal component loadings of environmental variables and LT_{50} for male and female flies

Principal component analysis (PCA)	Prin1 (57.5%)	Prin2 (15.8%)	Prin3 (10.9%)
T20-30°C & RH60-100%	0.954346	-0.030905	0.181506
T20-30°C	0.921278	-0.241807	0.224949
Average temperature (°C)	0.837885	-0.419570	0.265887
Female LT_{50}	0.827146	0.214348	-0.249415
Log_{10}(latitude)	-0.826659	0.380707	-0.131953
Male LT_{50}	0.727170	0.141419	-0.401798
log_{10} (precipitation)	0.547000	0.669968	0.132934
RH60-100%	0.622824	0.636057	-0.031578
Dispersal distance	-0.354985	0.349947	0.751843

Positive or negative loadings indicate a direct or inverse relationship with the principal component. Black or gray values denote strong or weak associations between LT_{50} and certain environmental variables, respectively.

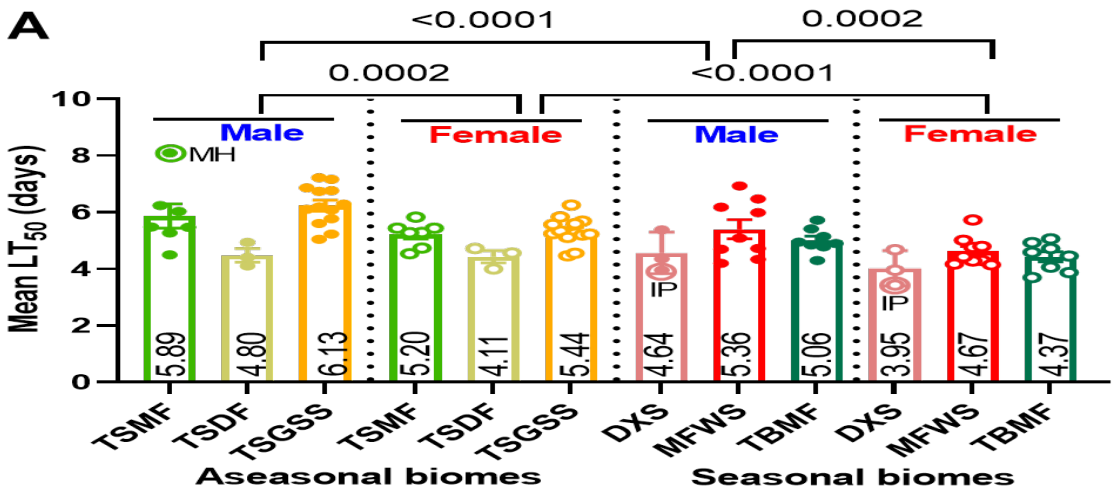
1.3.5 Abiotic and biotic stresses link ecological pressures to variations in disease resistance.

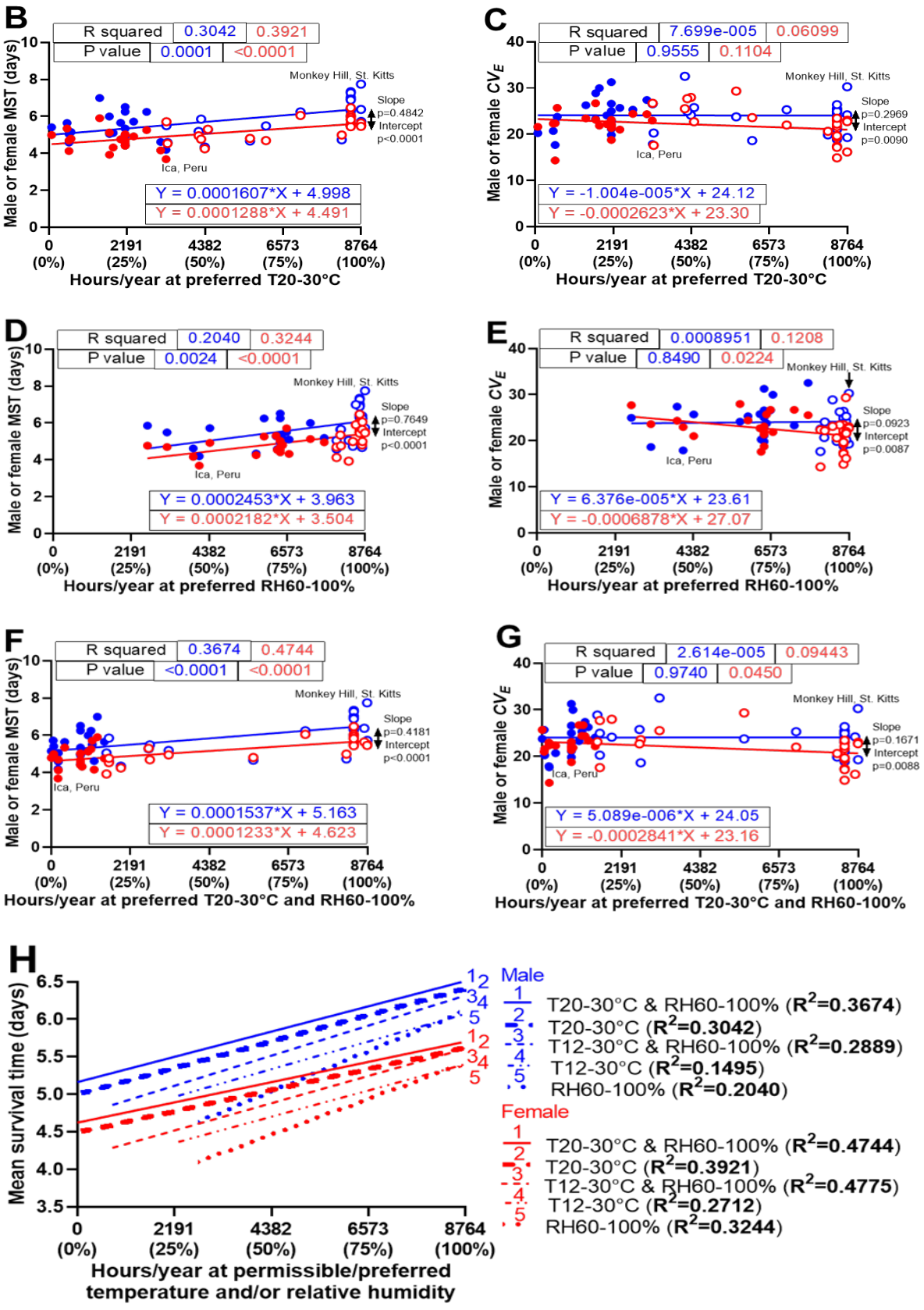
While annual temperatures, precipitation levels, and wettest month precipitation provide a general environmental overview, they may not capture short-term variations or extremes that ectotherms must endure[74, 75]. These variations will presumably be most pronounced in seasonal environments. Aseasonal biomes (TSMF, TSGSS, TSDF) typically have stable temperatures, high humidity, and abundant resources, whereas seasonal biomes (TBMF, MFWS, DXS) often experience greater environmental stresses. Aseasonal males (females) outlived seasonal males (females) by 1.15 (1.16)-fold (Figure 1.3A and S1.8C-D Table), and the most resistant lines from Monkey Hill (MH) and Africa were in the climactically uniform TSMF and TSGSS biomes.

Understanding biome-specific factors may reveal how abiotic and biotic stresses link ecological pressures to variations in resistance Temperatures from 12-30°C and/or relative humidity above 60% are required for *D. melanogaster* development and fertility[76, 77]. The optimal temperature for activity and development is 25°C[78, 79], and fluctuating temperatures have adverse effects on lifespan[80], so we assumed a narrower 20-30°C temperature range would be preferred over the permissible 12-30°C. To investigate the influence of time spent outside these thresholds on disease resistance, the average annual exposure (hours per year) within the permissible temperature (12-30°C) and preferred humidity ranges (60-100%) was calculated using NASA Power Data. We analyzed 52,584 hourly temperature and relative humidity data points at the 28 collection sites from 2005 to 2010, corresponding to most of the 43 fly lines collection dates (S1.3 Table). The hours spent per year within the 12-30°C permissible temperature and preferred 60-100% humidity ranges varied from 2,263 to 8,764 hours/year (Supplementary figure 1.5A-C) and 2,653-8,764 hours/year (Figure 1.3D, E), across collection sites, respectively. Linear regression analyses of the 43 fly populations revealed strong

correlations for both sexes between MST post-Ma549 infection and annual hours spent within 12-30°C or 60-100% humidity showing a trend for increasing disease resistance as climatic uniformity increases. Hours within the preferred 20-30°C range varied from 75 to 8,764 hours/year across collection sites (Figure 1.3B, C and Supplementary figure 1.5G). The results were similar to those for the 12-30°C range but with increased R^2 values.

Annual hours between 12-30°C that coincided with 60-100% humidity spanned 742 to 8,764 hours/year across sites (Supplementary figure 1.5D-F), while annual hours at 20-30°C and 60-100% humidity fluctuated more, ranging from 1 to 8,764 hours (Figure 1.3F-I and Supplementary figure 1.5I). Linear regression analyses of the 43 fly populations revealed that MST for both sexes was more strongly correlated with the annual hours at 20-30°C and 60-100% humidity than with annual hours at 12-30°C and 60-100% humidity, or with temperature and humidity considered separately. No correlation was observed between CV_E values and increasing climatic uniformity in males, while a negative relationship was found between CV_E values and annual hours at 60-100% humidity in females with (Figure 1.3G) and without (Figure 1.3E) considering annual time at 20-30°C, suggesting that favorably uniform humidity conditions do not select for plasticity in females.




B
R squared 0.3042 0.3921
P value 0.0001 <0.0001
Monkey Hill, St. Kitts
Slope p=0.4842
Intercept p<0.0001
Ica, Peru
Y = 0.0001607*X + 4.998
Y = 0.0001288*X + 4.491
Male or female MST (days)
Hours/year at preferred T20-30°C
C
R squared 7.699e-005 0.06099
P value 0.9555 0.1104
Monkey Hill, St. Kitts
Slope p=0.2969
Intercept p=0.0090
Ica, Peru
Y = -1.004e-005*X + 24.12
Y = -0.0002623*X + 23.30
Male or female CVE
Hours/year at preferred T20-30°C
D
R squared 0.2040 0.3244
P value 0.0024 <0.0001
Monkey Hill, St. Kitts
Slope p=0.7649
Intercept p<0.0001
Ica, Peru
Y = 0.0002453*X + 3.963
Y = 0.0002182*X + 3.504
Male or female MST (days)
Hours/year at preferred RH60-100%
E
R squared 0.0008951 0.1208
P value 0.8490 0.0224
Monkey Hill, St. Kitts
Slope p=0.0923
Intercept p=0.0087
Ica, Peru
Y = 6.376e-005*X + 23.61
Y = -0.0006878*X + 27.07
Male or female CVE
Hours/year at preferred RH60-100%
F
R squared 0.3674 0.4744
P value <0.0001 <0.0001
Monkey Hill, St. Kitts
Slope p=0.4181
Intercept p<0.0001
Ica, Peru
Y = 0.0001537*X + 5.163
Y = 0.0001233*X + 4.623
Male or female MST (days)
Hours/year at preferred T20-30°C and RH60-100%
G
R squared 2.614e-005 0.09443
P value 0.9740 0.0450
Monkey Hill, St. Kitts
Slope p=0.1671
Intercept p=0.0088
Ica, Peru
Y = 5.089e-006*X + 24.05
Y = -0.0002841*X + 23.16
Male or female CVE
Hours/year at preferred T20-30°C and RH60-100%
H
Mean survival time (days)
Hours/year at permissible/preferred temperature and/or relative humidity
Male
1 T20-30°C & RH60-100% (R2=0.3674)
2 T20-30°C (R2=0.3042)
3 T12-30°C & RH60-100% (R2=0.2889)
4 T12-30°C (R2=0.1495)
5 RH60-100% (R2=0.2040)
Female
1 T20-30°C & RH60-100% (R2=0.4744)
2 T20-30°C (R2=0.3921)
3 T12-30°C & RH60-100% (R2=0.4775)
4 T12-30°C (R2=0.2712)
5 RH60-100% (R2=0.3244)

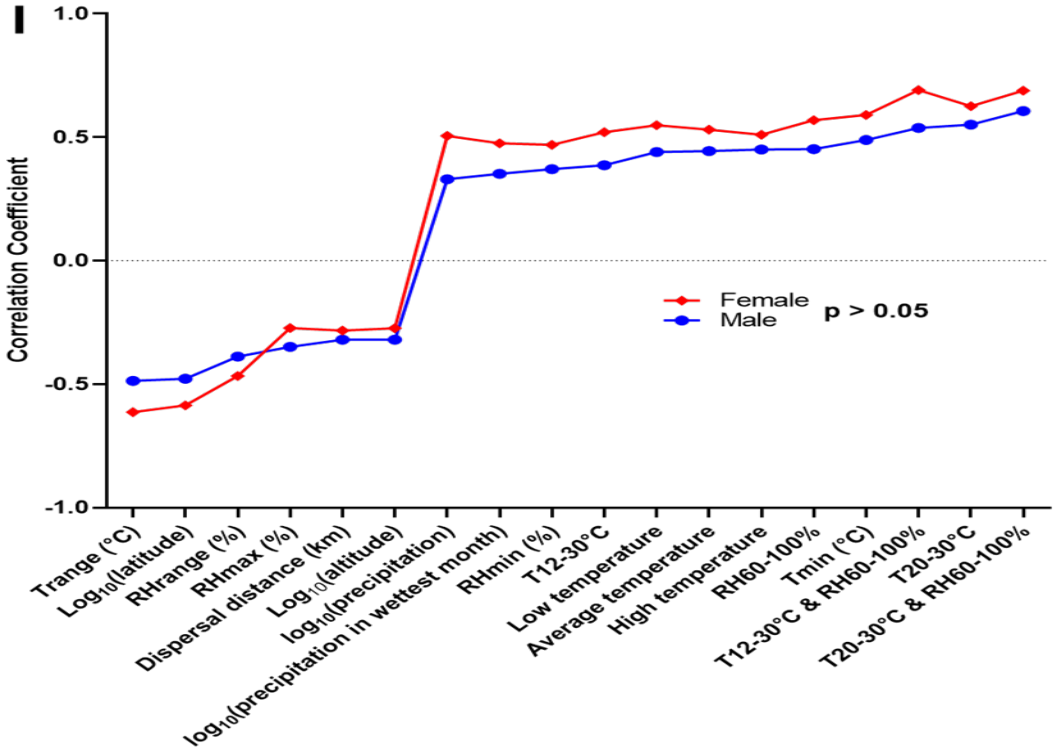


Figure 1.3 Biome variation in LT_{50} values among 43 global fly lines infected with Ma549 (**A**). The biome abbreviations and colors for the 43 worldwide fly lines match those in Figure 1.1A. LT_{50} values varied significantly across biomes and sexes, without a significant biome-sex interaction, as revealed by a two-way ANOVA. Significant p-values and LSM ± SEM from Tukey's multiple comparison tests are shown. Males of the highly resistant MH line in the TSMF biome and highly susceptible IP lines in the DXS biome are indicated. Results for preferred temperature with MST (**B**) and CV_E (**C**), preferred humidity with MST (**D**) and CV_E (**E**), and the combination of preferred temperature and humidity with MST (**F**) and CV_E (**G**), including R^2 values, p-values, and regression equations with slopes. Male (female) regression lines are blue (red). Aseasonal flies are depicted as circles, and seasonal flies as solid dots; this distinction is consistent in all figures. (**H**) Comparison of regression slopes (R^2). (**I**) Correlation coefficients (r) between environmental factors and disease resistance (MST) in male and female *Drosophila* populations included, from left to right, annual temperature range (Trange), latitude, annual relative humidity range (RHrange), annual precipitation, precipitation in the wettest month, annual minimum relative humidity (RHmin), hours/year at 12-30°C (T12-30°C), low (night) temperature, average temperature, high (day) temperature, hours/year at 60-100% relative humidity (RH60-100%), annual minimum temperature (Tmin), hours/year of T12-30°C and RH60-100%, hours/year at 20-30°C (T20-30°C), and hours/year of T20-30°C and RH60-100%.

We next analyzed populations from two ends of the resistance and biome spectrum to check the relationship between abiotic and biotic stresses. The males of the Monkey Hill, St. Kitts (MH) population show the highest disease resistance, whereas males and females of the Ica,

Peru (IP) population are the most susceptible (Figures 1.2D and 1.4A, S1.1A-B Table). MH males (females) demonstrate a 2.04 (1.54)-fold greater resistance to Ma549 infection than IP males (females) ($p < 0.0001$, Figure 1.4A). Relative to the mean LT_{50} values across strains for males (females) of 5.53 (4.84), MH males (females) were approximately 1.46 (1.08)-fold more resistant and IP flies 1.4-fold less resistant.

MH in the TSMF biome (Figure 1.4B) has dense vegetation and a uniform maritime climate, whereas IP in the DXS biome (Figure 1.4C) experiences arid conditions and sparse desert-adapted vegetation (Table 1.1, and S1.2-1.3 Tables). Using Lomb-Scargle periodograms (Supplementary figure 1.6A-B, and S1.3B Table), we confirmed that from 2005 to 2010, MH maintained near-ideal conditions (20-30°C temperature, 60-100% humidity) for fly development 24 hours a day. In contrast, IP mostly experienced arid and fluctuating conditions with preferred temperature and humidity conditions coinciding only 2.12% of the time (Supplementary figure 1.6C-J).

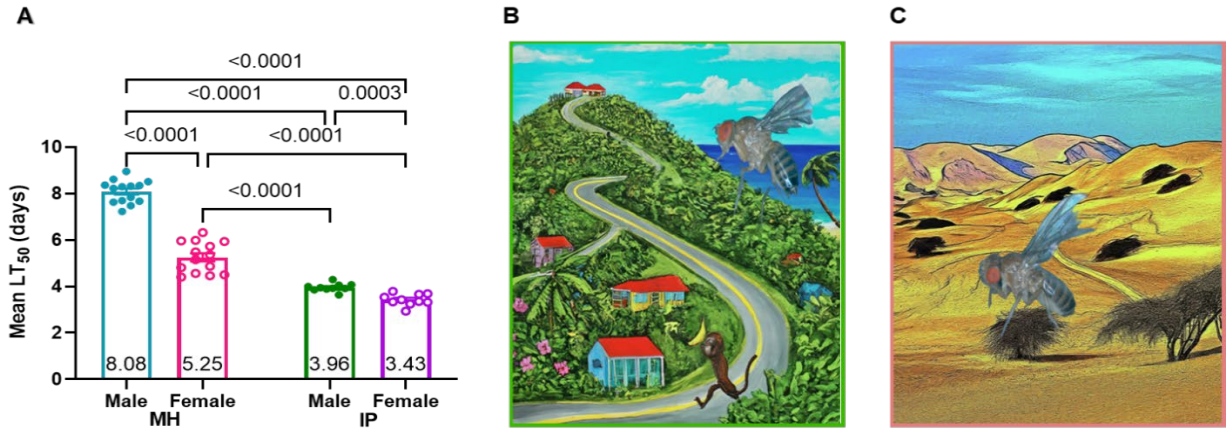
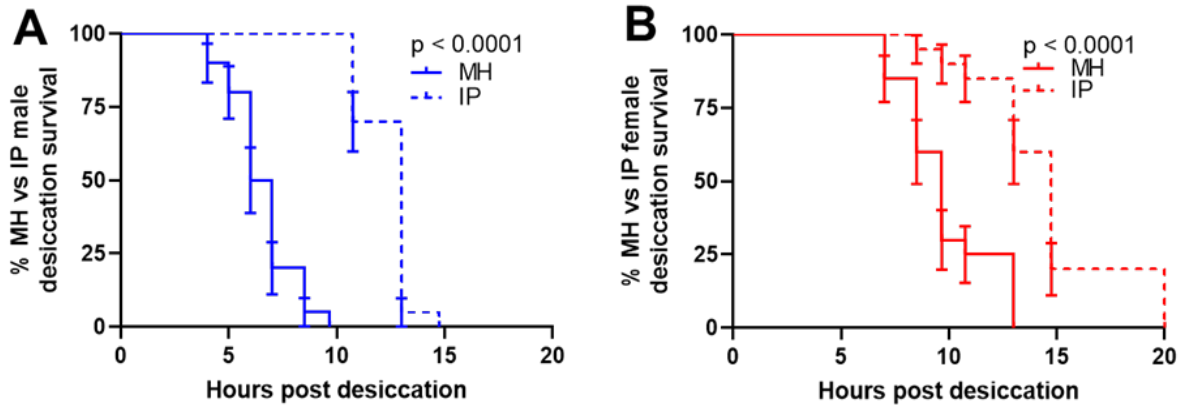


Figure 1.4 **(A)** Sexual dimorphism and longevity of infected Monkey Hill, St. Kitts (MH) and Ica, Peru (IP) fruit flies**:** The data presents the mean ± SEM LT_{50} values across 14 replicates, with 501 (373) MH females (males) from 3 experiments, and 10 replicates with 281 (339) IP females (males) from 2 experiments. p-values are derived from Welch's ANOVA tests with Dunnett's multiple comparisons. Cartoon drawings of Monkey Hills, St. Kitts **(B)** and Ica, Peru **(C),** contrast their distinct biomes, illustrating the landscapes shaped by each location's unique climatic environment.

We subjected male and female MH and IP flies to a desiccating environment to determine whether there might be trade-offs with disease resistance. Kaplan-Meier analyses demonstrated that IP males (females) were 2.00 (1.53)-fold more resistant to desiccation stress than MH males (females) ($p < 0.0001$, Figure 1.5A-B). Furthermore, females of both lines were more desiccation resistant than males, with IP and MH females outliving their male counterparts by 1.14 and 1.49-fold, respectively ($p \leq 0.0033$, Figure 1.5C-D) (S1.4A Table). These differences between IP and MH appear adaptive as only IP evolved in a dry environment; however, they totally reverse the direction of resistance to Ma549.

To examine the relationship between disease resistance and desiccation in a larger sample, we reanalyzed disease resistance in the DGRPs[49], and the desiccation resistance trait quantified in a later study of the DGRPs[81] (S1.4B Table). We found that resistance of DGRPs to desiccation positively correlates with female resistance to Ma549 ($r = 0.2334$, $p = 0.0028$), but not males ($r = 0.1241$, $p = 0.1157$) (Figure 1.5E). Moreover, similar to MH and IP, these DGRP lines exhibited a complete reversal in the direction of sexual dimorphism in these two resistance traits: males outlived females by 1.05-fold in Ma549 resistance, whereas females outlived males by 1.38-fold in desiccation resistance (Figure 1.5F).



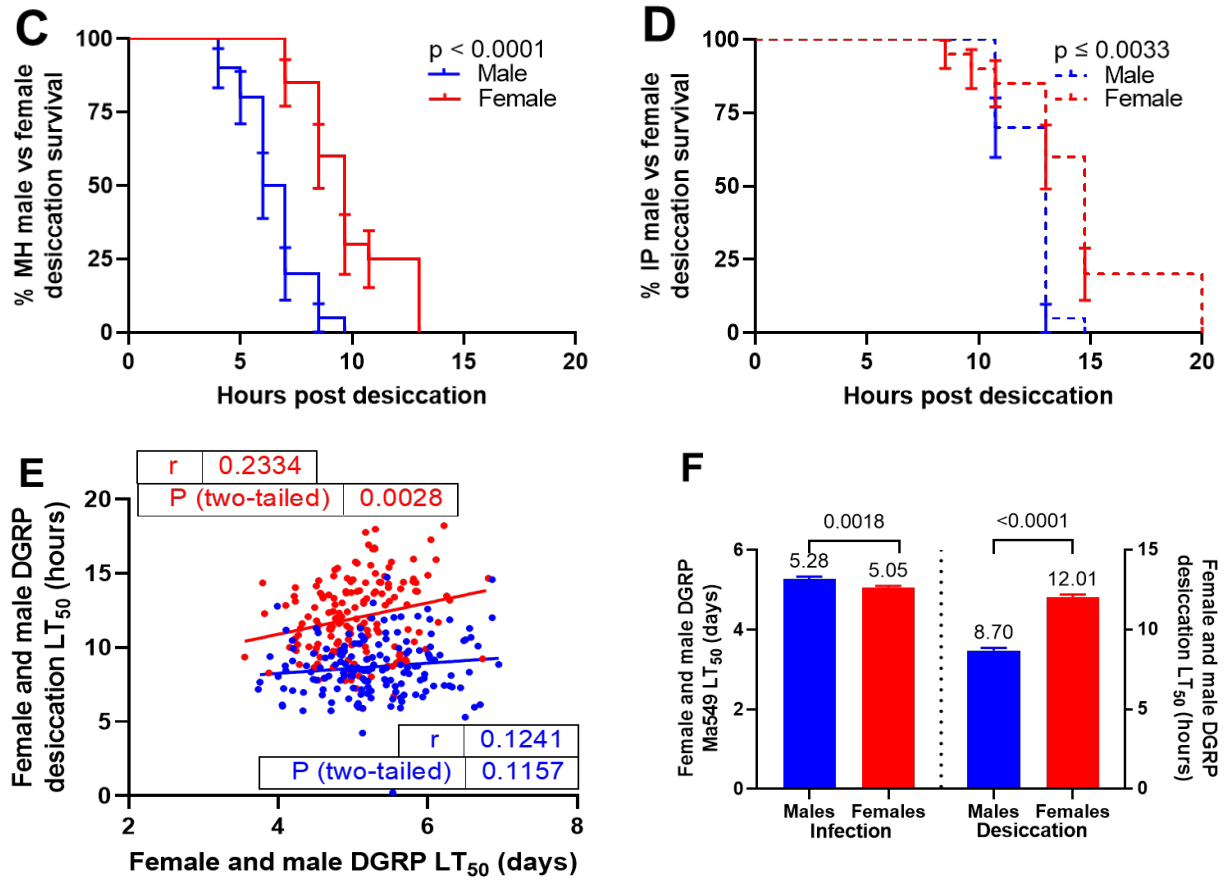


Figure 1.5 Differences in desiccation resistance. Kaplan-Meier plots illustrate survival differences between MH and IP lines **(A, B)**, and between sexes **(C, D).** MH males (females) from five replicates (10 flies each) across two trials are shown in turquoise (crimson), and IP males (females) in green (purple). Statistical significance was assessed using log-rank and Wilcoxon tests. **(E)** Correlation analysis between desiccation and Ma549 LT_{50} values of 162 DGRP females and males, with Pearson correlation coefficients (r) and p-values indicated. Males are represented by blue dots and females by red dots. **(F)** Sexual dimorphism in resistance to disease (left y-axis) and desiccation stress (right y-axis) for the 162 DGRP fly lines is represented as mean ± SEM LT_{50} values from disease resistance[49] and desiccation resistance data[81]. p-values are derived from Welch's t-tests.

1.3.6 Correlation between sleep and disease resistance

Using healthy females from 22 global populations[44], it was demonstrated that higher monthly average temperatures at collection sites correlate with increased sleep duration in the laboratory. Our current study found a correlation between collection site temperatures and longevity post-Ma549 infection, while our previous DGRP analysis linked disease resistance

with several sleep parameters[49] suggesting an avenue to explore the relationship between sleep indices and disease resistance in a global population.

Using the sleep behavior data provided for females from 22 global populations[44], we identified significant positive correlations between LT_{50} values and both 24h sleep duration (r = 0.5586, p = 0.0069, Figure 1.6A) and sleep bout length (r = 0.6230, p = 0.0020, Figure 1.6B), alongside a negative correlation with sleep bout number (r = -0.4703, p = 0.0272, Figure 1.6C). These results underscore a significant link between healthy consolidated sleep patterns and Ma549 infection outcomes.

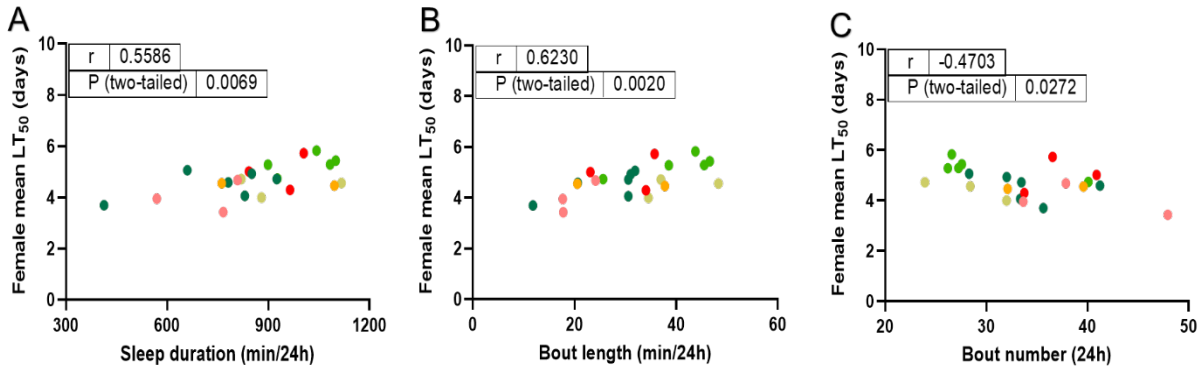


Figure 1.6 Correlations of LT_{50} values among Ma549-infected females and publicly available sleep indices for 22 of the lines[44] deployed in our study. LT_{50} values are positively correlated with sleep duration (**A**) and bout length (**B**), and negatively correlated with bout number (**C**). Individual Pearson correlation coefficients (r) and p-values are indicated in the figures.

1.3.7 Characterization of sickness sleep triggered by Ma549 infection

The significant correlation between healthy female daily sleep patterns and Ma549 infection outcomes could be because latitudinal and climatic gradients have acted independently on sleep and disease resistance or alternatively be because sleep and resistance are evolutionarily intertwined. We investigated post-infection sleep behaviors in MH (resistant) and IP (susceptible) flies using the *Drosophila* Activity Monitor (DAM2)[82] focusing on the first two days post-infection before the onset of overt symptoms. We also investigated a MFWS

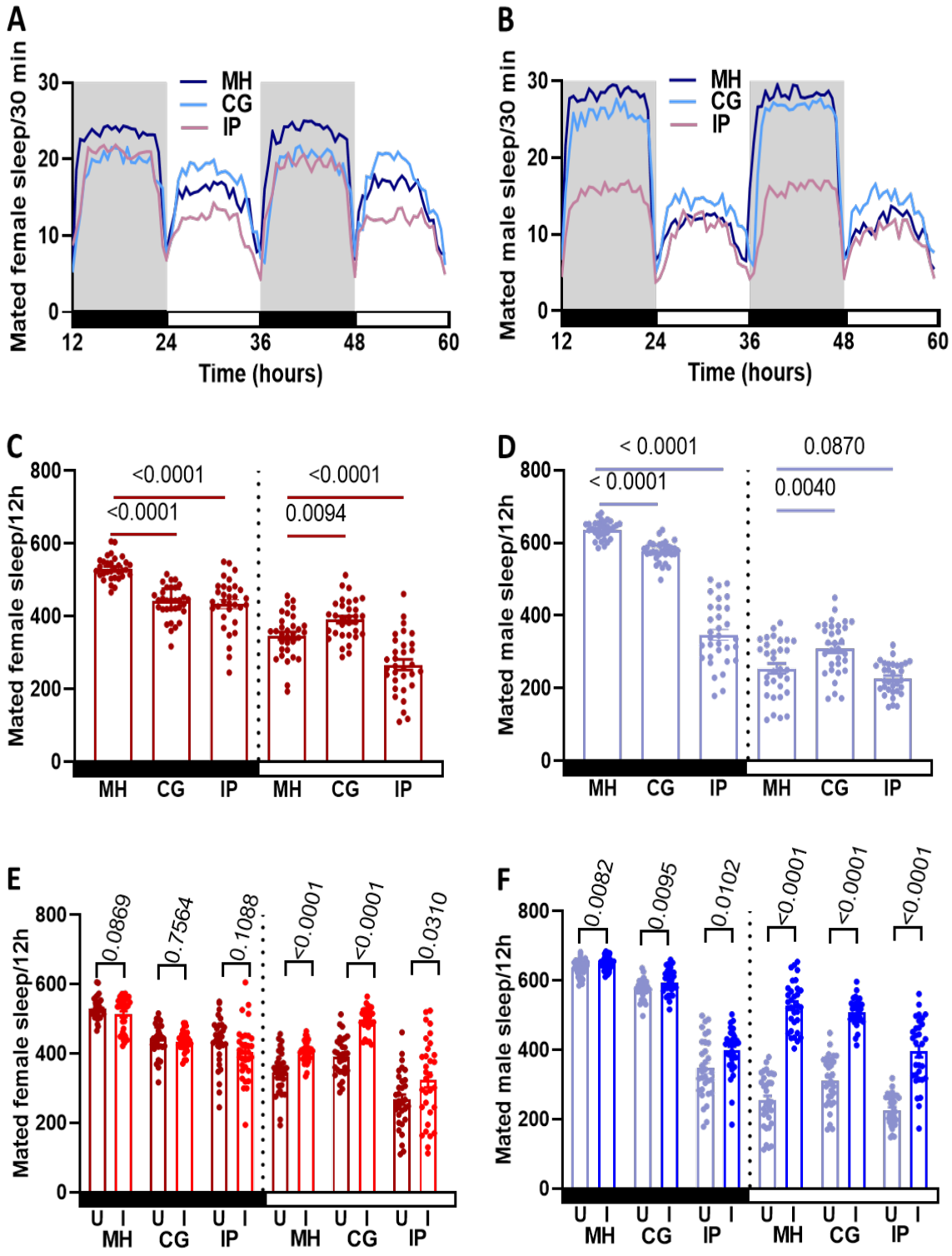
population from Crete, Greece (CG). Male (female) CG flies have LT_{50} values of 4.34 (4.30) days (Table 1.1), with only 0.04-day difference between the sexes (Figures 1.2D and 1.9A)[82].

The sleep data (S1.5 Table) showed that uninfected mated MH females had 16% less nighttime sleep, but 36% more daytime sleep than uninfected males ($p < 0.0001$, Figure 1.7A-D), which averaged out to females sleeping about 12 minutes less in a day. Ma549 infection did not disrupt the circadian rhythm in MH flies, but significantly increased daytime sickness sleep (extra sleep following infection as defined by Davis et al. 2017[83]) compared to uninfected controls ($p < 0.0001$, Figure 1.7E-H). Mated males showed the largest increase in daytime sleep (107%), followed by virgin males (68%) ($p < 0.0001$, Figure 1.7F, H). Infected mated (virgin) females showed only a 17% (14%) increase compared to uninfected females ($p < 0.0001$, Figure 1.7E, G). Nighttime sleep in females was unaffected by infection ($p \geq 0.0661$, Figure 1.7E, G) but increased by 2% in mated and virgin males ($p \leq 0.0119$, Figure 1.7F, H).

Compared to uninfected MH flies, CG females and males slept less at night and more during the day (Figure 1.7A-D). CG females slept 24% less at night and 26% more during the day than the males ($p < 0.0001$, Figure 1.7A-D). Infection with Ma549 increased daytime sleep: mated males by 64%, virgin males by 41%, mated females by 27%, and virgin females by 20% ($p < 0.0001$, Figure 1.7E-H). Similar to MH flies, nighttime sleep was minimally affected by Ma549 infection (Figure 1.7E-H).

The highly susceptible IP flies had shorter sleep durations than MH or CG flies with males being more active (slept less) at night (Figure 1.7A-D). Unlike MH and CG lines where males slept longer, IP females slept 25% longer at night ($p < 0.0001$, Figure 1.7A-D), and 18% longer during the day ($p = 0.0172$, Figure 1.7A-D). Ma549 infection increased daytime sleep in IP flies by 74% in mated males ($p < 0.0001$, Figure 1.7F), 81% in virgin males ($p < 0.0001$,

Figure 1.7H), 21% in mated females (p = 0.0310, Figure 1.7E), and 16% in virgin females (p = 0.0054, Figure 1.7G), with minimal effects on nighttime sleep (Figure 1.7E-H).



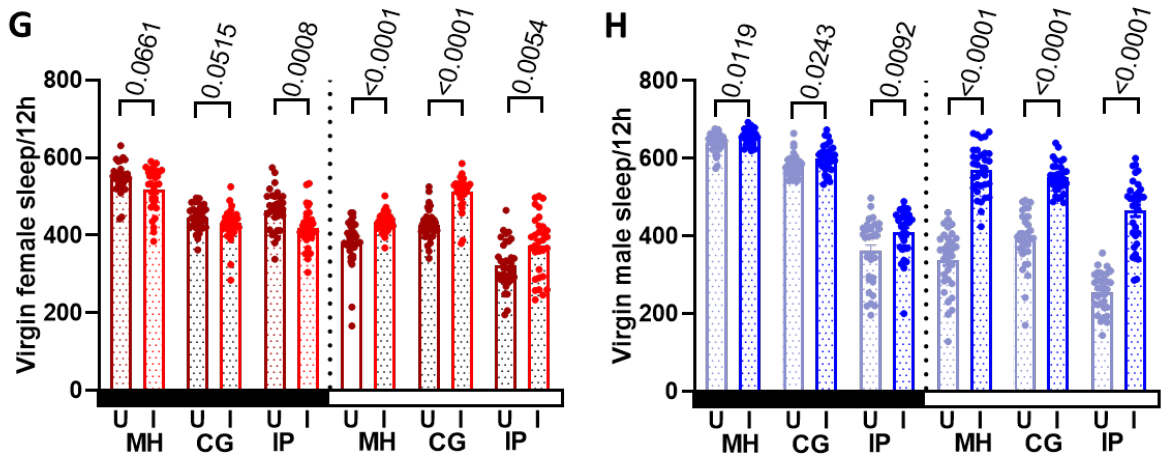


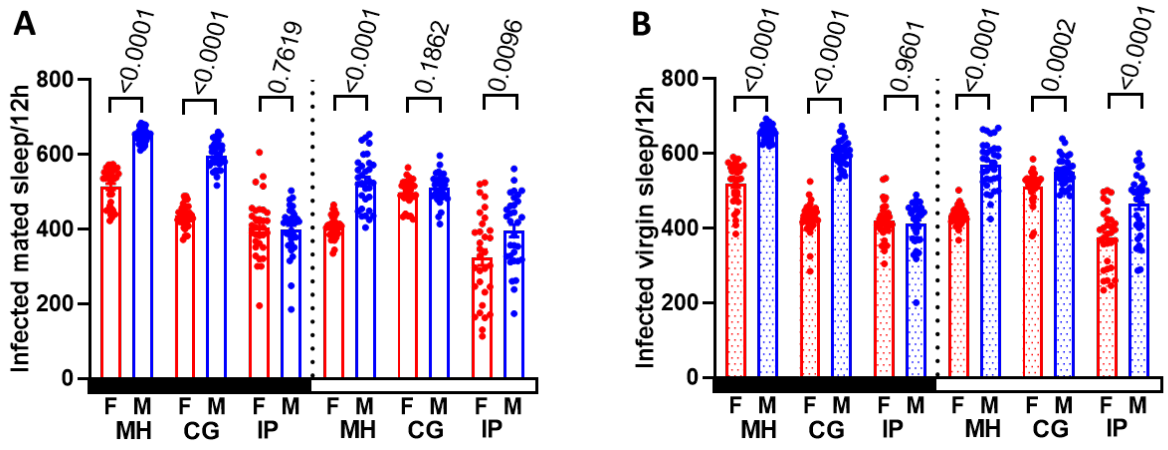
Figure 1.7 Ma549 infection affects sleep and survival. Sleep profiles depict sleep in min per 30 min from 12-60 h spanning two light-dark cycles for 32 flies pooled from three trials for uninfected mated MH, CG, and IP females **(A)** and males **(B)**, with gray shading indicating nighttime. Sleep comparisons were made between uninfected mated MH versus CG or IP females **(C)** or males **(D)**, and uninfected (U) versus infected (I) mated females **(E)**, mated males **(F)**, virgin females **(G)**, and virgin males **(H)** within the MH, CG and IP lines. Bars illustrate nighttime (black) and daytime (white) sleep for uninfected (dark red for females, sky blue for males) and infected (scarlet red for females, navy blue for males) mated and virgin (shaded) flies, with each data point representing the average sleep/12h of a single fly (n = 32) from 12-60 h post infection. In panels **(C)-(D)**, means ± SEM and p-values were obtained from one-way ANOVA. In panels **(E)-(H)**, Mann-Whitney U tests, Welch's t-tests, and unpaired Student's t-tests were used for various comparisons, with mean ± SEM and p-values indicated.

As males are generally more resistant than females, we investigated sexual dimorphism in sleep as a factor in disease resistance (Figure 1.8A-B, S1.5 Table). Ma549 infection significantly increased daytime sleep in mated and virgin males of MH (30% and 31%), IP (22% and 24%), and virgin CG (8%) lines ($p \leq 0.0096$). The effect was minimal in mated CG males (2%, $p = 0.1862$), as was the difference in longevity between mated CG males and females. Mated and virgin MH and CG males slept 27% and 40% longer at night, respectively ($p < 0.0001$), compared to females, while IP males did not differ significantly from females ($p \geq 0.7619$). These sleep pattern differences aligned with disease resistance, with mated (virgin) males outliving females by 53.8% (51.2%), 0.95% (8.3%), and 15.4% (13.80%) in the MH, CG, and IP lines, respectively ($p < 0.0001$, Figure 1.8E, Table 1.1 and S1.1C Table), suggesting that,

except for mated CG males, mated and virgin males exhibit longer sickness sleep and greater disease resistance than females following Ma549 infection.

Mating status did not affect nighttime sleep of infected females or males across all strains ($p \geq 0.2803$, Figure 1.8C, D and S1.5C Table), but sexual activity significantly decreased daytime sickness sleep irrespective of sex and infection status ($p \leq 0.0482$), with infection reducing daytime sleep by 3-15% in mated flies compared to virgins. Following infection, virgin females (males) exhibited 10.34-14.67% (8.47-20.20%) higher survival compared to mated counterparts ($p < 0.0001$, Figure 1.8E, Table 1.1 and S1.1C Table), highlighting the significant influence of sexual activity on sleep regulation and immunity in flies, regardless of genetic background or specific fly lines.

ANOVA was used to validate the effects of genetic line, sex, mating status, and infection on both nighttime and daytime sleep. The results are detailed in Table 1.7 (derived from data in S1.5B Table) and visualized in Supplementary figure 1.7A, B).



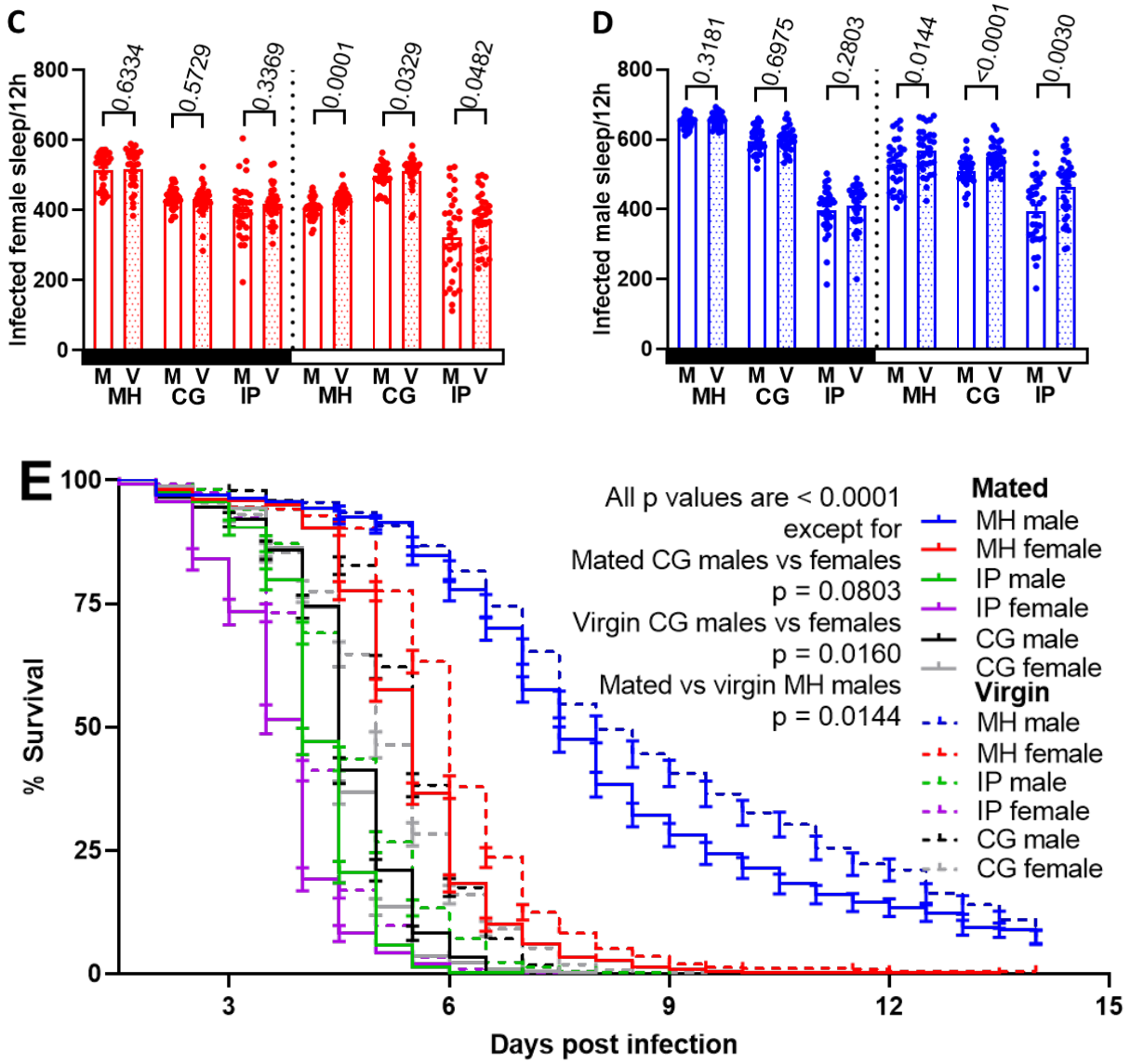
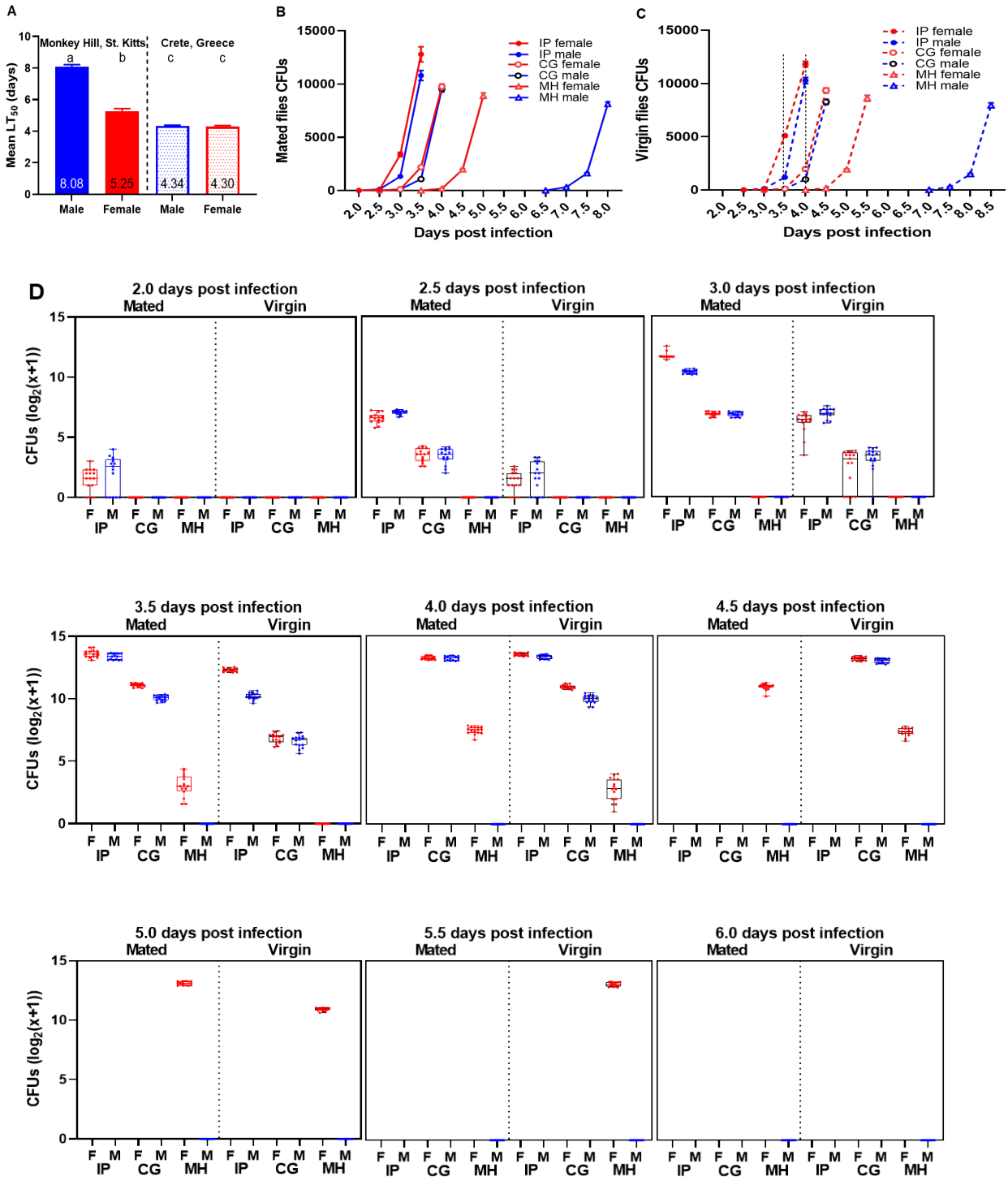


Figure 1.8 Sexual dimorphism in sleep was compared in mated **(A)** and virgin **(B)** MH, CG, and IP flies**.** Sleep was averaged per 12 h for each fly (n = 32) during day and night from 12-60 h post infection, pooled from three trials. Mann-Whitney U tests, Welch's t-tests, and unpaired Student's t-tests were used for various comparisons, with mean ± SEM and p-values indicated. Variations in nighttime and daytime sleep between mated and virgin infected female (**C**) and male (**D**) flies are shown, with each point representing a single fly's average sleep per 12 h (n = 32 pooled from three trials) from 12-60 h post-infection. Significance was assessed between mated and virgin females or males within each lines using various tests, with mean ± SEM and p-values indicated. Black (white) rectangles on the x-axis represent nighttime (daytime), and shaded bars denote virgin flies. Line abbreviations: V for virgin and M for mated. Kaplan-Meier plots **(E)** depict survival differences between female (n = 281-585) and male (n = 337-434) or between mated (n = 281-501) and virgin (n = 337-585) flies in each line post-infection. Log-rank and Wilcoxon tests were used for statistical comparisons, with p-values indicated for general comparisons and specific group comparisons.

1.3.8 The impact of host variation on fungal fitness

We analyzed the relationship between longevity and Ma549 colonization (measured as fungal load in colony forming units CFU) of the most resistant (MH), susceptible (IP), and representative average (CG) *D. melanogaster* lines. MH males (females) survived Ma549 infection 2.86 (2.28)-fold longer than CG males (females) ($p \leq 0.0006$, Figure 1.9A), with no significant differences between CG males and females ($p = 0.9897$). Host variation significantly impacted Ma549 fitness, with CFUs appearing 2.0 days post-infection in IP flies, and 4.0- and 7.0-days post-infection in virgin MH females and males, respectively. Mated IP females were the least able to restrain fungal growth with 12,791 ± 700.05 CFUs/fly on day 3.5, while virgin MH males had the lowest counts at 16 ± 1.21 CFUs/fly on day 7.0 (Figure 1.9B-D, S1.6 Table). Fungal loads increased rapidly near fly death in all lines, indicating a breakdown of host resistance. Males, regardless of mating status, had lower fungal loads than females (Figure 1.9B-D), and virgin females delayed fungal proliferation by approximately 0.5-days compared to mated flies (Figure 1.9C-D), aligning with differences in their LT_{50} values (Figures 1.4A and 1.9A, Table 1.1 and S1.1C Table). Correlation analyses showed that LT_{50} values (Table 1.1 and S1.1C Table) were strongly correlated with both the onset (initial time point at which fungal growth becomes detectable) and peak times (time point maximum fungal growth achieved) of CFUs ($r = 0.9910$, $p < 0.0001$, Figure 1.9E-F) across mated and virgin IP, CG, and MH females and males.

These findings confirm that a fly's line, sex and mating status all influence fungal invasiveness, with proliferation coinciding with and likely causing death due to an inability to tolerate high fungal levels (Figure 1.9).


A
Monkey Hill, St. Kitts
Crete, Greece
a
b
c
c
Mean LT50 (days)
8.08
5.25
4.34
4.30
Male
Female
Male
Female
B
Mated flies CFUs
IP female
IP male
CG female
CG male
MH female
MH male
Days post infection
C
Virgin flies CFUs
IP female
IP male
CG female
CG male
MH female
MH male
Days post infection
D
2.0 days post infection
2.5 days post infection
3.0 days post infection
3.5 days post infection
4.0 days post infection
4.5 days post infection
5.0 days post infection
5.5 days post infection
6.0 days post infection
Mated
Virgin
CFUs (log2(x+1))
F M
IP
CG
MH

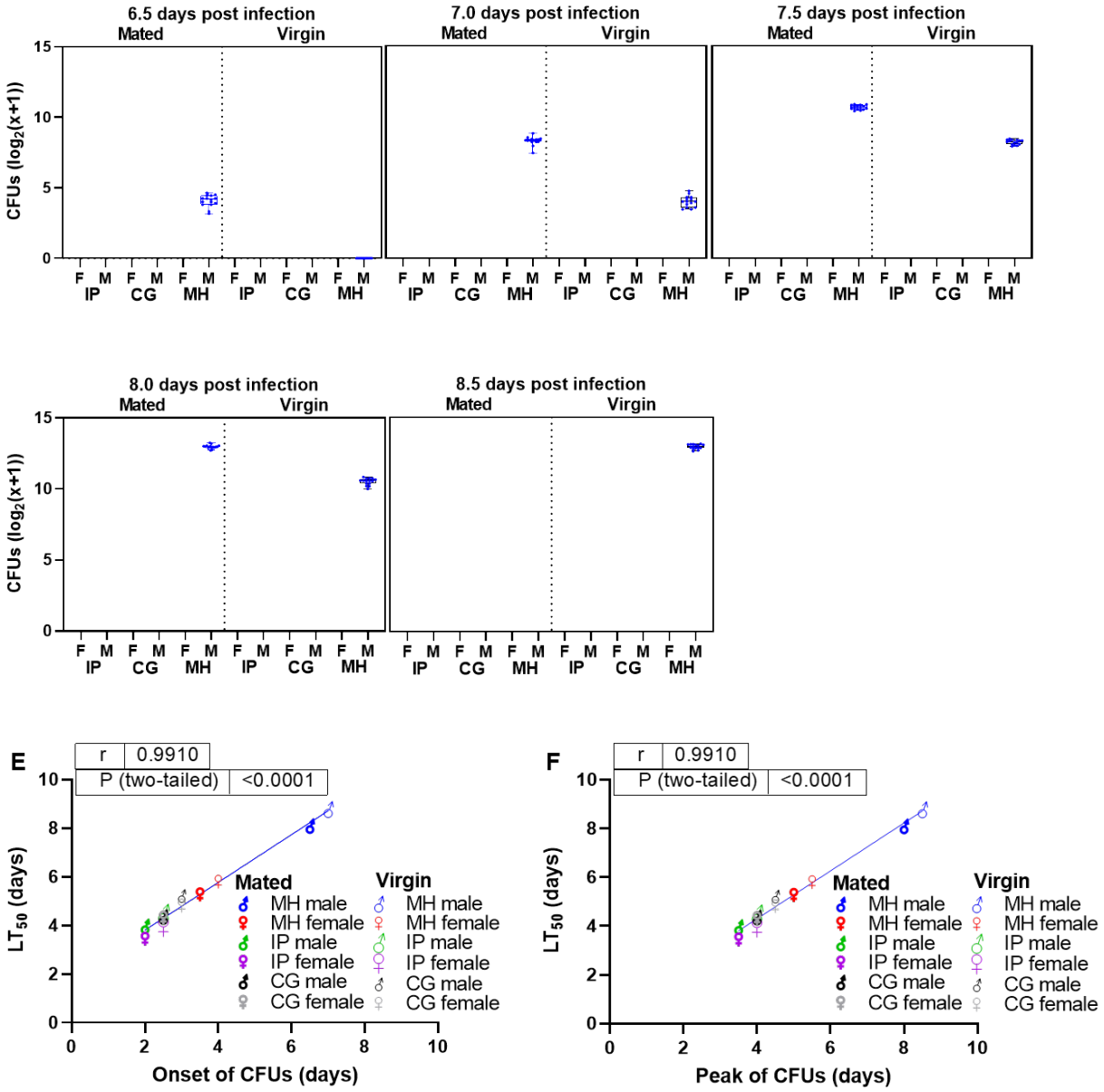


Figure 1.9 **(A)** Sexual dimorphism and longevity of infected Monkey Hill, St. Kitts (MH) and Crete, Greece (CG) fruit flies. The data display the mean ± SEM LT_{50} values from 14 replicates, with 501 MH females and 373 MH males across 3 experiments, and 422 CG females and 352 CG males across 2 experiments. Bars not connected by the same letter are significantly different. CFU production time course in mated **(B)** and virgin **(C)** CG, IP, and MH flies following Ma549 infection. Each data point represents the average of CFU values from 15 flies. Means ± SEM and p-values were obtained from two-way ANOVA. Time course of log-transformed Ma549 fungal loads in the hemolymph of CG, IP, and MH flies following Ma549 infection **(D).** Box plots depict each time point for each of the 15 individual replicates per sex, mating status, line, and time point across three repeated experiments, showing the mean ± SEM of CFUs. Correlations between LT_{50} values and CFU onset **(E)** or peak **(F)** were analyzed for infected mated and virgin

CG, IP, and MH females (red) and males (blue). Pearson correlation coefficients (r) and p-values indicated.

1.3.9 Potential adaptive differences between populations from Africa, France and Raleigh, North Carolina

Based on the observed longevity differences across geographic regions and biomes, we explored the underlying genetic basis by analyzing single nucleotide polymorphisms (SNPs) linked to disease resistance. Using SNPs from the North American DGRP[49], we investigated allele frequency differences that could indicate local adaptation to pathogens in the DGRP, African and French isolates (S1.7 Table). We previously found that most polymorphisms associated with Ma549 resistant DGRP lines were in the lower range of the allele frequency spectrum, with frequencies below 0.2 for 41% of the genes. These low-frequency alleles had larger effects on LT_{50} values than common alleles, resulting in the most Ma549 resistant DGRP lines having a preponderance of these alleles[49]. Given that African lines typically exhibit higher resistance than DGRP lines and are localized in an aseasonal biome (Supplementary figure 1.2), we hypothesized that these alleles may be more prevalent in Africa.

We defined subsets of rare SNPs (S1.7 Table) previously linked with quantitative traits (LT_{50} values or CV_E, micro-environmental plasticity as described[68]) in Ma549 resistant DGRP lines using their corresponding positions in African and European populations. We used the PopFly database[84] to download genome sequences from African lines corresponding to the 10-bp region flanking each SNP. Besides the 10 lines from Ghana, we included the Siavonga, Zambia (ZI) population, which consists of 207 individuals (large sample size) and is the most diverse among all sampled populations but with minimal admixture from non-African populations[84, 85].

The African populations exhibited higher frequencies of 11 out of 24 alleles affecting LT_{50} values in the DGRP lines. Variants typically showed modest 2- to 3-fold differences between African and American populations, but some SNPs displayed approximately 10-fold differences, mostly favoring African populations, resulting in a 22.30% higher average frequency of resistance alleles in African than American lines (Figure 1.10A). Sixteen out of 29 alleles influencing CV_E values were more prevalent in African lines, with frequency differences leading to twice as many CV_E variants for high plasticity in African genomes than in the DGRP lines (Figure 1.10B). Low frequency (<0.08) DGRP variants in *Rab26* (exocrine secretion), *Xpd* (DNA repair, UV damage) and at an intergenic site were the major alleles (>0.5) in African populations, whereas DGRP variants in two poorly understood genes, *CG32066* (DGRP frequency 0.1311) and *CG13229* (DGRP frequency 0.2327), were absent in Zambia and Ghana populations. An extended search in the PopFly database across additional African populations (136 Cameroon, 680 Ethiopia, 136 Gabon, 176 Kenya, 88 Malawi, 88 Nigeria, 136 Rwanda, 504 South Africa, 176 Uganda and 88 Zimbabwe) revealed the DGRP SNP variant of *CG32066* in single flies from South Africa (SD39N) and Uganda (UG19), but the DGRP *CG13229* variant was not found in Africa.

The DGRP is an admixture of African and European populations[86]. We extended our search to 172 French genomes in PopFly, finding that seven of the DGRP SNPs were absent. Most SNPs, except for those in *gem3, ash2, CG33136, Ddr, CCKLR-17D3* and *lar*, segregated at <0.1. All sites had missing data (>17%, < 96%) across the French genomes. Conversely, 34 out of 59 DGRP SNPs in the Zambian population had no missing data, and nine SNPs had less than 10% missing data. We also examined whether DNA near the SNPs showed adaptive functional differences between the DGRP and other populations using the F_{ST} (fixation index) estimator

embedded in PopFly. We estimated F_{ST} values for the one kb sequence surrounding SNPs with a minor allele frequency higher than 5% in DGRP and Zambian populations. The Ethiopian population was included as another ancestral African population. Mean pairwise comparisons yielded F_{ST} values of 0.154 ± 0.0153, and 0.179 ± 0.0292, respectively between DGRP and Zambian or Ethiopian populations, respectively. There was approximately 3-fold less differentiation between the American and French populations (F_{ST} = 0.0461 ± 0.01304, z = 4.67399, p < 0.00001, Mann-Whitney U test), showing that despite greater conservation of resistance associated SNPs with ancestral African populations, the DGRP shows less population differentiation with French than ancestral African populations in the surrounding DNA regions.

This is surprising since North American populations contain African and European DNA, but it aligns with comparative studies on microsatellites that showed mean pairwise comparisons between African and non-African populations produce high F_{ST} values ranging from 0.11 to 0.20, while European and American populations have average pairwise F_{ST} values of 0.071[87].

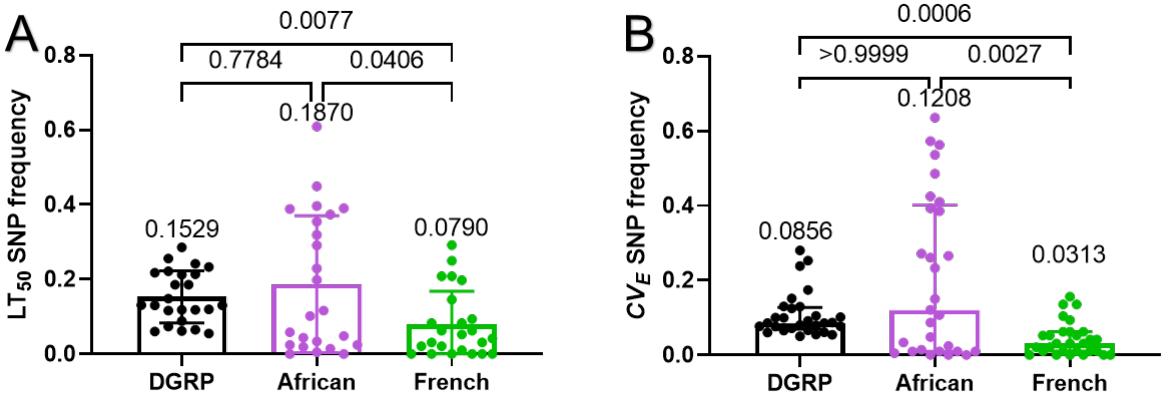


Figure 1.10 Frequency analysis of single-nucleotide polymorphism (SNP) for LT_{50} **(A)** and coefficient of variation (CV_E) **(B)** among DGRP, African, and French populations. Welch's ANOVA tests ($W_{(2, 42.24)}$ = 6.289, p = 0.0041) reveled significant differences in LT_{50} SNP frequency across the DGRP, African, and French groups. Dunnett's multiple comparisons test identified significant p-values and mean ± SEM (A). The Kruskal-Wallis test showed significant differences (H = 16.58, p = 0.0003) among the groups, with significant p-values and median ± IQR from Dunn's multiple comparisons test noted (B).

1.4 *Discussion*

Our study broadens previous research on disease resistance variation in the DGRP from North Carolina, United States, to global populations, reflecting *D. melanogaster* evolution as a human commensal. We used 43 global stocks established at different times, acclimated in the laboratory and assayed under standard laboratory conditions, and found persistent latitudinal clines for disease resistance based on their original collection sites. A caveat applying to this research is that lines are likely to suffer from laboratory adaptation and inbreeding. This may not be an issue as previous studies have found that laboratory-maintained inbred *Drosophila* populations exhibit similar trait correlations and plasticity in morphological traits and stresses as freshly caught populations[88], which is as expected for clinal traits shaped by natural selection[89], and we found no evidence of a relationship between stock age and disease resistance in our data. We also took the precaution of testing multiple stocks to provide sufficient independent data points. Finally, as noted by other authors using laboratory stocks to study clinal signals[90], if laboratory adaptation is present at all, it should act against (or even erase) the clinal signals we have detected here. However, we look on this study as being a precursor to additional studies, ideally conducted under natural settings as far as feasible, which can confirm ecological relevance.

Our data indicate that disease resistance is linked to collection sites with proximity to the equator and with higher average annual temperatures, as described previously for sleep[44]. To the 22 strains[44], we added 21 lines, including African populations to provide representation from near the ancestral range of the species (Accra, Ghana). These Ghana lines[85] cluster with Zimbabwe and Zambia populations and contributed to the discovery that sub-Saharan

populations are more diverse than other populations[85, 86, 91]. Accra, due to its equatorial location within the TSGSS biome, experiences minimal seasonal temperature and humidity fluctuations (96% of the year falling within the preferred 20-30°C temperature and 60-100% humidity range), closely resembling the stable, aseasonal Monkey Hill environment. An alternative to latitudinal clines in disease resistance is the possibility that distance (in any direction) away from the range center is associated with altered species interactions and reduced genetic diversity, which may translate to reduced disease resistance. Under this "range center" distance from Africa should negatively correlate with disease resistance to a similar extent as latitudinal clines, which it does not even though latitude is a component of distance. The tendency of lower latitude flies to exhibit higher resistance persisted over large distances suggesting that latitude or climatic variables are more important at predicting disease resistance than a population's position relative to the species range.

Given the African origin of *D. melanogaster*, the non-African population diversity is expected to be a subset of African diversity[40]. However, 12(13) of the 15 African male (female) lines were among the 50% most resistant. The exceptions were flies from Madagascar and Zimbabwe and male flies from Malawi, which experience greater seasonal fluctuations in temperature and especially humidity. This indicates that African genomic diversity did not translate into phenotypic diversity in resistance to Ma549 as they were generally resistant, perhaps reflecting their aseasonal habitat. Similarly, four African *D. melanogaster* lines were more resistant to the fungal pathogen *Beauveria bassiana* than two non-African populations[50]. The authors noted that if such variation in pathogen susceptibility also exists in pest insects, it could lead to the evolution of resistance to fungi developed as biopesticides [50]. The frequency of SNPs associated with resistance to Ma549 in the DGRP was lower than that in African genomes,

despite nearby DNA resembling European populations. This could be due to African SNPs being favored in the DGRP or even *de novo* mutations in America, as most were rare or absent in France.

African populations, as well as other aseasonal (tropical) populations from uniform climates, such as MH, are generally more disease resistant than seasonal (temperate) populations that endure intermittent thermal and humidity stress. Relatively little is known about clines whose evolution may be governed by interspecific interactions. However, the differences we found between *Drosophila* populations align with studies linking climate variables to malaria risk; uniform temperatures and high humidity support *Anopheles gambiae* vector populations and influence malaria transmission patterns[92], emphasizing the importance of aseasonal regions in disease dynamics. Moreover, extensive clinal variation exists within *A. gambiae* populations along an aridity gradient[93]. *D. melanogaster* thrives within an optimal temperature range crucial for activity and development[78, 79], while fluctuating temperatures can shorten the lifespan of *D. melanogaster*[80]. Relative humidity above 60% also significantly contributes to fly longevity, particularly in females[94]. Several studies have found that *Drosophila* fly populations increase with rainfall[95, 96], and fungal insect diseases, including Ma549, are strongly favored by humid conditions[97], suggesting a connection between precipitation and increased disease exposure. Aseasonal regions with stable climates and abundant food resources host a majority of terrestrial biodiversity and species interactions[98, 99], and their greater species richness and microbial prevalence may select for enhanced host defenses[100]. This ecological context of a simultaneous increase of fungal infestations and conditions favorable to fungal infections may act as a stronger selective force at lower latitudes. Similarly, genetically based plant defenses against herbivory are higher at lower latitudes corresponding to greater levels of herbivory in the tropics[101].

Resistance to abiotic stress is likely to be a larger component of fitness at high latitudes. Stress responses are costly, and surviving abiotic stresses requires nutrients and may even reduce immune function[102]. Thus, alternatively, or in addition, while adapting to abiotic stresses is crucial for the persistence of temperate populations, these adaptive processes could be traded-off against alleles imparting greater resistance to disease. Hoffmann et al. have proposed a similar evolutionary trade-off where an individual fly cannot be both cold resistant and starvation resistant, with starvation resistance being selected for in warm climates[103]. Our previous DGRP analysis found an association between starvation resistance and disease resistance[49], suggesting that it could be one of the factors contributing to adaptive differences in disease resistance. Starvation resistance is linked with metabolic storage and the ability to maintain homeostasis, which could help deal with abiotic and biotic stressors. However, high resource availability and pathogen prevalence in tropical climates could also select for disease resistance. Most *D. melanogaster* genotypes reared at 17°C have larger bodies than flies reared at 28°C, exemplifying adaptive body size plasticity, as larger bodies are supposedly better at conserving heat [104]. Another study of the DGRPs found significant correlations between abdomen size at 28°C and chill coma recovery and between abdomen size at 17°C and resistance to Ma549[104], suggesting that disease resistance is linked to developmental plasticity that help organisms cope with environmental heterogeneity. Most of the variation in the DGRP that predicted resistance affected the general robustness of the flies, and in particular genes involved in development, nutrition and sleep indices[49].

In this study, we used previously acquired desiccation and disease resistance data from DGRP lines[49, 81] to demonstrate a significant link between them in females, indicative of cross-resistance to multiple stresses rather than tradeoffs, and therefore common mechanisms for some

of the variation in these traits. The resistance of DGRP lines to the bacterium *Pseudomonas aeruginosa* and the narrow host range *Entomophthora muscae* (Entomophthoromycota) are correlated with resistance to Ma549, indicating the presence of general (non-specific multipurpose) defenses and that resistance to one pathogen is not traded off with increased susceptibility to a different pathogen[49, 105]. Whether increased resistance to fungi and bacteria influences defense against parasitoids and viruses remains to be seen. We have assumed that disease resistance is linked to adaptation to local environments. Local adaptation seems to be the case for desiccation resistance as laboratory conditions that mimic the low humidity of IP favor IP flies over MH flies. However, if some insects have evolved an increased generalized resistance to multiple stressors, then resistance to Ma549 may be a co-incidental side effect of cross-resistance to other stressors and not derived from an evolutionary history with pathogens. The fly immune system is known to have functions beyond defense against pathogens with cold stress in particular known to activate immune genes and increase resistance to *B. bassiana*[106]. Likewise, reactive oxygen species activate some innate immune mechanisms[107], as well as sleep, which protects against oxidative stress[108], so not surprisingly, the resistance of DGRP lines to oxidative stress is correlated with resistance to Ma549[49].

It is unknown whether this multi-stress resistance is still advantageous when insects simultaneously encounter abiotic and biotic stresses. This scenario is most likely to occur in seasonal regions due to arid or thermal stresses. Although desiccation and disease resistance are associated in females, disease resistance peaks in stable regions where desiccation is not a major threat. Flies from IP and males from MH represent the range of variation in disease resistance. Monkey Hill's stable, warm, near-optimal temperature and humidity (marine ecoregion) presumably imposes less abiotic stress than the seasonally variable, drier desert climate of IP

(DXS biome), while pathogen pressure is likely to be low in an arid desert. Relaxation of selective pressure could have led to degeneration of immune defenses in IP flies, similar to *D. sechellia* that lives in an "enemy-free space"[48]. Conversely, IP flies display high desiccation resistance with the highly disease-resistant MH males being the most susceptible to desiccation[109]. This reversal in the direction of sexual dimorphism aligns with the DGRP lines (Figure 1.5E-F) and a recent study on sexual dimorphism in 15 *Drosophila* lines, which found that males generally live longer but females are more resistant to starvation and desiccation, with greater variation between lines[110]. These findings indicate that the relationship between desiccation and disease resistance is prioritized differently in males and females.

We found that the association between African populations and DGRP was particularly strong for SNPs linked to the phenotypic micro-environmental plasticity of DGRP lines. DGRP lines exhibit significant variability in their plasticity to disease[49] and thermal stress[104], showing that even within a local population, genotypes can produce flies with different plasticity. However, it is unclear to what extent a lines responsiveness to environmental conditions in nature is associated with between individual differences found within vials (micro-environmental plasticity) and quantified with coefficient of variation CV_E[104]. Nevertheless, males usually showed greater variation in disease resistance both within and across populations than females. Plasticity is generally costly and only selected for in heterogenous environments[111], which implies males may be more adaptable or experience a greater diversity of pathogens. Using CV_E, we found that selection favors the plasticity of disease resistance in males equally in seasonal and aseasonal populations, whereas in females, there was a trend for reduced plasticity in stable climates. This implies that aseasonal females may be less resilient to changes in disease exposure. Despite a positive correlation between male and female post-infection survival, males

generally exhibited higher resistance. Female fitness is more critical for population persistence and growth than male fitness[112], so these differences may affect species survival when confronted with global climate change and infectious diseases[64].

A central problem in infection biology is determining why two individuals exposed to the same pathogen have different outcomes. We found that the resistant MH, the highly susceptible IP, and a line (CG), which represents the global average, had marked differences in their ability to control Ma549 growth and colonization rate, and thus pathogen invasiveness. Because variation in survival is linked to fly robustness and resistance to stressors, as well as physiology and behaviors such as sleep, we had expected flies to differ in their ability to tolerate extensive colonization. Instead, all lines showed low fungal loads until the day before death, suggesting that they die at a specific fungal load. Thus, variation in survival stems from variations in the ability to repress fungal growth, which is most likely determined by the immune system rather than the ability to tolerate extensive colonization. The anti-microbial peptides (AMPs) Bomanins only found in *Drosophila* spp are candidates for this role as unlike other *Drosophila* AMPs they have activity against Ma549[97]. Classical virulence theory suggests that the extra resistance demonstrated by MH could drive selection for higher pathogen virulence, while tolerance, if it had occurred, could have led to eventual pathogen-host co-existence[113].

Using sleep data for healthy females from 22 lines[44], we demonstrated that variation in disease resistance was positively correlated with consolidated sleep (sleep duration and bout length) and negatively correlated with fragmented sleep (bout number). This suggests that flies that sleep more while healthy are better at resisting infection. Because of the sexual dimorphism in disease resistance, we bioassayed both sexes in our study. In addition to the diurnal rhythm sleep observed in healthy animals, *Drosophila* increases sleep in response to infection, akin to

rabbits reacting to fungal infection[114]. This "sickness sleep" precedes symptoms like reduced feeding and the upregulation of the immune gene *Drosomycin* during Ma549 infection[19, 97]. Our sleep analysis showed Ma549 infection increased daytime sickness sleep in MH, CG, and IP flies, especially in males, most notably MH males who succumbed the slowest to Ma549. The benefits of sleep for diseased organisms are unclear, but the GWAS using the DGRP found overlapping alleles affecting resistance to Ma549 and sleep, some of which are sex-dependent, impacting sleep and/or disease resistance in only one sex[49]. The differences between male and female MH, CG, and IP flies may be a phenotypic expression of this phenomenon. Males exhibited a lower fungal burden than females, consistent with sex-specific immune investment strategies. Sexual activity reduced sleep and the ability to delay fungal growth in both sexes, consistent with trade-offs between reproduction and immunity[115-117], even though males do not experience the energy drain of egg production[118].

The mechanisms involved in disease resistance have typically been studied independently, limiting our understanding of the interplay between biotic and abiotic stress responses and their relation to disease resistance and ecology. The issue is particularly relevant for clines in sleep, stress resistance, and defense, as these traits are closely linked to fitness. Growing recognition exists that stress and defense traits are interconnected with each other, and with sleep, yet the general issue of their joint clinal evolution remains unresolved. Given the anticipated climate change and the importance of rapid evolution in invasive species, this is likely to change, and the *Drosophila*-Ma549 model appears to be well-suited for these studies. Sleep and stress responses may provide an evolutionary mechanism for optimizing host defenses, but it is unclear whether selection primarily shapes host defenses through changes in non-specific stress responses and variations in physiological and behavioral traits such as sleep or

vice versa whether the selection is on stress responses or sleep with disease resistance as a secondary factor. IP females exhibit greater desiccation resistance than males, but the high nocturnal activity (low sleep) of IP males might feasibly be a behavioral adaptation to their arid environmental conditions and unrelated to disease resistance. Our findings emphasize the critical role of environmental conditions on fly lifespan post-infection, highlighting the complex relationship between ecological context and disease resistance.

1.5 *Materials and Methods*

1.5.1 Fly stocks, geographic and climate data, and biomes

Twenty-two fly populations were a gift from Dr. Alex Keene's lab (Texas A&M University)[44] and an additional 21 fly populations [Ghana (Mataheko or Dansoman, Accra), Mauritius (Le Reduit), Malawi (Lujeri), Madagascar (Tananarive), France (Montpellier), and the United Kingdom (Sussex)]) were acquired from the *Drosophila* Species Stock Center (Cornell University, Ithaca, NY) with all stock numbers given in Table 1.1. *Drosophila* were cultured on cornmeal-molasses media supplemented with yeast, agar, Tegosept, and propionic acid at 25°C and ~70% humidity under a 12-hour light:dark cycle. Virgin flies of each genotype were collected under light CO_2 anesthesia within 8 h of eclosion and housed in separate vials for 2–3 days to mature.

Latitude, longitude, and altitude measurements of each collection site in Table 1.1 were obtained from Google Earth Pro and converted from degrees, minutes, seconds to decimal degrees. Latitude and altitude measurements were transformed into absolute values, followed by conversion to $\log_{10}$ for regression analysis. The 43 fly populations were categorized into six

biomes based on the latitude and longitude of each collection site using ArcGIS Pro (2022, Version 3.0.1, Esri Inc., Redlands, CA, USA), and 14 biomes defined in Ecoregions 2017[59]. Because latitude, altitude, and precipitation measurements were not normally distributed, we calculated the $\log_{10}$ values for the Ma549 LT_{50} regression analyses. The geographic data in Figure 1.1A were generated using ArcGIS Pro.

Monthly average, high (daytime), and low (nighttime) temperature datasets of land and ocean in a 1° × 1° latitude-longitude grid covering the Earth's surface from 1850 to the present were obtained from the Berkeley Earth website (http://berkeleyearth.org/data/) and verified using the visualization tool L3HARRIS IDL software (https://www.l3harrisgeospatial.com/Software-Technology/IDL). Temperature data based on collection year of all 28 collection sites were in a gridded NetCDF format and extracted with a self-coded program script (S1.2B Table), as provided in S1.2A Table. The annual average, high, and low temperatures were computed by summing the respective monthly averages, highs, and lows and dividing each sum by 12.

The monthly precipitation data within a 0.5° grid for each collection site and collection year in the S1.2A Table were extracted from a large database of up to 10 different weather stations at the World Meteorological Organization[119]. The annual mean precipitation was determined by averaging the monthly data across all 12 months. The month with the highest precipitation value among the 12 months was identified as the wettest month.

Data on 52,584 hourly temperatures and relative humidity's from the years 2005-2010 for all 28 collection sites defined by latitude and longitude were obtained from the Prediction of Worldwide Energy Resource (POWER) Project, Langley Research Center, National Aeronautics and Space Administration. To cover all 43 fly populations, we used NASA POWER data to analyze hourly temperature and humidity from 2005 to 2010 at 28 collection sites, aligning with

the collection dates of most fly lines (S1.3 Table). This data was utilized to analyze the effects of environmental factors on fly longevity. The total hours during which temperatures fell within specific ranges and relative humidities within 60-100% from 2005-2010 across all 28 collection sites were calculated using 52,584 hourly temperature and humidity data points. The total hours were then divided by six years to calculate the annual hourly duration (hours/year) for 43 fly lines, which were used in simple linear regression analyses.

1.5.2 Fungal strains, and longevity bioassays

Metarhizium anisopliae 549 (Ma549) was acquired from the ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) (Ithaca, NY). Ma549 cultures were thawed from −80˚C stock vials and grown for 10 to 14 days at 27 °C on potato-dextrose-agar media plates.

Bioassays using *M. anisopliae* 549 transformed to express green fluorescent protein (Ma549-GFP) were performed according to a previous report[19] on 43 worldwide fly populations. Briefly, fly populations were infected at the same time of day and survival was monitored after topical inoculation in groups of five replicates (20-30 flies each) per sex per line. Each line was screened three times. To prepare inoculum, conidia were suspended in sterile distilled water, vortexed for 2 minutes and filtered through Miracloth (22-25µm) (Andwin Scientific). Flies (two to four days old) were vortexed with spore suspensions (20 ml, $2.5x10^4$ conidia/ml) for 30 seconds, collected by filtering through Miracloth, and transferred into vials containing fly stock food without Tegosept and propionic acid. Flies were cultured at 27°C and ~85% relative humidity. Male and female flies were housed in separate food vials to examine sexual dimorphism and avoid an interaction between the effects of sex and age in response to infection.

Less than 10% of flies vortexed with water alone (mock-infected), or conidial suspensions died within one day, and there were no significant differences between lines; therefore, flies succumbing within one day post infection were deleted from the infection data. The flies were flipped into fresh food vials daily. The number of dead flies was recorded twice daily for 10-14 days. Host survival differences were measured as LT_{50} values (median lethal time in days at which 50% of the flies died) calculated using R 4.1.0 (R Core Team 2021). This method was highly reproducible, with a mean LT_{50} value for control *Drs*-GFP flies of 4.682 ± 0.029 (N = 126). The inoculum load of spores per male and female *Drosophila* was approximately 200 colony-forming units[19]. To quantify micro-environmental plasticity in mean survival time (MST) as described in a previous report[68], we used either the untransformed or transformed within-line standard deviation (σ_E) and coefficient of environmental variation (CV_E) (S1.1B Table), depending on normality tests. σ_E has the advantage of being measured in days post-infection, the same scale as the trait mean, while CV_E is calculated as the standard deviation divided by the mean and multiplied by 100.

1.5.3 Desiccation assays

Ten age-matched (three to six days old) flies were sorted by sex using light CO_2 anesthesia and placed into a 25 x 95 mm tube, covered at the top with gauze, and joined with parafilm to another 25 x 95 mm tube containing 10 g of silica gel desiccant beads (Wisedry B01MPYB16J) at the bottom to achieve a desiccated relative humidity of 5-10%. Two replicates per sex per line were used for each experimental trial. Control chambers contained 5 ml of a supersaturated NaCl solution to maintain a stable relative humidity of approximately 70%. The

flies were monitored at one-hour intervals for 20 hours for death, as indicated by failure to right themselves or to move their legs when their vials were tapped or inverted[120].

1.5.4 Fungal colonization of hosts measured by colony forming units (CFUs)

A time-course bioassay of fungal growth in the hemolymph of mated and virgin MH, CG, and IP females and males was conducted using previously described protocols[19]. Fifteen flies per sex, mating status, and line at each time point across three experiments were individually homogenized with 45 µl of 0.1% Tween 80. For the resistant MH line, the entire 45 µl homogenate was spread onto *Metarhizium* selective medium (Rose Bengal Agar plates supplemented with oxbile, CTAB, oxytetracycline, streptomycin, penicillin, chloramphenicol, and cycloheximide[121]. For susceptible lines, 5 µl of a 10-fold dilution of the homogenate was spread onto the plates. Colony forming units (CFUs) were counted using the ImageJ Cell Counter after up to 7-days incubation at 25°C.

1.5.5 Sleep assays and data analysis

The *Drosophila* Activity Monitor (DAM2) from Trikinetics, Waltham, MA, was used to monitor locomotor activity and analyze sleep patterns in MH (resistant), IP (susceptible), and CG (average longevity) flies. Flies were individually housed in 5 x 65 mm monitor tubes containing 5% sucrose and 2% agar after CO_2 anesthesia, with any initial mortalities replaced within 12 hours to minimize acclimation effects. Monitoring occurred over seven days under a 12-hour light/dark (LD) cycle (lights on at 9:00 AM EDT, lights off at 9:00 PM EDT). Sleep data, defined as periods of 5 minutes or more of inactivity[82], were processed using DAMFileScan113

software (Trikinetics) and analyzed using custom software (Insomniac3, RP Metrix, Skillman, NJ; gift of Dr. Julie Williams, University of Pennsylvania). The DAM2 output was converted to total daily, nighttime, and daytime sleep duration, represented by the number of minutes when a fly was asleep in 24 or 12 hours. Sleep patterns were examined from 12 to 60 hours post-infection with Ma549, spanning two light-dark cycles. This period precedes the onset of most visible symptoms, such as reduced feeding and reproductive behavior, and the upregulation of the immune gene *Drosomycin* in response to Ma549 infection[19, 97]. The sleep profiles of mated CG and IP flies were compared with those of MH flies under normal conditions, and infected flies were compared with uninfected peers to assess the impact of infection within each line. Sex-specific differences in sleep and disease outcomes were also investigated along with the influence of mating status on disease resistance and sleep patterns. Sleep differences were quantified using the net percentage differences between the experimental groups (S1.5 Table).

1.5.6 Statistical analyses on interactions among clinal cues, geographic variables, longevity, and sleep parameters

We conducted simple linear regression analyses between LT_{50} values and environmental cues or geographic variables (altitude and latitude) at collection sites for 43 global (subdivided into 23 aseasonal and 20 seasonal) populations. We also correlated LT_{50} values with sleep parameters data for 22 female fly populations[44]. Pearson's correlation analyses were performed in GraphPad Prism 10 (GraphPad Software, Boston, MA, USA).

To assess the survival rates of flies following Ma549 infection, we conducted Kaplan-Meier survival analysis. Survival curves for each group (MH, CG, and IP), including variations by sex (male or female) and mating status (mated or virgin), were generated using GraphPad

Prism 10. Statistical significance between survival curves was determined using the log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test. Percentage survival and standard errors were calculated and compared across different genotypes and conditions to assess the impact of genetic variation and infection on fly survival.

We evaluated data normality using the D'Agostino-Pearson and Shapiro–Wilk normality tests, known for their robustness across various statistical distributions[122]. For data sets passing at least one of normality test and showing statistically similar standard deviations (SDs), we employed the unpaired Student's t-test for two group comparisons and one-way ANOVA (Tukey's multiple comparisons) for three or more groups. If variances significantly differed ($p < 0.05$), we used Welch's correction (Welch's t-test) for two groups and the one-way Welch's ANOVA (Dunnett's multiple comparisons) for multiple groups. For data sets failing normality tests, we used the Mann–Whitney U test for two groups and the Kruskal-Wallis test with Dunn's post hoc test for three or more groups. Reported values included p-values, median ± IQR (interquartile range: the difference between the 75th and 25th percentile) from the Kruskal-Wallis test with Dunn's post hoc multiple comparisons test, and mean ± SEM from ordinary ANOVA or Welch's test. Statistical significance was set at $p < 0.05$.

JMP® Pro, version 17.1 (SAS Institute Inc., Cary, NC, USA) was utilized for the forward stepwise regression analysis on the impact of geography and biome on resistance levels (Table 1.4), and principal component loadings of environmental variables and LT_{50} for male and female flies (Table 1.5), as well as ANOVA involving four factors and generating interaction profiles (Table 1.7 and Supplementary figure 1.7).

1.6 *Supplementary tables*

S1.1 Table: Survival data following Ma549 infection for 43 global mated females and males, as well as virgin CG, IP, and MH females and males.

S1.2 Table: Monthly average, high, and low temperatures (°C), precipitation and precipitation in wettest month at the collection year of each of 43 lines, along with the ncPick script.

S1.3 Table: Hourly temperature and relative humidity data from the years 2005-2010 for 28 locales or 43 fly strains.

S1.4 Table: Desiccation data for IP and MH strains, along with disease and desiccation resistance values in 162 DGRP lines in Figure 1.5.

S1.5 Table: Sleep data in Figures 1.7-1.8.

S1.6 Table: CFU data in Figure 1.9B-D.

S1.7 Table: LT_{50} and CV_E SNP frequency in Figure 1.10.

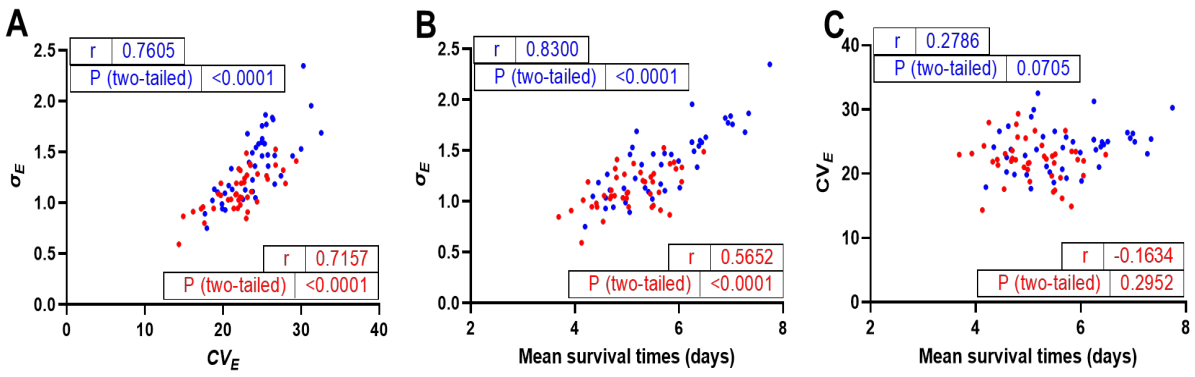
S1.8 Table: ANOVA summaries on main effects and interactions.

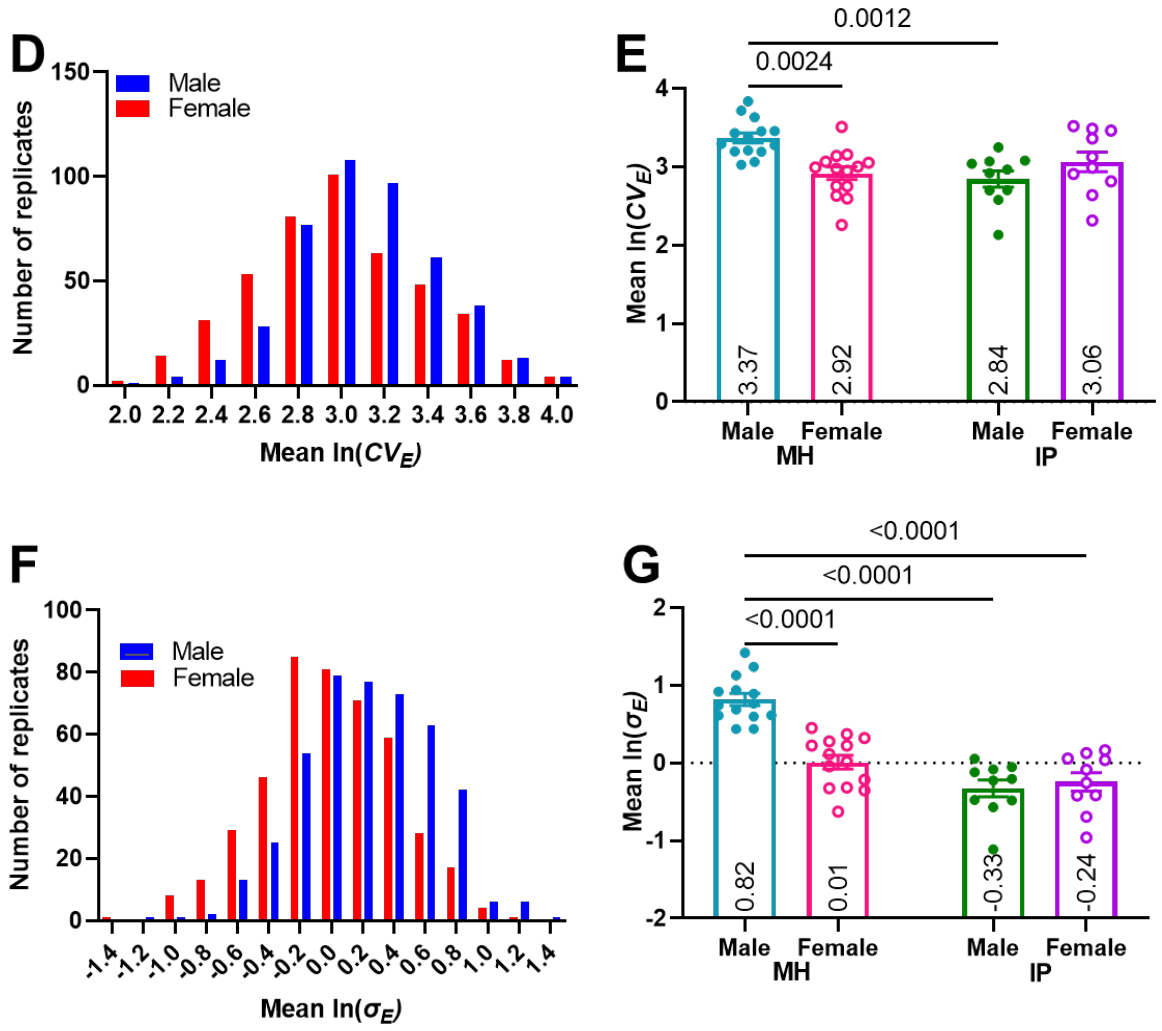
Table 1.6 Comparison of mean and variation in disease resistance between males and females

Line	**t-test for equality of means**			**Longevity CV_E**			**Levene's test for equality of variances**	
	t	**p value**	**Δmean**	**Male**	**Female**	**ΔCV_E**	**F ratio**	**p value**
Accra, Ghana.163	9.01	<0.0001	0.98	24.99	19.71	5.28	27.51	<0.0001
Accra, Ghana.165	9.41	<0.0001	0.94	24.54	21.69	2.85	13.62	0.0002
Accra, Ghana.167	9.41	<0.0001	0.84	21.06	17.43	3.63	26.86	<0.0001
Accra, Ghana.169	6.72	<0.0001	0.86	25.42	22.99	2.43	15.63	0.0001
Accra, Ghana.171	11.44	<0.0001	1.33	23.11	22.22	0.90	18.22	<0.0001
Accra, Ghana.173	13.30	<0.0001	1.46	25.54	17.20	8.34	72.48	<0.0001
Accra, Ghana.175	6.17	<0.0001	0.68	23.75	19.38	4.37	17.27	<0.0001
Accra, Ghana.177	6.17	<0.0001	0.58	24.94	14.91	10.03	45.92	<0.0001
Accra, Ghana.179	12.38	<0.0001	1.44	26.41	19.69	6.72	41.95	<0.0001
Accra, Ghana.181	1.96	0.0500	0.19	18.87	23.50	-4.63	0.93	0.3352
American Samoa	2.36	0.0184	0.21	19.30	23.05	-3.75	0.72	0.3953
Athens, Greece	9.02	<0.0001	1.00	25.33	23.25	2.08	11.56	0.0007
Bahia, Brazil	1.08	0.2816	0.09	24.14	27.99	-3.84	0.02	0.8801
Bermuda	-1.66	0.0980	-0.14	23.78	29.36	-5.58	22.95	<0.0001
Blacksburg, Virginia	7.92	<0.0001	0.78	29.98	21.88	8.11	29.19	<0.0001
Bogota, Colombia	-1.83	0.0673	-0.20	26.60	26.72	-0.12	1.43	0.2320
Cape Town, South Africa	8.52	<0.0001	1.09	26.29	23.45	2.84	10.05	0.0016

Cebu, Philippines	-3.68	0.0003	-0.25	19.89	20.65	-0.76	0.11	0.7420
Chiapas, Mexico	1.94	0.0529	0.23	32.56	24.96	7.59	13.06	0.0003
Crete, Greece	0.75	0.4540	0.05	20.28	17.62	2.66	7.52	0.0062
Fukushima, Japan	11.75	<0.0001	1.13	28.89	23.15	5.74	34.75	<0.0001
Ica, Peru	7.93	<0.0001	0.51	17.92	22.97	-5.05	4.69	0.0307
Israel	5.15	<0.0001	0.46	27.41	24.34	3.07	6.68	0.0099
Kariba Dam, Zimbabwe	9.39	<0.0001	0.79	18.64	23.60	-4.97	4.41	0.0361
Le Reduit, Mauritius	7.87	<0.0001	0.74	24.20	16.17	8.03	50.55	<0.0001
Lujeri, Malawi	-0.23	0.8179	-0.02	25.77	22.70	3.07	5.23	0.0225
Monkey Hill, St. Kitts	18.60	<0.0001	2.29	30.29	22.74	7.55	100.58	<0.0001
Montpellier, France.139	14.80	<0.0001	1.50	25.02	21.71	3.31	28.95	<0.0001
Montpellier, France.140	4.22	<0.0001	0.32	21.16	18.79	2.37	3.17	0.0754
Montpellier, France.141	7.64	<0.0001	0.55	19.87	21.37	-1.50	0.02	0.8803
Montpellier, France.142	13.93	<0.0001	1.69	31.29	23.13	8.16	81.37	<0.0001
Montpellier, France.143	3.32	0.0010	0.26	22.84	21.99	0.85	0.60	0.4401
Montpellier, France.144	0.48	0.6305	0.04	26.61	22.20	4.41	0.77	0.3795
Ogasawara Islands, Japan	1.72	0.0865	0.19	25.31	22.02	3.29	5.83	0.0160
Plainville, Connecticut	8.81	<0.0001	0.86	23.33	26.73	-3.40	0.64	0.4255
Pyrenees, Spain	3.82	0.0001	0.38	23.77	25.29	-1.52	0.78	0.3789
Queensferry, Scotland	4.98	<0.0001	0.42	20.24	21.60	-1.36	2.43	0.1198
Queensland, Australia	9.92	<0.0001	1.08	24.99	27.71	-2.72	2.10	0.1475
San Luis Potosi, Mexico	8.01	<0.0001	0.80	25.72	21.02	4.70	38.20	<0.0001
Southwest Harbor, Maine	3.30	0.0010	0.31	20.72	22.32	-1.59	1.96	0.1616
Sussex, United Kingdom.161	7.51	<0.0001	0.46	22.54	14.35	8.19	44.75	<0.0001
Sussex, United Kingdom.162	5.04	<0.0001	0.35	17.68	22.37	-4.70	0.40	0.5261
Tananarive, Madagascar	10.03	<0.0001	0.90	24.07	22.18	1.89	1.75	0.1861
Cross strain							**t-test:**	**t = 2.755**
Average CV_E				**24.07**	**22.05**			**p = 0.0072**

(Δmean = male mean -female mean and ΔCV_E =male CV_E -female CV_E)



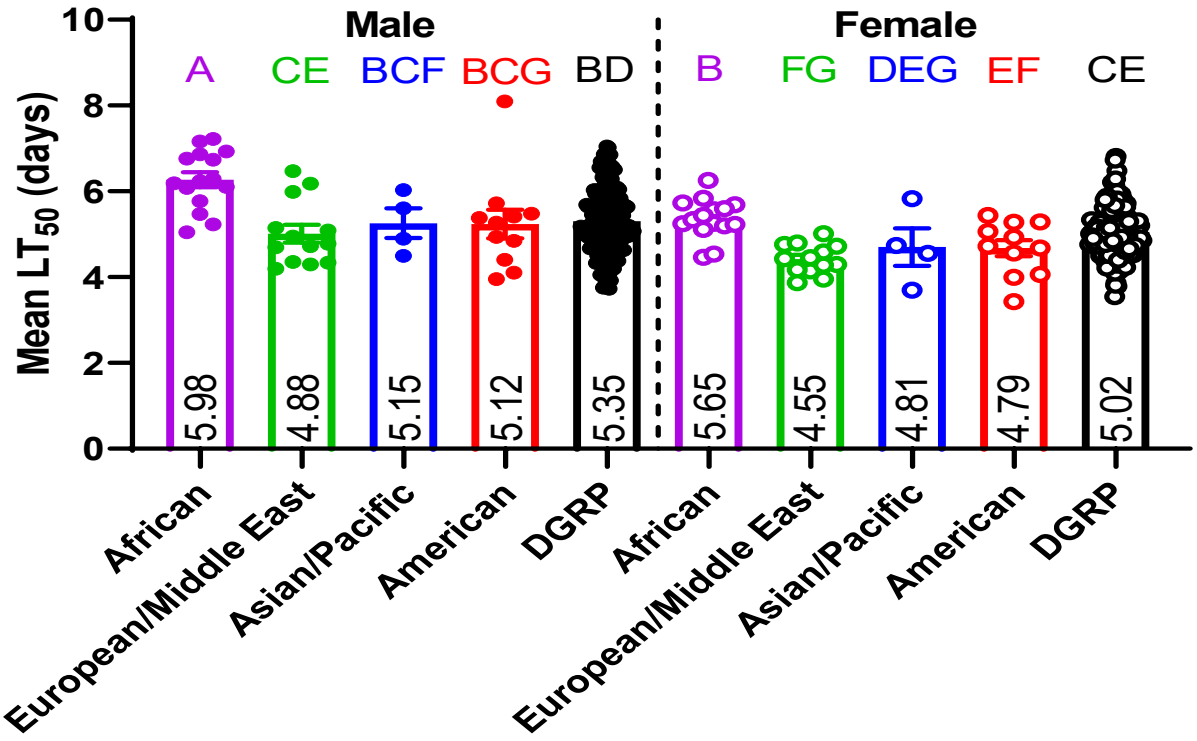


Supplementary figure 1.1 Correlation analyses of plasticity in global lines exposed to Ma549 are presented for male and female σ_E versus CV_E **(A)**, σ_E versus MST **(B)**, and CV_E versus MST **(C)**, with corresponding r and p-values. Blue and red dots in these figures represent males and females from all 43 lines, respectively. Plasticity distribution **(D, F)** and bar graph **(E, G)** of ln(CV_E) **(D, E)** and ln(σ_E) **(F, G).** 443 global male (blue) and female (red) replicates are represented in panels D and F, while 14 replicates with 373 MH males (turquoise) and 501 females (crimson) from three trials, 10 replicates with 344 IP males (green) and 281 females (purple) from two trials are depicted in panels E and G. The transformed ln(σ_E) and ln(CV_E) show a normal distribution in D and F. Panels E and G use two-way ANOVA (Tukey's multiple comparisons) to analyze MH and IP plasticity, presenting mean ± SEM and p-values.

Geographic variables impact host susceptibility to infection

Two-way ANOVA demonstrated significant main effects on fly longevity, with sex accounting for 5.549% ($F_{(1, 456)} = 29.37$, $p < 0.0001$), and geographical origins for 8.296% ($F_{(4,}$

$_{456)}$ = 10.98, p < 0.0001) of the total variation (S1.8B Table). DGRP males (females) were 1.09 (1.10)-fold more resistant than European/Middle Eastern males (females) (p = 0.0207).

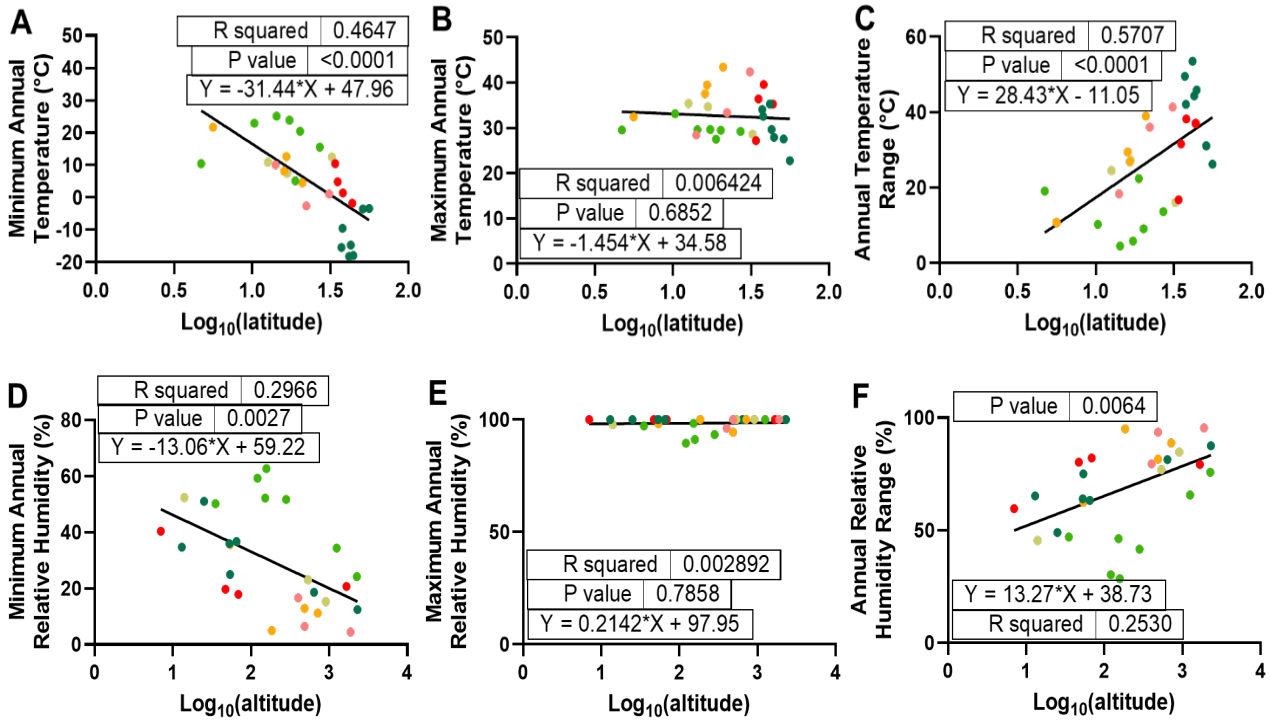


Supplementary figure 1.2 Geographic variation in LT_{50} values among worldwide fly lines infected with Ma549. The 43 worldwide fly lines are depicted with dots: pink for African, green for Europe/Middle East, blue for Asian/Pacific, and red for American regions. LT_{50} values were averaged for each of the 43 male and female lines, with data obtained from 10-15 replicates (averaging 35 flies each) per sex per line across 2-3 experiments. Data from previously published DGRP lines are shown in black[49]. A two-way ANOVA was conducted to evaluate the main effects of geographic region and sex on disease resistance. The analysis revealed no significant interaction between region and sex ($F_{(4, 456)}$ = 2.261, p = 0.0617). Least squares means (LSM) ± SEM from Tukey's multiple comparison test are shown, with a compact letter display (CLD) positioned at the top of individual bars, with statistical significance where bars are not connected by the same letter.

Linear regression analyses of latitude, altitude, and climate variables for global fly collection sites (2005-2010)

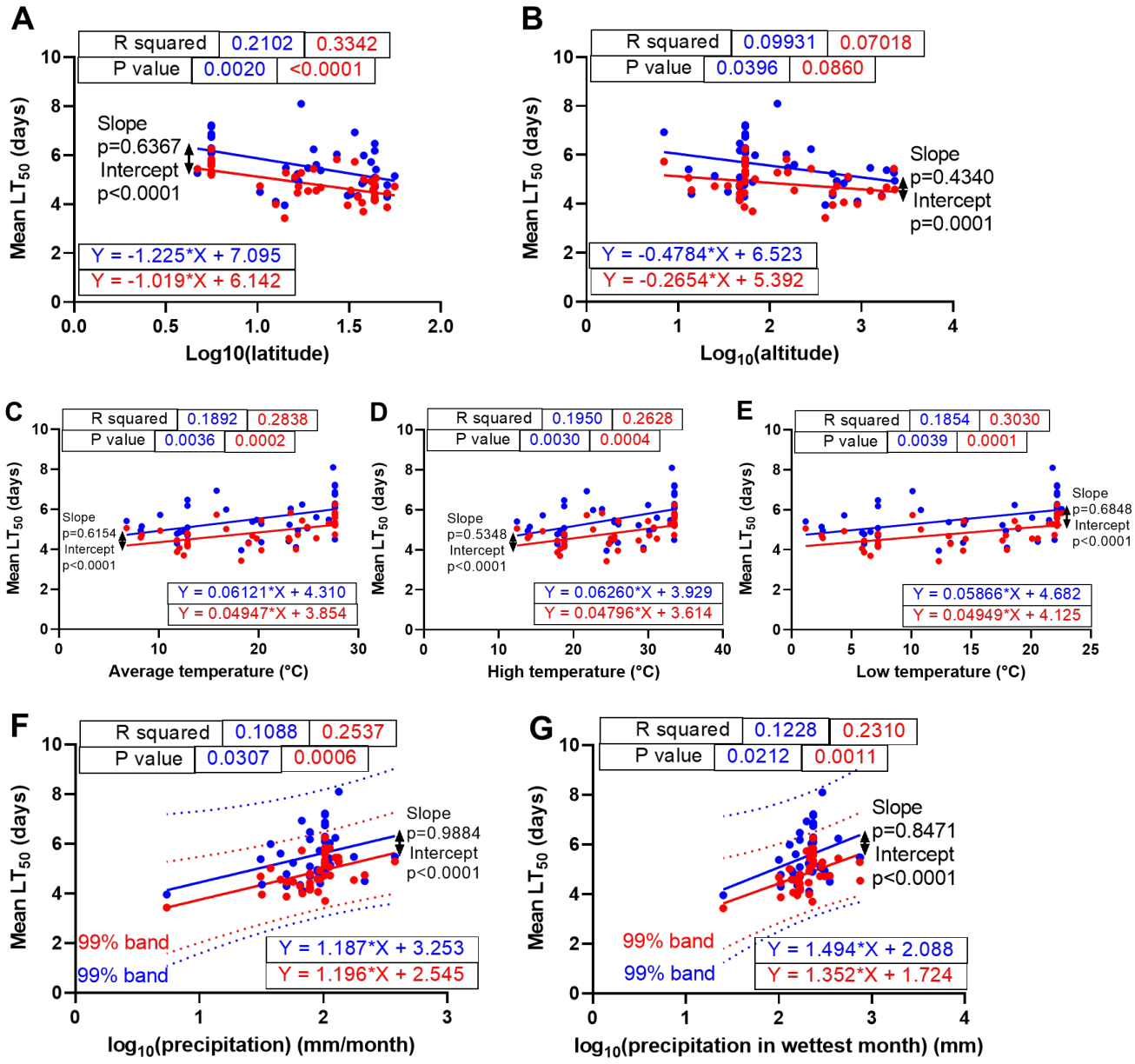
Using NASA Power Data, we analyzed hourly temperature and relative humidity from 2005 to 2010 at 28 collection sites, corresponding to the collection dates of most of the 43 fly

lines (S1.3 Table). Using NASA Power Data to analyze hourly temperature, we are able to detect very short interludes with extreme conditions, such as a reading of 43.46°C in Australia. We recorded mean annual hourly minimum, maximum, and range (maximum - minimum) values. Linear regressions show strong correlations between collection site latitude (Table 1.1) and minimum annual temperature (Tmin) (A) and annual temperature range (Trange) (C), but not maximum annual temperature (Tmax) (B). Altitude (Table 1.1) moderately correlates with minimum annual relative humidity (RHmin) (D) and annual relative humidity range (RHrange) (F), but not maximum annual relative humidity (RHmax) (E). The narrow ranges of maximum annual temperature and humidity explain the lack of correlations with latitude or altitude.



Supplementary figure 1.3 Simple linear regression analyses between latitude and annual temperatures or altitude and relative humidities for the global fly population collection sites: Biome colors across 28 geographic locations (2005-2010) match those in Figure 1.1A.The figures show results for minimum annual temperature **(A)**, maximum annual temperature **(B)**, annual temperature range **(C)**, minimum annual relative humidity **(D)**, maximum annual relative humidity **(E)**, and annual relative humidity range **(F)**, including R^2 values, p-values, and regression equations with slopes.

Simple linear regression analyses correlating Ma549 LT_{50} values with latitude, altitude, annual temperatures or precipitations at collection sites for global fly populations



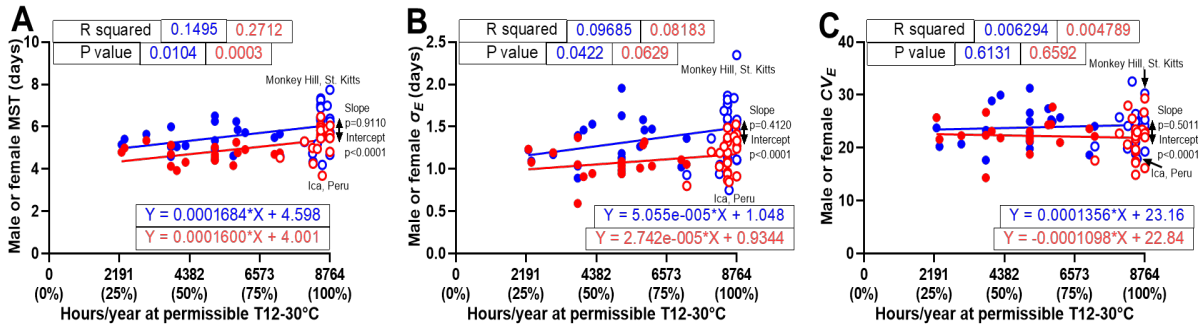
Supplementary figure 1.4 Simple linear regression analyses correlating Ma549 LT_{50} values with latitude, altitude, annual temperatures or precipitations at collection sites for global fly populations: Temperature and precipitation data were extracted from all 28 collection sites based on the original collection year for each fly line. Results for latitude (**A**), altitude (**B**), annual average temperatures (**C**), high temperature (**D**), low temperature (**E**), annual mean precipitation (**F**), and precipitation in wettest month (**G**) are shown, featuring R^2 values, p-values, and regression equations including slopes. Male (female) regression lines are in blue (red), with p-

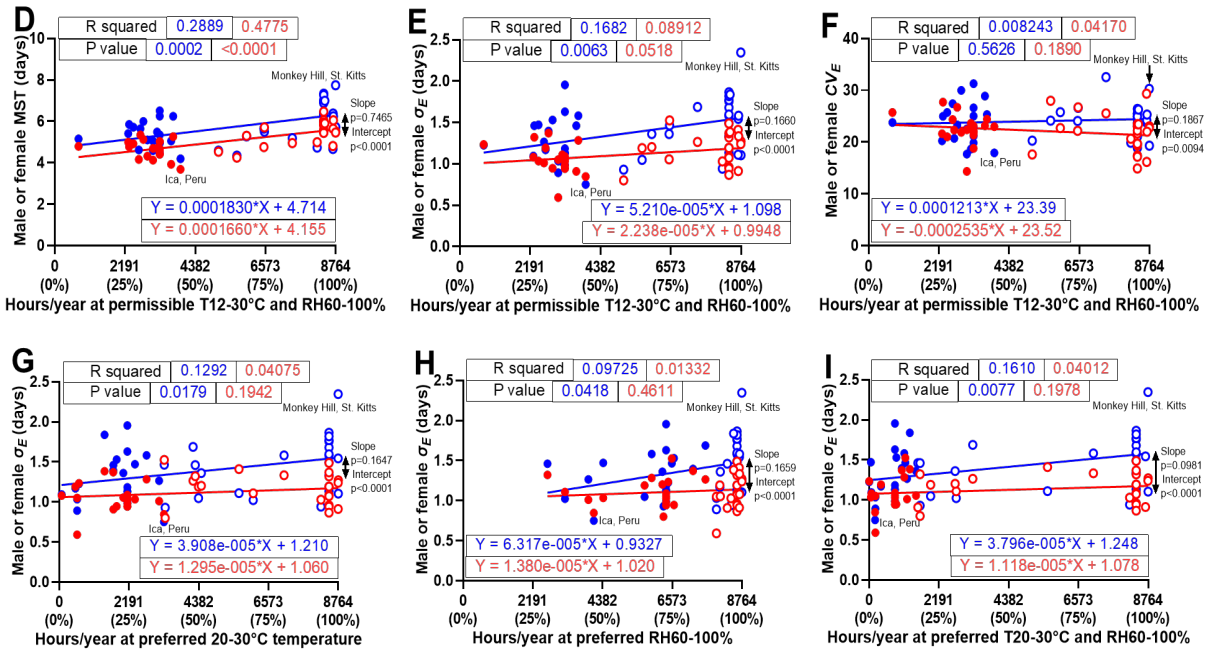
values for comparing slopes and intercepts between female and male lines using analysis of covariance (ANCOVA).

The effects of annual temperature and relative humidity on female and male LT_{50} and plasticity values

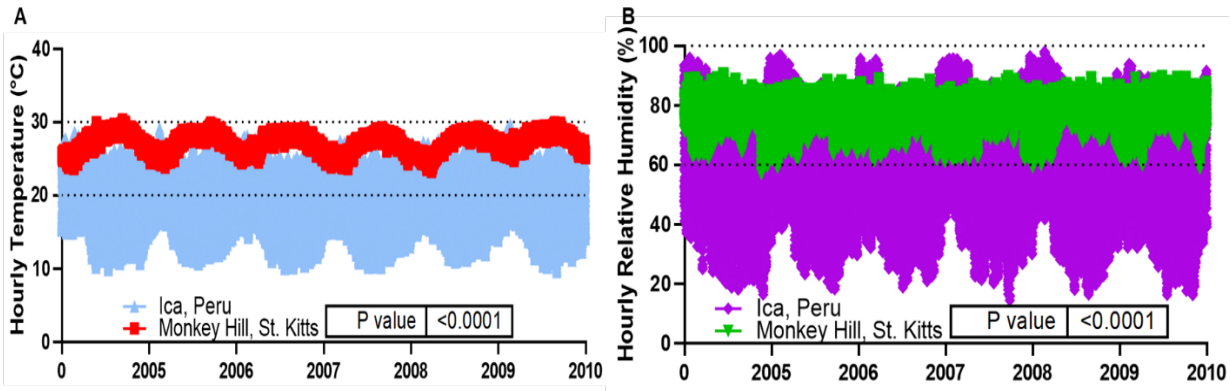
To investigate disease resistance, we calculated the average annual exposure to the preferred temperature (20-30°C) using 52,584 hourly data points from 2005 to 2010 (S1.3A Table). Exposure at collection sites ranged from 75 to 8,764 hours/year (A-C).

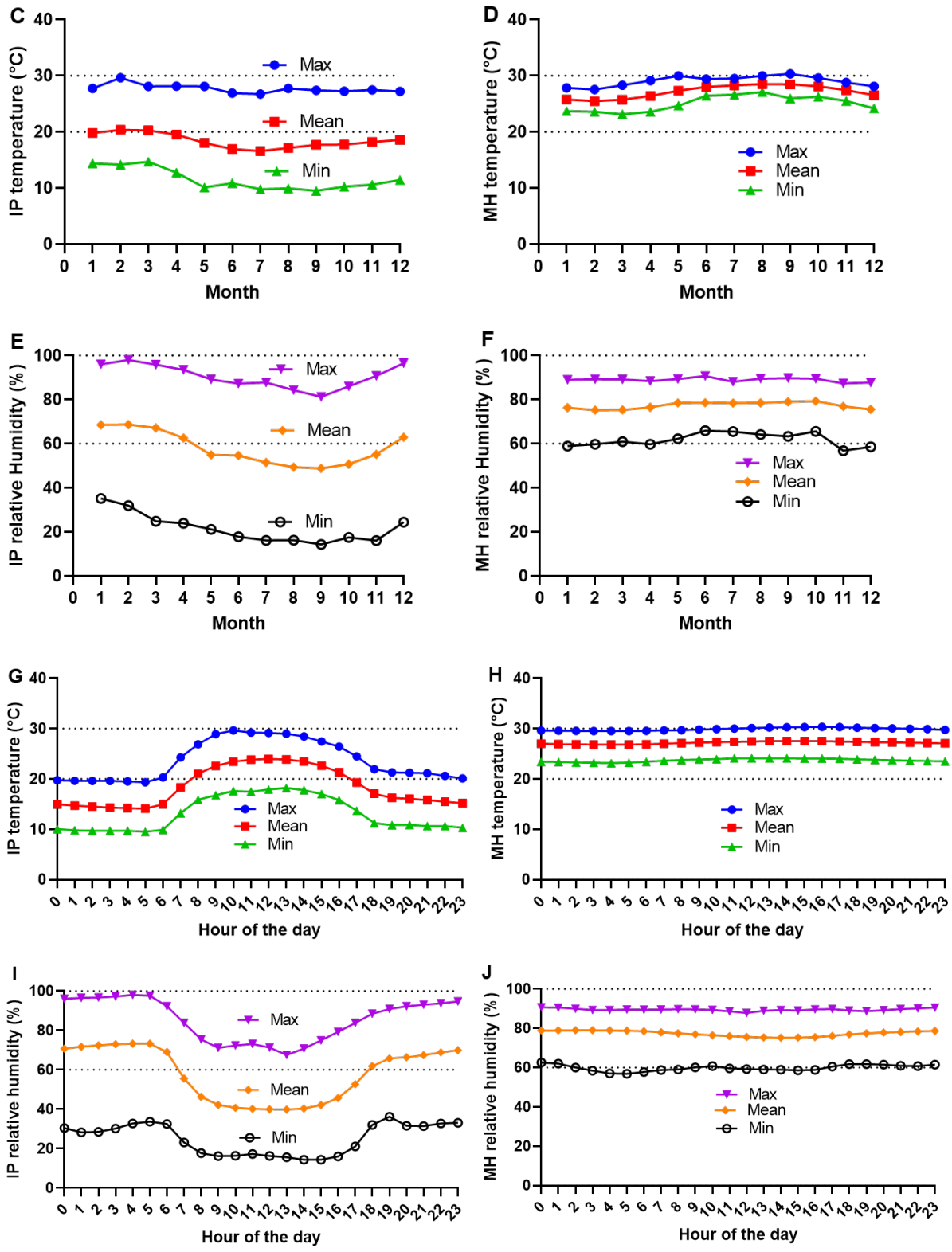
Linear regression analyses for the 43 fly populations showed strong correlations between mean survival time (MST) in response to Ma549 for males and females and experiencing longer aseasonal uniformity measured as the hours per year spent within preferred temperature ranges (A). Positive correlations were also found between σ_E and increasing aseasonal uniformity in males, but not females (B). We observed no evidence of correlation between CV_E values and increasing aseasonal uniformity in males and females (C). Despite differing intercepts due to males' greater resistance, the MST values for both sexes trended similarly with temperature, resulting in nearly parallel regression slopes. Higher R^2 values for females suggest that temperature have a greater impact on female disease resistance.





Supplementary figure 1.5 Simple linear regression analyses correlating Ma549 MST, σ_E, and CV_E values with hours of permissible temperature (12-30°C) with and without relative humidity (60-100%) or σ_E values with preferred temperature (20-30°C), or preferred relative humidity (60-100%) or preferred temperature (20-30°C) coinciding with preferred relative humidity (60-100%) at collection sites for global fly populations. The analysis uses 52,584 hourly data points from 2005-2010 from all 28 sites for each fly line (S1.3A Table)**.** Results are shown for permissible temperature with MST (**A**), σ_E (**B**), and CV_E (**C**), and for permissible temperature coinciding with relative humidity with MST (**D**), σ_E (**E**), and CV_E (**F**), as well as σ_E values with preferred temperature (**G**), or preferred relative humidity (**H**) or preferred temperature coinciding with preferred relative humidity (**I**) at collection sites for global fly populations, including R^2 values, p-values, and regression equations with slopes. Male (female) regression lines are in blue (red). Male and female regression lines are blue and red, respectively. Aseasonal flies are depicted as circles, and seasonal flies as solid dots; this distinction applies to all subsequent figures. Males exhibited a positive correlation between σ_E plasticity and exposure to permissible or preferred conditions (B, E, G-I).





Supplementary figure 1.6 Hourly temperature **(A)** and relative humidity **(B)** data for Monkey Hills, St. Kitts (MH) and Ica, Peru (IP) are shown through Lomb-Scargle periodograms, capturing hourly fluctuations from January 01, 2005, to December 31, 2010 (n=52,584).

Comparisons were made using Welch's t-test with mean ± SEM and p-values indicated. Monthly temperature **(C, D)** and relative humidity **(E, F)** plots for IP **(C, E)** and MH **(D, F)** display maximum, mean, and minimum values across months from 2005-2010 (n=52,584 hourly data points), identifying seasonal trends and temperature-humidity interactions. Hourly temperatures (G, H) and relative humidity **(I, J)** plots for IP **(G, I)** and MH **(H, J)** illustrate daily patterns of maximum, mean, and minimum temperatures and relative humidity from 2005-2010 (n=52,584 hourly data points). These visuals help identify seasonal variations and trends in temperature and humidity interactions.

Effects of line, sex, mating status, and infection on sleep

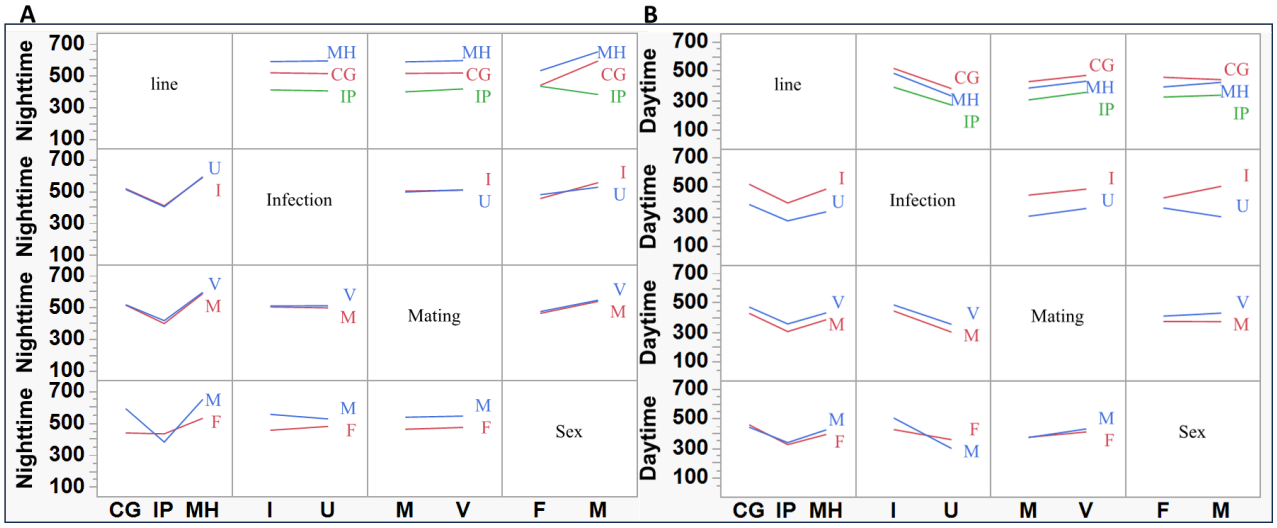
ANOVA was used to validate the effects of genetic line, sex, mating status, and infection on nighttime (Supplementary figure 1.7A) and daytime (Supplementary figure 1.7B) sleep, as detailed in Table 1.1 (derived from the data in S1.5B Table). The analysis confirmed that infection status significantly affected daytime sleep ($p < 0.0001$) but not nighttime sleep ($p = 0.5364$), highlighting infection's role in altering sleep at different times. Additionally, mating status had a much stronger impact on daytime sleep. A significant three-way interaction among line, infection, and sex ($p \leq 0.0020$) was observed for both sleep periods, indicating that the influence of one factor on sleep can be significantly modified by the others.

Table 1.7 Effects of line, sex, mating status, and infection as well as their interactions on night and daytime sleep.

Source	Npar m	DF	Nighttime sleep			Daytime sleep		
			Sum of Squares	F Ratio	p value	Sum of Squares	F Ratio	p value
line	2	2	4286291.9	874.5660	<0.0001	1876451.8	219.7682	<0.0001
Infection	1	1	937.4	0.3825	0.5364	3622098.1	848.4331	<0.0001
Mating	1	1	17285.3	7.0537	0.0081	422367.3	98.9345	<0.0001
Sex	1	1	1024007.7	417.8727	<0.0001	17504.2	4.1001	0.0432
line*Infection	2	2	4138.2	0.8444	0.4302	34266.2	4.0132	0.0185
line*Mating	2	2	8143.3	1.6615	0.1906	2587.0	0.3030	0.7387
line*Sex	2	2	1519311.5	309.9971	<0.0001	73329.8	8.5883	0.0002

Infection*Mating	1	1	2616.3	1.0676	0.3018	7005.3	1.6409	0.2006
Infection*Sex	1	1	132418.2	54.0366	<0.0001	915803.3	214.5160	<0.0001
Mating*Sex	1	1	694.8	0.2835	0.5945	22777.7	5.3354	0.0212
line*Infection*Mating	2	2	90.1	0.0184	0.9818	21402.3	2.5066	0.0822
line*Infection*Sex	2	2	30723.9	6.2688	0.0020	99378.2	11.6391	<0.0001
line*Mating*Sex	2	2	1820.1	0.3714	0.6899	15843.5	1.8556	0.1571
Infection*Mating*Sex	1	1	1845.4	0.7531	0.3858	162.7	0.0381	0.8453
line*Infection*Mating*Sex	2	2	6.5	0.0013	0.9987	15603.2	1.8274	0.1615

Source: This is a list of the tested factors and interactions. Nparm: Number of parameters associated with the effect. DF: degrees of freedom for each source of variation. Sum of Squares: sum of squares (SS) for each source of variation, along with the total from all sources. F Ratio: the mean square of the factor (lot) divided by the mean square of the error. A p-value less than 0.05 means that the factor or interaction has a statistically significant effect.



Supplementary figure 1.7 Interaction profiles of line, sex, mating status, and infection status on nighttime **(A)** and daytime **(B)** sleep (min/12h) across MH, CG, and IP females and males. These profiles were derived from the sleep data of 768 individual flies, considering three genetic lines (MH, CG, IP), both sexes (females and males), two mating statuses (mated and virgin), and two infection statuses (infected and uninfected). Abbreviations: sex (F = female, M = male), mating status (M = mated, V = virgin), and infection status (I = infected, U = uninfected).

Chapter 2: Exploring the relationship between disease resistance and sleep in the *Drosophila melanogaster* Sleep Inbred Panel

(Prepared for submission)

2.1 *Abstract*

Closely related individuals can exhibit significant differences in sleep duration and infection outcomes. Research using the *Drosophila* Genetic Reference Panel (DGRP) suggested that a shared genetic architecture between sleep duration and infection outcomes with *Metarhizium anisopliae* (Ma549) could contribute to this variability. We investigated sleep's impact on resistance to fungal infection using flies from the Sleep Inbred Panel (SIP), bred for extreme long or short nighttime sleep durations. Trends seen with SIP lines mirrored those in the DGRP but were more pronounced. Short-sleepers, with more fragmented sleep when healthy, outlived long-sleepers post-Ma549 infection, and daytime sleep bout numbers correlated with dimorphic disease resistance. Resistance differences were sex specific: males were generally more resistant among short-sleepers, and females among long-sleepers. Using one short- and one long-sleeping SIP fly lines as examples, Ma549 infection was found to increase daytime sleep in both short- and long-sleepers, so that short-sleeping males slept nearly as long as long-sleepers. We also found that regardless of sleep status, virgins slept more and survived infection longer, indicating that sex, mating status, and sleep traits affect disease resistance. This study promotes the idea that both sleep and host defense traits are closely tied to fitness, and therefore intertwined targets of natural selection.

2.2 *Introduction*

Sleep and disease resistance are both remarkably conserved across various species, including humans and *Drosophila*[51, 52], but can also vary greatly from individual to individual

within a species, including in humans and *Drosophila*[31, 53]. However, the reasons for this variation among individuals are not well understood, despite having clear genetic components. The *Drosophila* fruit fly with its wealth of available tools offers unique opportunities to investigate sleep, infection and host defense[16, 17, 123].

Over one billion people are infected by fungal diseases globally, causing an estimated 1.7 million deaths each year[26]. Fungi cause most insect diseases and infect insects by penetrating their cuticles using a combination of cuticle-degrading enzymes and mechanical pressure[29, 30]. The entomopathogenic fungus *Metarhizium anisopliae* is distributed almost worldwide, and strain ARSEF549 (Ma549) is pathogenic to *Drosophila*[19, 34]. Unlike many other entomopathogen species, Ma549 does not produce toxins that inhibit innate immune responses[36]. Previous research has shown that about 9% of mutant *D. melanogaster* lines have altered disease resistance to Ma549[19], and using 188 lines from the *Drosophila* Genetic Reference Panel (DGRP) of fully sequenced fly lines[124] demonstrated that flies have varying longevity after Ma549 infection due to a complicated network of physiological, particularly sleep-related genes[49].

To study the impact of sleep variability on resistance to Ma549 infection, we utilized the *Drosophila* Sleep Inbred Panel (SIP), which consists of 39 inbred *Drosophila melanogaster* lines with extreme long (19 lines) and short (20 lines) sleep duration[125, 126]. As described by Serrano Negron, 2018[125], the progenitors, consisting of the five longest-night and five shortest-night sleeping DGRP lines, were used as the starting genetic material and allowed to cross at random for 21 generations to produce an outbred population, referred to as the intermediate Sleep Advanced Intercross Population (SAIP)[126]. The SAIP populations were divided into four inbred populations that were artificially selected for extreme long (L1 and L2) or short (S1 and S2)

night sleep creating 10 lines each from the L1, S1, and S2 populations, and 9 lines from the L2 population (for a total of 39 lines). The nightly sleep duration for these lines ranged from 68.61 ± 8.55 minutes to 697.14 ± 2.66 minutes[125]. These lines are homozygous or near-homozygous to preserve the extreme long- and short-sleeping phenotypes for further analysis. It was found that the SIP lines retained their sleep phenotypes after several years, demonstrating the success of this strategy[125, 127, 128], whereas short sleeping flies that were not inbred and had genetic variation reverted to normal sleep patterns when selection was relaxed due to natural selection forces[89].

Here, the connection between sleep length and resistance to Ma549 infection was investigated by measuring longevity [median lethal time (LT_{50})] post infection in SIP lines. Results showed significant difference in longevity post-infection, with short-sleepers having more fragmented sleep when healthy living longer. Furthermore, the correlation between disease resistance and sleep, both of which exhibited sex-specific differences, was primarily driven by daytime sleep bout numbers. Increased daytime sleep after infection with Ma549 (sickness sleep[83]) contributed to the longevity of SIP lines. Although general trends were similar, stronger correlations were observed between resistance to Ma549 infection and sleep parameters in SIP lines compared to DGRP lines. Overall, our findings demonstrated that selecting populations exclusively for long or short periods of night sleep was sufficient to alter their susceptibility to a ubiquitous pathogen. Thus, short-sleeping S1_7 males, which were the longest-lived flies post-infection, exhibited predominantly daytime sleep patterns compared to long-sleeping L1_6 males. Virgin flies, regardless of their sex, showed prolonged sleep and increased resistance to infection, suggesting a complex interplay between sex, mating status, and sleep in modulating disease resistance. This work promotes the idea that sleep and host defense traits are closely tied to fitness, and therefore intertwined targets of natural selection.

2.3 *Results*

2.3.1 SIP lines preserve reliable activity patterns

In 2021, we verified that the previously observed activity patterns of SIP lines[125] remained consistent after three years of maintenance, using uninfected long-sleeping L1_6 and short-sleeping S1_7 lines as examples. We analyzed daytime, nighttime, and daily activity counts over a 48 hour period as described[82], and discarded the first 12 hours of activity data to mitigate acclimation effects. Healthy L1_6 and S1_7 flies exhibited a bimodal locomotor activity profile with peaks of activity appearing just before dawn and dusk as previously reported[125] (Figure 2.1, S2.1 Table). Compared to L1_6 flies, S1_7 females (males) showed 61.78% (80.07%) more overall daily activity, with nighttime activity increasing 117.62% (216.33%) and daytime activity increasing 26.55% (49.32%) ($p < 0.0001$). Our findings align with the previous reports by Serrano Negron et al., 2018[125] and are further supported by Thompson et al., 2020[127].

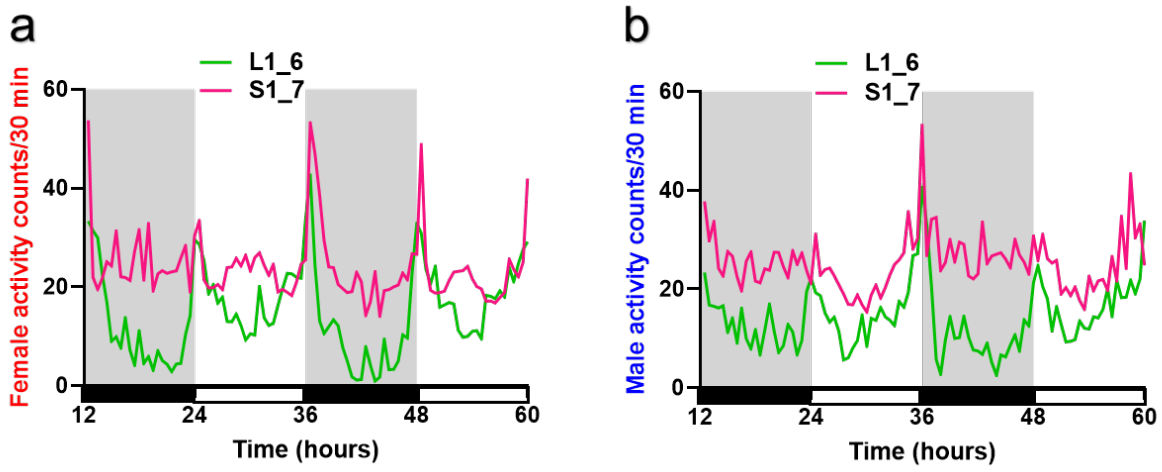


Figure 2.1 Comparison of activity patterns between long-sleeping and short-sleeping lines. The profiles depict activity in min per 30 min from 12-60 h spanning two light-dark cycles for 32 flies pooled from three trials for uninfected L1_6 (green) and S1_7 (pink) females (**a**) and males (**b**), with gray shading indicating nighttime. Either unpaired or Welch's t test were performed for daily, daytime and nighttime sleep comparisons.

2.3.2 The SIP and DGRP populations vary in their resistance to Ma549 infection

The 38 out of 39 SIP lines available to us are divided into two groups of long-sleepers (L1, L2) and two groups of short-sleepers (S1, S2), each containing either nine or ten lines (Table 2.1). To identify sexual dimorphism, we measured resistance to Ma549 separately for a total of 10,917 males and 11,166 females from the SIP lines. Infection was performed with age-matched flies via the natural route of applying spores to the body's surface. Median lethal times (LT_{50}) were measured using three replicates (~30 flies each) per sex per line across three experiments. We compared the Ma549 longevity data for the 38 SIP populations[125] with data from our previous analysis of 188 DGRP lines[49, 129], which included the original five long-sleeping progenitors (LP) and short-sleeping progenitors (SP) of the SIP populations.

Table 2.1 The fly lines, stock numbers, female and male Ma549 LT_{50}s, standard error of the mean (SEM), and sample size (N) of 38 SIP and 10 progenitor populations.

Fly lines	Stock	Female			Male		
		LT_{50}	± SEM	N	LT_{50}	± SEM	N
SIP_L1_1	76271	3.80	0.05	287	4.88	0.08	268
SIP_L1_2	76272	3.23	0.09	302	3.54	0.03	284
SIP_L1_3	76273	6.08	0.10	290	5.29	0.15	249
SIP_L1_4	76274	4.44	0.12	242	3.09	0.10	252
SIP_L1_5	76275	5.41	0.04	331	4.78	0.06	289
SIP_L1_6	76276	4.56	0.16	270	2.83	0.10	237
SIP_L1_7	76277	5.13	0.09	292	4.93	0.08	278
SIP_L1_8	76278	4.83	0.12	291	4.86	0.15	277
SIP_L1_9	76279	5.31	0.06	266	4.30	0.11	286
SIP_L1_10	76280	4.28	0.10	276	2.85	0.07	214
SIP_L2_1	76291	5.05	0.06	297	4.41	0.06	296
SIP_L2_2	76292	4.39	0.12	252	4.71	0.14	293
SIP_L2_3	76293	5.36	0.06	309	5.23	0.10	281
SIP_L2_4	76294	4.05	0.10	259	5.34	0.10	283
SIP_L2_5	76295	4.90	0.13	315	4.91	0.14	306
SIP_L2_6	76296	4.53	0.10	301	3.88	0.09	298
SIP_L2_7	76297	5.33	0.12	290	4.87	0.07	296

SIP_L2_8	76298	4.26	0.12	296	4.54	0.09	323
SIP_L2_10	76299	3.81	0.10	321	4.49	0.14	251
SIP_S1_1	76281	5.19	0.20	250	5.71	0.12	292
SIP_S1_2	76282	5.28	0.20	237	5.48	0.20	230
SIP_S1_3	76283	5.03	0.10	286	4.59	0.11	243
SIP_S1_4	76284	6.03	0.12	253	5.72	0.14	259
SIP_S1_5	76285	5.55	0.13	322	5.73	0.10	281
SIP_S1_6	76286	6.49	0.13	289	7.06	0.15	277
SIP_S1_7	76287	4.20	0.07	297	6.30	0.08	290
SIP_S1_9	76289	6.29	0.10	302	6.24	0.11	247
SIP_S1_10	76290	5.30	0.17	309	6.62	0.15	280
SIP_S2_1	76300	4.97	0.15	243	6.80	0.15	276
SIP_S2_2	76301	4.76	0.09	323	5.92	0.13	263
SIP_S2_3	76302	5.29	0.09	307	7.58	0.08	298
SIP_S2_4	76303	6.06	0.16	250	7.60	0.16	293
SIP_S2_5	76304	5.81	0.09	276	5.14	0.11	221
SIP_S2_6	76305	4.47	0.11	232	5.16	0.12	256
SIP_S2_7	76306	4.43	0.14	237	6.94	0.14	283
SIP_S2_8	76307	5.94	0.05	229	6.30	0.16	269
SIP_S2_9	76308	5.46	0.19	233	7.52	0.14	247
SIP_S2_10	76309	5.70	0.13	233	6.44	0.14	327
DGRP_235_LP	28275	4.90	0.06	180	5.41	0.05	225
DGRP_313_LP	25180	4.79	0.27	67	4.51	0.24	73
DGRP_335_LP	25183	4.27	0.41	71	4.05	0.02	75
DGRP_338_LP	28173	5.97	0.24	87	5.20	0.25	85
DGRP_379_LP	25189	5.47	0.28	74	4.89	0.06	85
DGRP_38_SP	28125	6.48	0.30	78	7.05	0.45	89
DGRP_310_SP	28276	6.04	0.15	78	6.67	0.21	91
DGRP_365_SP	25445	4.78	0.04	69	4.98	0.07	78
DGRP_808_SP	28238	6.21	0.41	74	6.95	0.10	89
DGRP_832_SP	28245	4.72	0.07	65	4.19	0.16	74

The average LT_{50} of the 38 SIP lines for males (females) was 5.33 (5.03) days, which was similar to that of the 10 progenitors and the entire DGRP ($p < 0.05$, Figure 2.2a). The average LT_{50} per SIP line ranged from 2.83 (3.23) to 5.34 (6.08) for long-sleeping males (females) and from 4.59 (4.20) to 7.60 (6.49) for short-sleeping males (females) (Table 2.1 and S2.4a Table). The range for SIP females (3.26 days) was similar to that for the entire DGRP.

However, the range for SIP males was greater (4.77 days) than that for the DGRP males (3.32 days), despite the DGRP having almost five times more lines as the SIP (Table 2.1, Figure 2.2b-c).

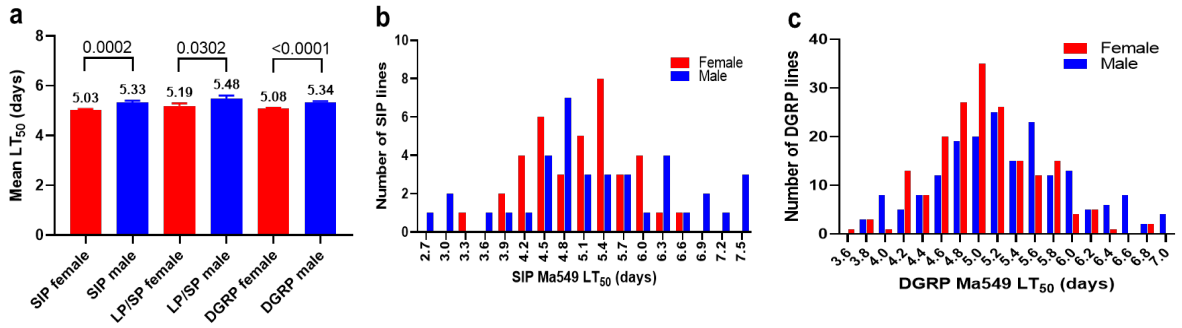


Figure 2.2 Sexual dimorphism and longevity of infected females and males of 38 SIPs, 10 progenitors (LP/SP), and 188 DGRP populations (**a**). Separate bars were generated for male (blue) and female (red) flies. The data display the mean ± SEM LT_{50} values from 342 SIP replicates, 42 LP/SP replicates, and 424 DGRP replicates[49]. Welch's t-tests were performed to compare female and male longevity in SIP and DGRP groups, with the Mann-Whitney U test for progenitors. p-values are indicated in the figures. Distribution of female (red bars) or male (blue bars) longevities of 38 SIP (**b**) and 188 DGRP (**c**) populations. The data were subjected to D'Agostino & Pearson and Shapiro-Wilk tests ($p > 0.05$), confirming their normal distribution and suitability for further parametric analyses.

To identify sexual dimorphism, we measured resistance to Ma549 separately for males and females. Cross-sex survival correlations were moderate for the SIP lines, with ($r = 0.5877$, $p < 0.0001$) or without ($r = 0.5302$, $p = 0.0006$) inclusion of their 10 progenitors (Figure 2.3a-b). In comparison, correlations were higher for the 188 DGRP lines ($r = 0.7374$, $p < 0.0001$, Figure 2.3c)[49], consistent with greater sexual dimorphism in SIP lines.

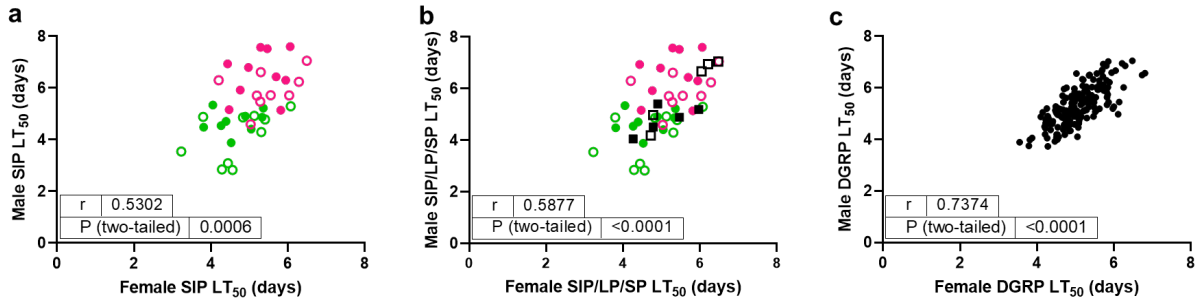


Figure 2.3 Correlation analyses comparing male and female LT_{50} values following infection with

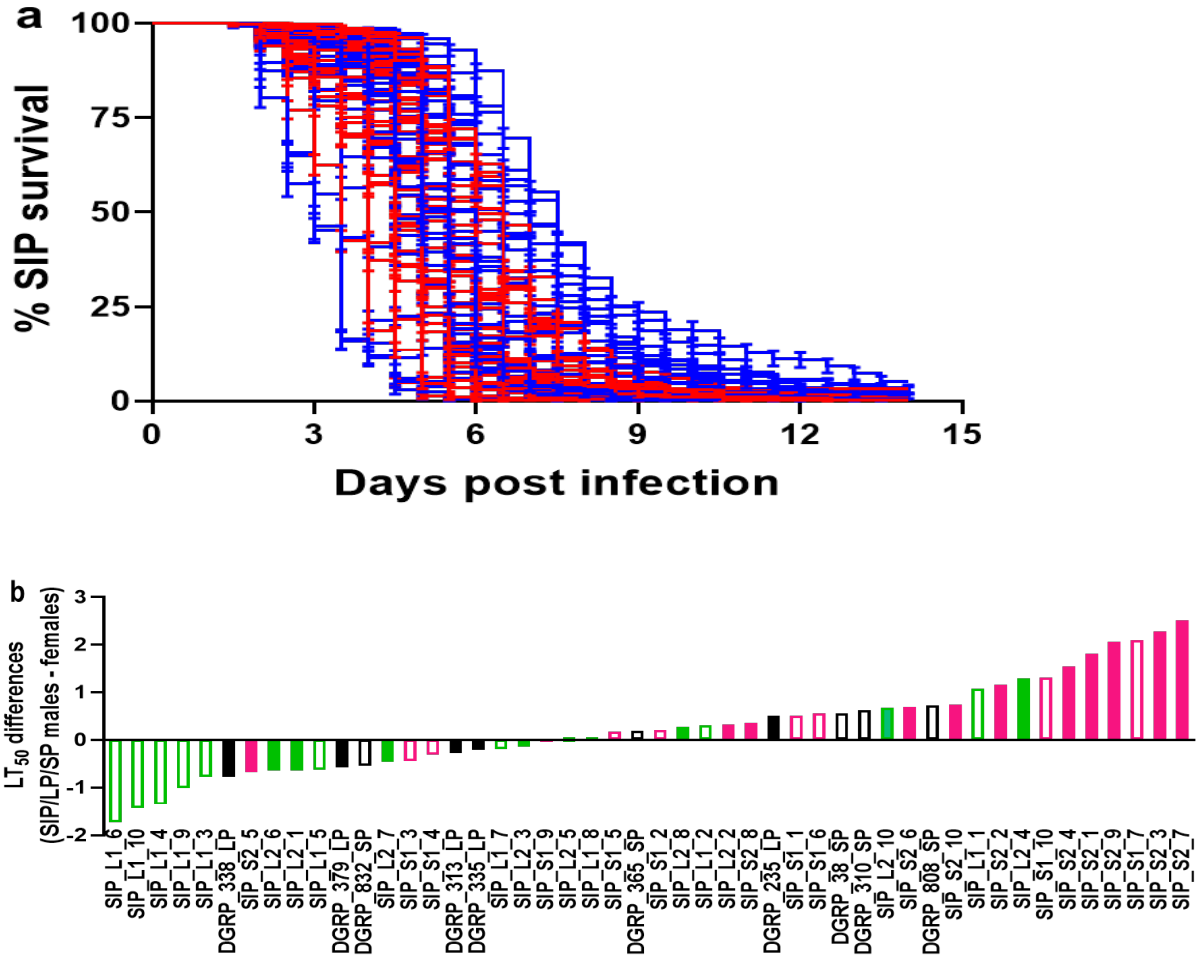
Ma549 from 38 SIPs (**a**), 38 SIPs plus their 10 progenitors (**b**), and 188 DGRPs[49] (**c**). In panels (a) and (b): L1 - open green circles, L2 - solid green circles, S1 - open pink circles, S2 - solid pink circles. In panel (b) only, LP - solid black squares, SP - open black squares; and in panel (c) only, DGRP populations - all black circles. Pearson correlation coefficients (r) and p-values are indicated in the figures.

Analyses using two-way ANOVA revealed significant interaction effects between genetic factors/lines and sex on mean survival time (MST) (S2.4 Table) ($F_{(37, 608)} = 36.37$, $p < 0.0001$), accounting for 23.14% of the total variation, indicating that sex's impact on survival post-infection was influenced by genetics. Genetic factors were the most impactful ($F_{(37, 608)} = 101.7$, $p < 0.0001$), explaining 64.71% of the total variation, while sex only accounted for 1.70% ($F_{(1, 608)} = 98.95$, $p < 0.0001$), as short-sleep males were more resistant than females, while the reverse was true for long-sleep lines neutralizing each other (Figure 2.4a and S2.6 Table).

Males from the 38 SIP lines showed an average 1.06-fold higher resistance to Ma549 than females ($p = 0.0002$), which is similar to the results obtained with both the 10 DGRP progenitors ($p = 0.0302$) and the entire DGRP ($p < 0.0001$) (Figures 2.2a, 2.4a). However, infected females outlived males in 57 out of 188 DGRP lines, one of which is the long-sleeping progenitor (LP) DGRP line 338 (Figure 2.4c)[49]. In 20 of the 48 SIP and progenitor DGRP lines, females also outlived males, with 15 of these 20 lines belonging to long-sleeping (L1, L2, and LP) groups. In the remaining 28 lines, where males outlived females, 19 were short sleepers in S1, S2, and the five short-sleeping DGRP progenitor (SP) lines (Figure 2.4b, Table 2.1). Four out of five long-sleeping DGRP progenitor lines (LP) lines (338, 379, 313, 335), except 235, showed greater resistance in females, while four out of five SP lines, (808, 310, 38, 365, but not 832), showed greater resistance in males (Figure 2.4b).

As previously reported[125], the genetic composition of the SIP lines reflects the sleep phenotypes of their founder DGRP lines, with long-sleeping DGRP lines largely contributing to

L1 and L2 SIP lines, and short-sleeping DGRP lines influencing S1 and S2 SIP lines. The results indicate that the differences in disease resistance between males and females depend on particular sleep phenotype under selection. Specifically, selecting for short sleep is more likely to increase disease resistance in male individuals, while selecting for long sleep may reflect the LP ancestry in females. However, selecting for sleep phenotypes significantly magnified sexual disparities. The most notable disparities within a single SIP line were observed in L1_6 (females outlived males by 1.73 days) and S2_7 (males outlived females by 2.52 days) (Figure 2.1b and Table 2.1). In contrast, the 10 DGRP progenitors exhibited less pronounced survival differences between sexes, with DGRP 338 females outliving males by 0.78 days and DGRP 808 males outliving females by 0.73 days.



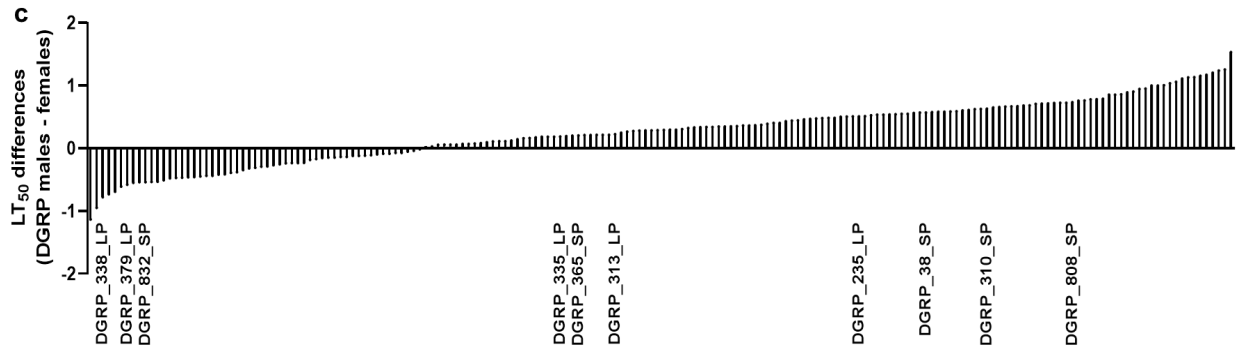
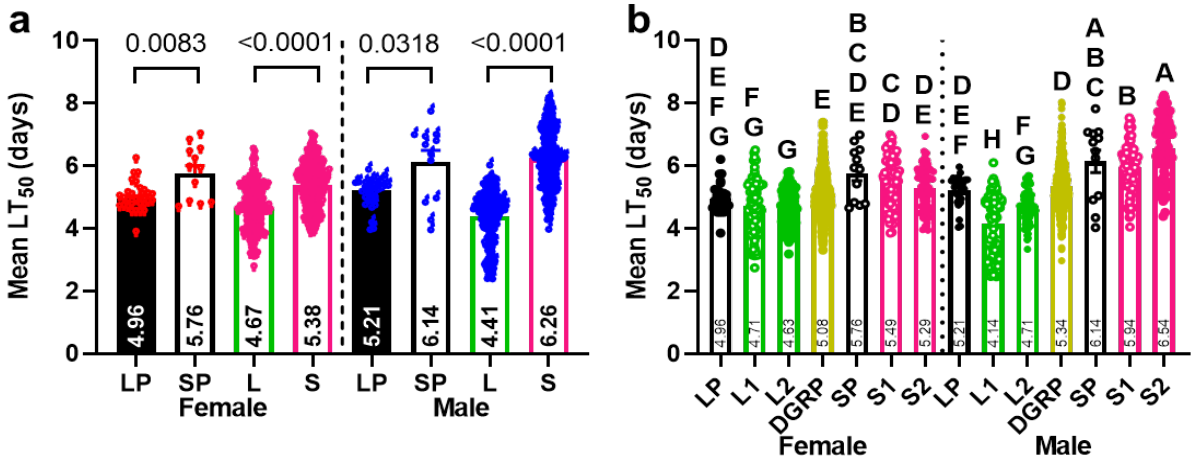


Figure 2.4 (**a**) Percent survival of 38 SIP populations infected with Ma549. Survival curves showing the mean % survival ± SEM were generated separately for each of the 38 female (red, n = 229-331) and male (blue, n = 214-327) lines from 9 replicates (averaging 30 flies each) per sex per line across three trials. (**b**) Sex-based disparities in Ma549 LT_{50} by SIP lines. The values were calculated by subtracting the female LT_{50} values from the male LT_{50} values for each of the 38 lines, averaged from 9 replicates (each containing an average of 30 flies), per sex per line across 3 experiments. Positive values indicate greater resistance in males, whereas negative values indicate greater resistance in females. L1 - open green bars, L2 - solid green bars, S1 - open pink bars, S2 - solid pink bars, and their corresponding progenitors (LP – solid black bars and SP – open black bars), and (**c**) Sex-based disparities in Ma549 LT_{50} by DGRP populations in black bars. The values were calculated by subtracting the female LT_{50} values from the male LT_{50} values for each of the 188 DGRP lines[49]. Positive values indicate greater resistance in males, whereas negative values indicate greater resistance in females.

The 38 SIP lines, divided into short- and long-sleep groups, along with their 10 respective LP and SP progenitors, were statistically analyzed for disease resistance. Overall, infected short-sleeping (S) females (males) outlived long-sleepers (L) by 1.15 (1.42)-fold, and females (males) of the five short-sleeping DGRP progenitor lines (SP) outlived the five long-sleeping progenitor lines (LP) by 1.16 (1.18)-fold ($p \leq 0.318$, Figure 2.5a and S2.4 Table). DGRP males and females displayed intermediate longevity between short- and long-sleeping lines (Figure 2.5b and S2.4 Table). Ranking individual male LT_{50} values from least to most resistant to Ma549 infection showed that L1 and L2 long-sleepers included predominantly shorter-lived lines, while S1 and S2 short-sleepers were mostly longer-lived except for S1_3 males (Figure 2.5c and S2.4 Table). Additionally, the shortest-sleeping DGRP line, DGRP_38, and the longest-sleeping DGRP line,

DGRP_335, were the longest- and shortest-surviving among the 10 DGRP progenitors, respectively.

We estimated phenotypic plasticity using the coefficient of environmental variation (CV_E) to remove the mean-variance association[69]. The CV_E ranged from 18.53 to 36.50 in males and 17.38 to 31.07 in females. Levene's test was used to compare sex-based variations in disease resistance (Table 2.5). Of the 38 strains, 24 had higher CV_E in males, with 17 showing significant differences per Levene's test. There was a significant difference in CV_E between short- and long-sleeping males ($t = 2.493$, $p = 0.0174$), suggesting greater variation in more resistant males. Despite significant longevity differences, CV_E did not vary between long- and short-sleeping females. These results suggest that sleep duration impacts phenotypic plasticity more in males than females. Additionally, no significant differences in CV_E were found between long- or short-sleeping males and females, aligning with the overall data across the 38 SIP lines ($t = 1.822$, $p = 0.0725$, Table 2.5). This contrasts with longevity data, where females outlived males in long-sleeping lines and males outlived females in short-sleeping lines, highlighting the varied impact of sleep duration on phenotypic plasticity and disease resistance across sexes and strains.



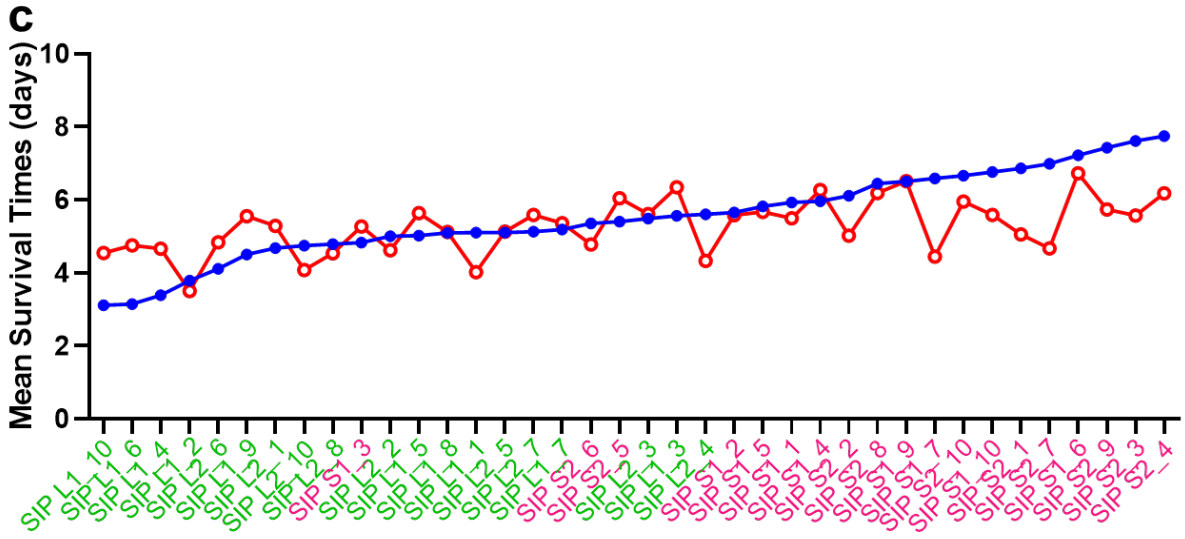


Figure 2.5 Sexual dimorphism and longevity of infected long- and short-sleeping progenitor and SIP groups **(a).** The data presents the mean ± SEM LT_{50} values across 171 long (L) and short (S)-sleeping SIP replicates of 19 line each across 3 trails, 30 long-sleeping progenitor (LP) replicates and 12 short-sleeping progenitor (SP) replicates[49]. p-values are derived from either unpaired (L vs S males or females) or Welch's t-tests (LP vs SP females) or the Mann-Whitney U test (LP vs SP males). Comparison of female and male LT_{50} values within two progenitor (LP, SP) and four SIP (L1, L2, S1, S2) groups (**b**). The SIP, their progenitor, and DGRP fly lines are depicted with open green dots for L1, solid green dots for L2, open pink dots for S1, solid pink dots for S2, solid black dots for LP, open black dots for SP, and gold dots for DGRP females and males. LT_{50} values were averaged for 5 LP lines (30 replicates), 5 SP lines (12 replicates), 10 L1 or S2 lines (90 replicates), 9 L2 or S1 lines (81 replicates), and 188 DGRP[49] (424 replicates) lines. A two-way ANOVA was conducted to evaluate the main effects of genetic factor/line and sex on disease resistance. Least squares means (LSM) ± SEM from Tukey's multiple comparison test are shown, with a compact letter display (CLD) positioned at the top of individual bars, with statistical significance where bars are not connected by the same letter. Mean survival times (MST) for males and females across all 38 SIP lines (**c**). The male MST was ranked from least to most resistant to Ma549 infection. The MST metric exhibits normal distributions, validated by both D'Agostino-Pearson and Shapiro-Wilk normality tests[122]. Male data points are depicted as blue dots, while female data points are shown as red circles across all 38 SIP lines.

2.3.3 Correlation between sleep parameters and infection

Using previously published DGRP sleep indices of healthy flies[129], Wang et al., 2017[49] showed that in male DGRP flies infected with Ma549, LT_{50} values were negatively correlated with daytime bout length and sleep duration, while the values for female flies were not significant. In addition, DGRP flies of both sexes showed a positive correlation between LT_{50}

values and the number of nighttime sleep bouts ("naps") and a negative correlation with nighttime sleep duration (Table 2.2, Figure 2.6).

To further examine the relationship between infection and sleep, we correlated our Ma549 longevity data with seven previously collected SIP sleep indices from healthy flies[125] (Table 2.2).

Table 2.2 Correlation analyses of sleep indices and Ma549 longevity for SIPs with or without progenitors (LP/SP) and DGRPs.

Lines	**156 DGRP**				**48 SIP+LP+SP**				**38 SIP**			
Sex	**Male**		**Female**		**Male**		**Female**		**Male**		**Female**	
Sleep indices\correlations	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Nighttime bout length (min)	-0.09	0.2609	-0.21	0.0086	-0.69	<0.0001	-0.37	0.0088	-0.74	<0.0001	-0.43	0.0076
Nighttime bout number (cts)	0.25	0.0018	0.24	0.0028	0.40	0.0053	0.43	0.0022	0.34	0.0344	0.44	0.0063
Nighttime sleep duration (min)	-0.32	<0.0001	-0.28	0.0004	-0.72	<0.0001	-0.40	0.0050	-0.76	<0.0001	-0.43	0.0077
Daytime bout length (min)	-0.19	0.0193	-0.05	0.5545	-0.52	0.0001	-0.32	0.0275	-0.74	<0.0001	-0.41	0.0108
Daytime bout number (cts)	0.12	0.1229	0.16	0.0430	0.27	0.0611	-0.25	0.0847	0.17	0.3041	-0.34	0.0357
Daytime sleep duration (min)	-0.20	0.0113	0.04	0.5960	-0.66	<0.0001	-0.37	0.0089	-0.71	<0.0001	-0.45	0.0043
Daily sleep duration (min)	-0.30	0.0001	-0.13	0.0938	-0.71	<0.0001	-0.40	0.0047	-0.75	<0.0001	-0.44	0.0055

Sleep data for SIP[125] and DGRP[129] flies were from previous studies. Susceptibility to Ma549 and some sleep correlation analyses for DGRP flies were from Wang et al. 2017[49]. r: Pearson correlation coefficient, p: p value.

Table 2.3 Significance testing of correlation coefficient differences for SIPs with or without progenitors (LP/SP) and DGRPs.

Lines	**r diff. (SIP vs DGRP)**				**r diff. (SIP vs SIP+LP+SP)**			
Sex	**Male**		**Female**		**Male**		**Female**	
Sleep indices\correlations	**z-score**	**p**	**z-score**	**p**	**z-score**	**p**	**z-score**	**p**
Nighttime bout length (min)	4.55	0.0000	1.29	0.1958	0.42	0.6740	0.28	0.7826
Nighttime bout number (cts)	-0.56	0.5759	-1.19	0.2331	0.27	0.7891	-0.02	0.9847
Nighttime sleep (min/12hr)	3.52	0.0004	0.90	0.3667	0.37	0.7111	0.14	0.8861
Daytime bout length (min)	4.01	0.0001	2.06	0.0391	1.59	0.1108	0.46	0.6424
Daytime bout number (cts)	-0.26	0.7967	2.77	0.0055	0.47	0.6364	0.44	0.6598
Daytime sleep (min/12hr)	3.60	0.0003	2.83	0.0046	0.41	0.6800	0.42	0.6717
Daily sleep (min/24hr)	3.49	0.0005	1.81	0.0705	0.35	0.7291	0.22	0.8270

As shown in Tables 2.2-2.3, the LT_{50} values for SIP males and females positively correlated with the number of nighttime sleep bouts (Figures 2.6a, d), but negatively correlated with both nighttime and daytime bout length, as well as total daily sleep duration (Tables 2.2-2.3, Figures 2.6b, e). The nighttime bout length in both sexes positively correlated with nighttime sleep duration ($r \geq 0.854$, $p < 0.0001$). However, daytime and nighttime sleep duration were negatively correlated with disease resistance (Figures 2.6c, f). Adding the 10 progenitors to the 38 SIPs slightly weakened correlations (except the number of bouts in males), though the reductions in r were not statistically significant (Tables 2.2-2.3).

Unlike DGRP lines[125], SIP females showed correlations between disease resistance and both daytime sleep bout length and duration, as well as daily sleep duration. Additionally, SIP males exhibited a correlation between nighttime sleep bout length and disease resistance.

Disease resistance in SIPs, with or without progenitors, showed stronger correlations with sleep indices than DGRPs, with significant r differences in SIP males for all sleep indices except night and daytime bouts, and in females for daytime sleep indices (Tables 2.2-2.3). The results also demonstrated that compared to females, disease resistance in males was more negatively correlated with daytime bout length (Figures 2.6b, e), daytime sleep (Figures 2.6c, f), and 24-hour daily sleep (Tables 2.2-2.3), indicating that infected SIP males had experienced more fragmented sleep when healthy.

Next, we explored the relationship between disease resistance and sleep indices of healthy flies by examining sexual dimorphism. Our data showed positive correlations between the disparities in LT_{50} and daytime sleep bout numbers across sexes, with SIP lines exhibiting the strongest correlations both with ($r = 0.5747$, $p = 0.0002$) and without ($r = 0.5302$, $p = 0.0001$) progenitors, followed by DGRPs ($r = 0.1831$, $p = 0.0221$). However, no significant correlations

were found between LT_{50} differences and other sleep indices in any of the lines, indicating that the sexually dimorphic correlation between disease resistance and sleep was specific to daytime sleep bout numbers.

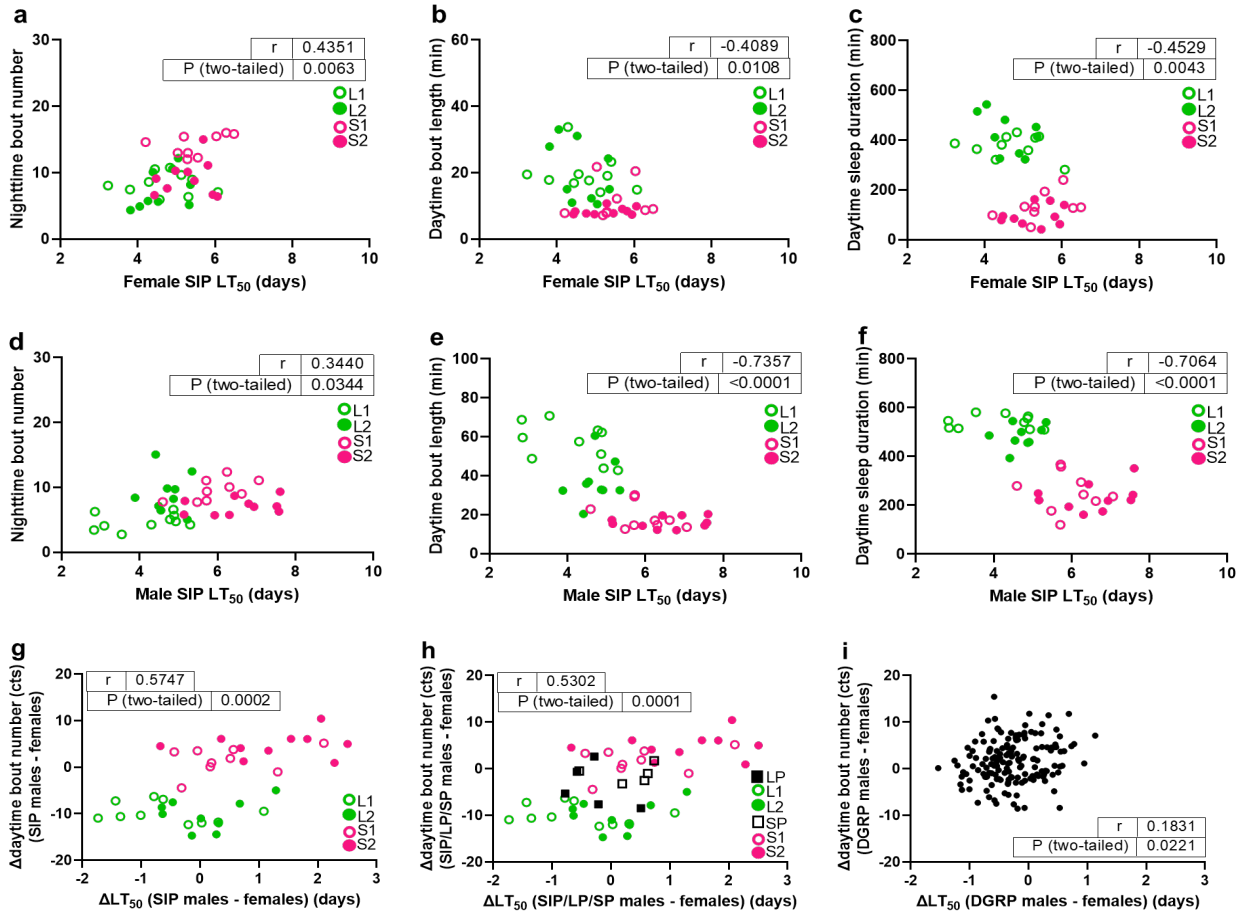


Figure 2.6 Correlations of LT_{50} values for Ma549-infected females and males with publicly available sleep indices for 38 SIP lines[125]. SIP LT_{50} values are positively correlated with nighttime bout number (**a, d**) and negatively correlated with daytime bout length (**b, e**) and sleep duration (**c, f**). Individual Pearson correlation coefficients (r) and p-values are indicated in the figures. Correlations of the differences in LT_{50} and daytime sleep bout numbers across sexes in 38 SIP lines without (**g**) and with (**h**) 10 progenitors, as well as in DGRP lines (**i**). LT_{50} values are positively correlated with daytime sleep bout numbers. Individual Pearson correlation coefficients (r) and p-values are indicated in the figures.

2.3.4 Characterization of sickness sleep triggered by Ma549 infection

Given the significant correlation between healthy sleep indices and the outcomes of Ma549 infection, we investigated post-infection sleep behaviors using the *Drosophila* Activity

Monitor (DAM2)[82] in S1_7 and L1_6 flies. These populations were selected from among the 38 SIP fly populations due to the notable survival rate disparities between males and females post-infection, with L1_6 females outliving males by 1.73 days and S2_7 males outliving females by 2.52 days (Figure 2.4b).

We determined that post-infection mated (virgin) L1_6 females outlived mated (virgin) S1_7 females by 1.09 (1.07)-fold. In contrast, mated (virgin) S1_7 males outlived mated (virgin) L1_6 males by 2.23 (1.99)-fold ($p \leq 0.0208$, Figure 2.7a and S2.4 Table). Post-infection, mated (virgin) L1_6 females outlived males by 1.61 (1.50)-fold, while mated (virgin) S1_7 males outlived mated (virgin) females by 1.50 (1.42)-fold ($p < 0.0001$, Figure 2.7a), indicating a reverse sex dimorphism in disease resistance between L1_6 and S1_7 lines. Additionally, virgin females (males) outlived their mated L1_6 and S1_7 counterparts by 1.16 (1.24)-fold and 1.17 (1.11)-fold, respectively ($p < 0.0001$, Figure 2.7a, Table 2.1 and S2.4c Table), highlighting the significant impact of mating status on sleep regulation and immunity, independent of genetic background.

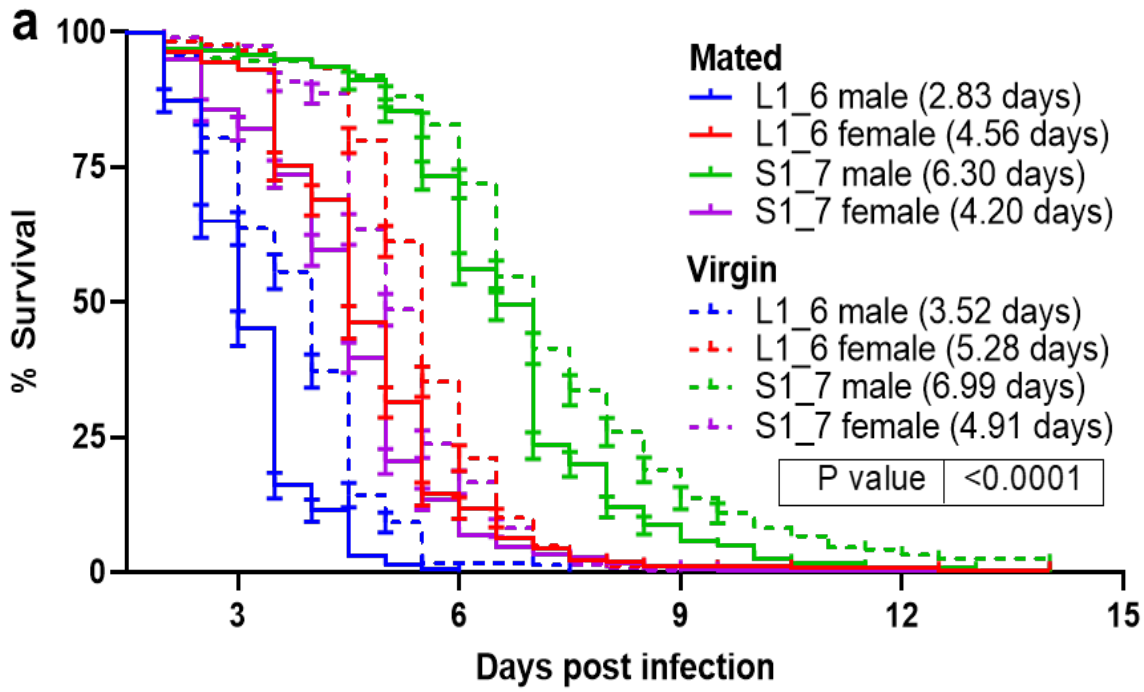
Sleep patterns were examined from 12 to 60 hours post-infection with Ma549, spanning two light-dark cycles. This period precedes the onset of visible symptoms, such as reduced feeding and reproductive behavior, and the upregulation of the immune gene *Drosomycin* in response to Ma549 infection[19, 97].

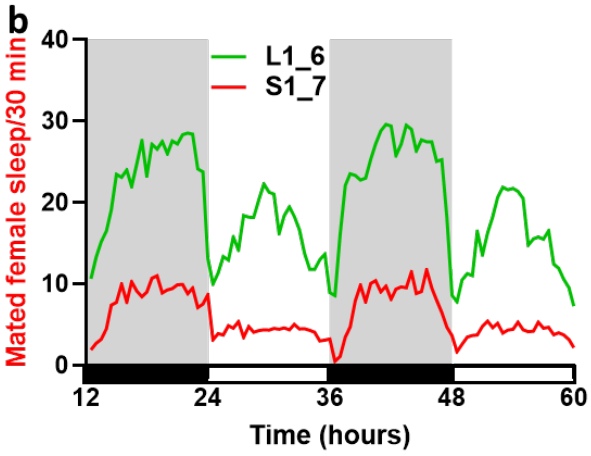
In L1_6 flies, sleep data indicated that uninfected mated males and females had similar nighttime sleep patterns ($p = 0.3691$) but males slept for 21.71% longer during the day ($p = 0.0004$, Figure 2.7b-e and S2.1-2.3 Tables). Interestingly, Ma549 infection did not disrupt the circadian rhythm but significantly increased daytime sickness sleep (extra sleep following infection as defined by Davis et al. 2017[83]) compared to uninfected controls ($p < 0.0001$, Figure

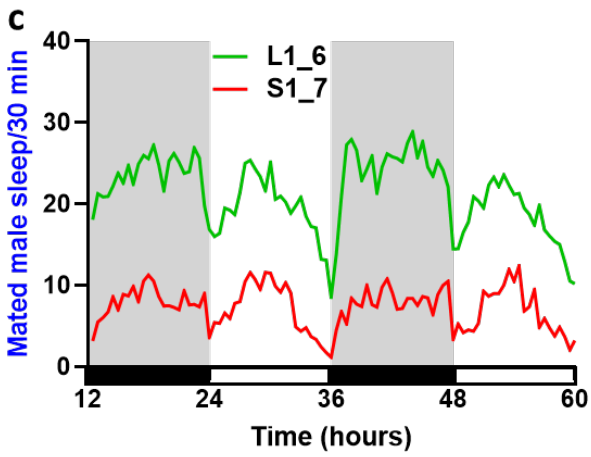
2.7f-i and S2.2 and S2.3c Tables). Mated females showed the largest increase in daytime sickness sleep (51.68%), followed by virgin females (36.86%). Infected mated (virgin) males showed a 33.41% (17.83%) increase compared to uninfected females ($p < 0.0001$, Figure 2.7f-i). Despite uninfected L1_6 females sleeping less than males, their larger increase in daytime sickness sleep meant they slept almost as much during the day as infected males, potentially contributing to the comparative longevity of females in L1 populations. Nighttime sleep was unaffected by infection ($p \geq 0.3653$, Figure 2.7f-i).

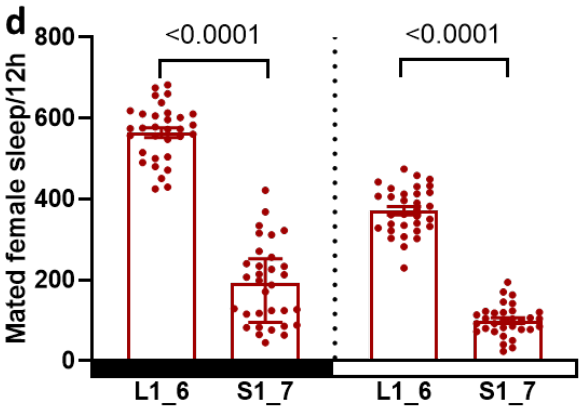
Uninfected S1_7 females and males slept 63-73% less than L1_6 flies during both night and day ($p < 0.0001$, Figure 2.7b-e and S2.1b Table). S1_7 males slept as much as females at night ($p = 0.9872$, Figure 2.7b-e), but 67.05% more during the day ($p = 0.0053$, Figure 2.7b-e). Infection with Ma549 increased daytime sleep in S1_7 flies by 169.60% in mated males, 123.58% in virgin females, 120.99% in virgin males, and 83.17% in mated females ($p < 0.0001$, Figure 2.7f-i and S2.2 and S2.3c Tables). Unlike L1_6 flies, Ma549 infection modestly (18-32.5%) increased nighttime sleep in all S1_7 flies, regardless of sex or mating status ($p < 0.05$). Therefore, infected short-sleeping males showed particularly large increases in sickness sleep, potentially contributing to their exceptional longevity (Figure 2.7f-i). With 447 minutes of daytime sleep (averaged over two days), infected S1_7 males slept for 26% less than infected L1_6 males (602.9 minutes), In contrast, with 165 minutes of daytime sleep, healthy S1_7 males slept for 63% less than healthy L1_6 males (451.9 minutes).

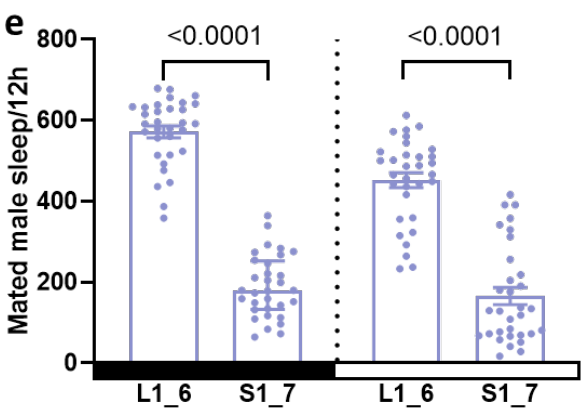
This suggests that selecting for short-night sleeping either did not select against sickness-induced daytime sleep or that disease eliminated their 'short-sleeper' traits.


a
% Survival
100
75
50
25
0
3
6
9
12
15
Days post infection
Mated
L1_6 male (2.83 days)
L1_6 female (4.56 days)
S1_7 male (6.30 days)
S1_7 female (4.20 days)
Virgin
L1_6 male (3.52 days)
L1_6 female (5.28 days)
S1_7 male (6.99 days)
S1_7 female (4.91 days)
P value
<0.0001


b
Mated female sleep/30 min
40
30
20
10
0
L1_6
S1_7
12
24
36
48
60
Time (hours)


c
Mated male sleep/30 min
40
30
20
10
0
L1_6
S1_7
12
24
36
48
60
Time (hours)


d
Mated female sleep/12h
800
600
400
200
0
<0.0001
<0.0001
L1_6
S1_7
L1_6
S1_7


e
Mated male sleep/12h
800
600
400
200
0
<0.0001
<0.0001
L1_6
S1_7
L1_6
S1_7

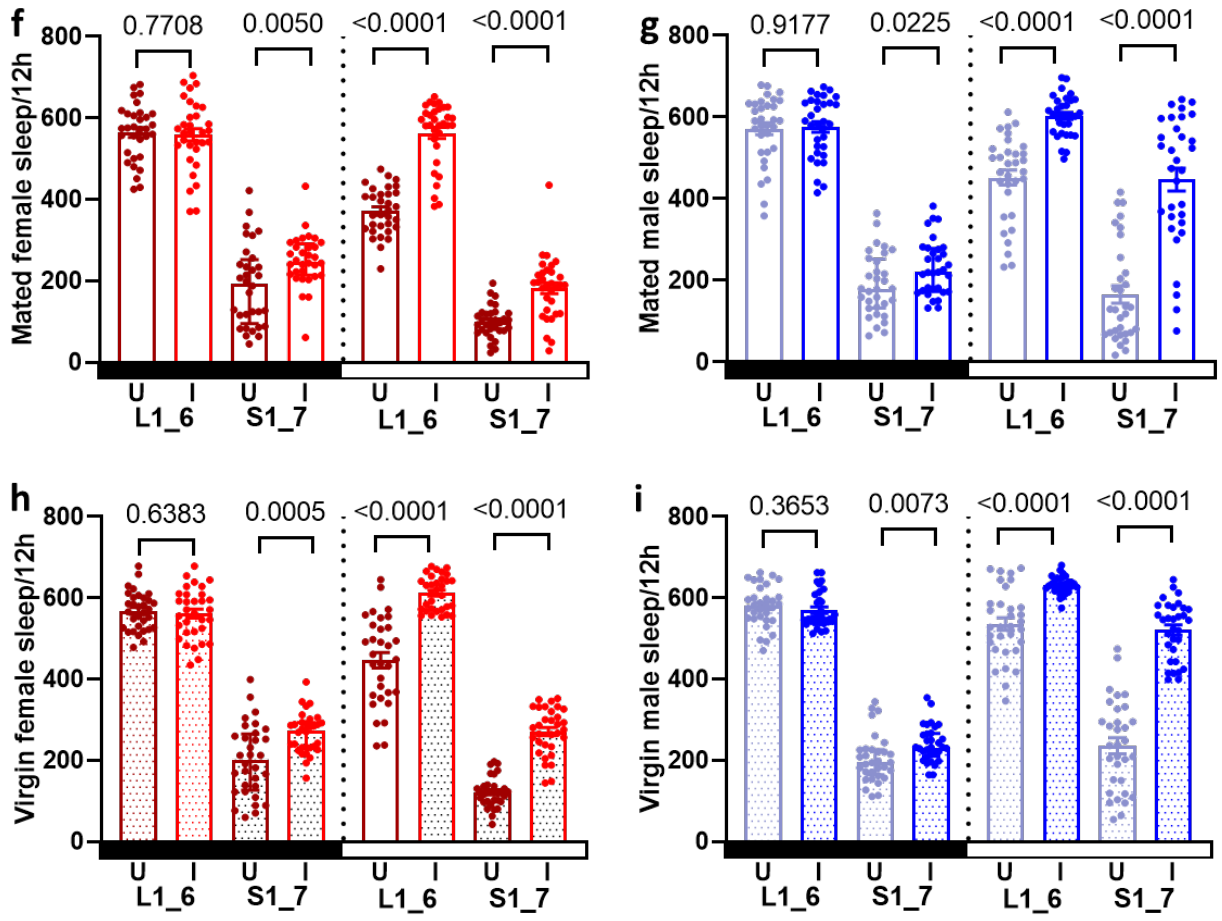


Figure 2.7 Kaplan-Meier plots (**a**) depict survival disparities between L1_6 and S1_7 in mated females (n = 270 for L1_6 and n = 297 for S1_7), mated males (n = 237 for L1_6 and n = 290 for S1_7), virgin females (n = 289 for L1_6 and n = 282 for S1_7), and virgin males (n = 239 for L1_6 and n = 285 for S1_7), with statistical significance determined using the log-rank and Wilcoxon tests for intergroup comparisons. Ma549 infection affects sleep and survival. Sleep profiles depict sleep in min per 30 min from 12-60 h spanning two light-dark cycles for 32 flies pooled from three trials for uninfected mated L1_6 and S1_7 females (**b**) and males (**c**), with gray shading indicating nighttime. Sleep comparisons were made between uninfected mated L1_6 versus S1_7 females (**d**) or males (**e**), and uninfected (U) versus infected (I) mated females (**f**), mated males (**g**), virgin females (**h**), and virgin males (**i**) within the L1_6 and S1_7 lines. Bars illustrate nighttime (black) and daytime (white) sleep for uninfected (dark red for females, sky blue for males) and infected (scarlet red for females, navy blue for males) mated and virgin (shaded) flies pooled from three trials, with each data point representing the average sleep/12h of a single fly (n = 32) from 12-60 h post infection. Means ± SEM and p-values were obtained from Welch's t test (d) and unpaired t test (**e**). In panels (f)-(i), Mann-Whitney U tests, Welch's t-tests, and unpaired Student's t-tests were used for various comparisons (S2.3c Table), with mean ± SEM and p-values indicated.

We investigated sexual dimorphisms in total daytime or nighttime sleep between Ma549-infected males and infected females of S1_7 and L1_6 (Figure 2.8a-b, S2.2 and S2.3d Tables).

Infected S1_7 males showed 145.87% (mated) and 92.15% (virgin) more daytime sleep than infected females ($p < 0.0001$), while L1_6 males slept slightly more than females ($p \leq 0.0491$). At night, virgin S1_7 males slept 9.97% less than females ($p = 0.0283$), while no significant differences were found in mated S1_7 males and L1_6 males compared to females ($p \geq 0.2395$). The substantially higher daytime sleep in S1_7 males, alongside their extended survival, suggests a pronounced sexual dimorphism in the response to Ma549 infection, with enhanced daytime sleep potentially contributing to male longevity.

Considering mating status (mated vs virgin), we found that mating status did not affect nighttime sleep of infected females or males in either line ($p \geq 0.2560$, Figure 2.8c-d, S2.2 and S2.3e Tables) but mating significantly decreased daytime sickness sleep regardless of sex and infection status ($p \leq 0.0240$).

Overall, our results showed that Ma549 infection affected both sleep and survival outcomes in a manner that indicated they were linked. Significant positive correlations were observed between longevity and sleep parameters across the two fly lines, including mated and virgin females and males (Figure 2.8e-g, Table 2.1, S2.2 and S2.4 Tables). LT_{50} values positively correlated with changes in both minutes ($R^2 = 0.6674$, $p = 0.0133$) and percentages ($R^2 = 0.5507$, $p = 0.0350$) of daytime sickness sleep, suggesting that increased sleep following infection is linked to enhanced survival.

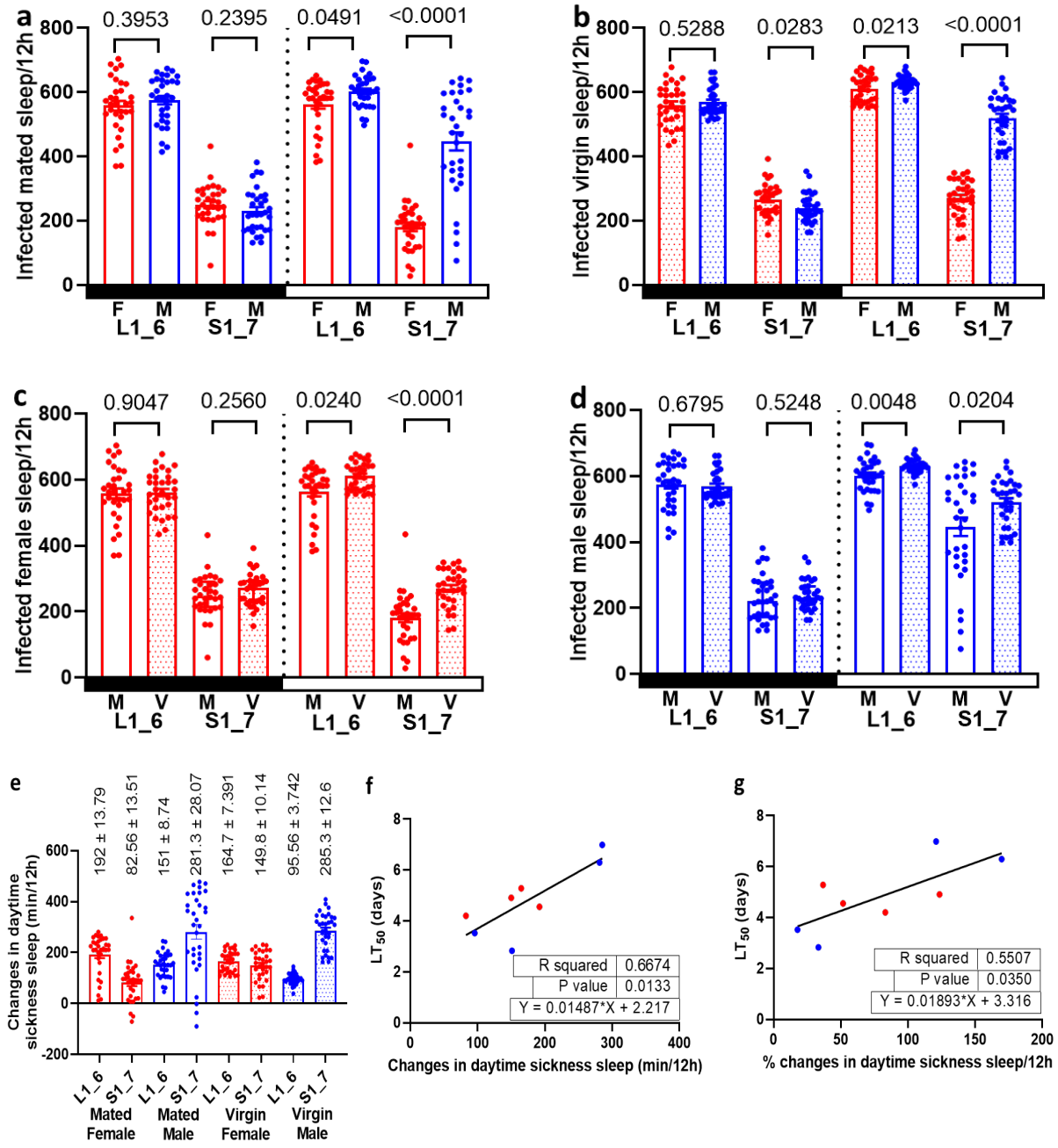


Figure 2.8 Sexual dimorphism in sleep was compared in mated (**a**) and virgin (**b**) L1_6 and S1_7 flies. Sleep was averaged per 12 h for each fly (n = 32) during day and night from 12-60 h post infection pooled from three trials. Mann-Whitney U tests, Welch's t-tests, and unpaired Student's t-tests were used for various comparisons (detailed in S2.3d Table), with mean ± SEM and p-values indicated. Mating status impacts sleep and longevity after Ma549 infection in L1_6 and S1_7 flies. Variations in nighttime and daytime sleep between mated and virgin flies in infected female (**c**) and male (**d**) groups are shown. Each point represents a single fly's average sleep per 12 h (n = 32 pooled from three trials) during nighttime and daytime from 12-60 h post-infection. Statistical significance was assessed between mated and virgin females or males within the L1_6

and S1_7 lines. Mann-Whitney U tests, Welch's t-tests, and unpaired Student's t-tests were used for various comparisons (detailed in S2.3e Table), with mean ± SEM and p-values indicated. Black (white) rectangles on the x-axis signify nighttime (daytime), with shaded bars denoting virgin flies. Line abbreviations: V for virgin and M for mated. Sickness sleep post-Ma549 infection (**e**) is represented, detailing net minute changes for mated and virgin females (red, hatched red) and males (blue, hatched blue) from L1_6 and S1_7 lines, with mean ± SEM provided. Linear regression analyses correlating LT_{50} values with changes in both minutes (**f**) and percentages (**g**) of daytime sickness sleep in mated and virgin L1_6 and S1_7 females (red) and males (blue), showing R^2 values, p-values, and regression slopes. Percent changes in sickness sleep during daytime were calculated as sleep difference between infected and uninfected flies, divided by sleep in uninfected flies, and multiplied by 100%.

2.3.5 Effects of line, sex, mating status, and infection on sleep

ANOVA confirmed the effects of genetic line, sex, mating status, and infection on nighttime (Figure 2.9a) and daytime (Figure 2.9b) sleep (Table 2.4 derived from the data in S2.2 Table). Genetic line ($p < 0.0001$) significantly affected both night and daytime sleep, confirming distinct sleep behaviors among L1_6 and S1_7. Males and females showed significant differences only in daytime sleep ($p < 0.0001$). Infection status significantly affected both daytime sleep and nighttime sleep ($p \leq 0.0001$), with a more pronounced impact on daytime (F ratio = 556.16) than nighttime sleep (F ratio = 14.98). Mating status significantly impacted daytime sleep ($p < 0.0001$) but not nighttime sleep ($p = 0.2125$). Significant interactions were found between line and infection for both night and daytime sleep ($p \leq 0.0011$), while interactions between line and sex, and infection and sex were significant only for daytime sleep ($p \leq 0.0002$). Three-way interactions among line, infection and sex, and among line, mating, and infection were significant for daytime sleep ($p \leq 0.0099$) but not for nighttime sleep ($p \geq 0.2619$), indicating that the influence of one factor on sleep can be significantly modified by the others.

Table 2.4 Effects of line, sex, mating status, and infection as well as their interactions on night and daytime sleep.

Source	Nparm	DF	Nighttime sleep			Daytime sleep		
			Sum of Squares	F Ratio	p value	Sum of Squares	F Ratio	p value
Line	1	1	15433526	3288.551	<0.0001	9427974.6	1333.291	<0.0001
Mating	1	1	7313	1.5582	0.2125	482990.9	68.3039	<0.0001
Line*Mating	1	1	4033	0.8594	0.3544	786.3	0.1112	0.7389
infection	1	1	70313	14.9821	0.0001	3932697.8	556.1568	<0.0001
Line*infection	1	1	100969	21.5143	<0.0001	76575.0	10.8291	0.0011
Mating*infection	1	1	621	0.1324	0.7161	260.9	0.0369	0.8477
Line*Mating*infection	1	1	406	0.0865	0.7688	47422.4	6.7064	0.0099
Sex	1	1	8	0.0017	0.9671	1711654.7	242.0599	<0.0001
Line*Sex	1	1	15842	3.3756	0.0668	436440.7	61.7209	<0.0001
Mating*Sex	1	1	95	0.0201	0.8872	885.9	0.1253	0.7235
Line*Mating*Sex	1	1	1.7578125	0.0004	0.9846	3930.0	0.5558	0.4563
infection*Sex	1	1	3650	0.7777	0.3783	100422.0	14.2015	0.0002
Line*infection*Sex	1	1	5920	1.2614	0.2619	394688.4	55.8163	<0.0001
Mating*infection*Sex	1	1	1210	0.2578	0.6119	16761.7	2.3704	0.1243
Line*Mating*infection*Sex	1	1	29	0.0061	0.9378	2452.2	0.3468	0.5562

Source: This is a list of the tested factors and interactions. Nparm: Number of parameters associated with the effect. DF: degrees of freedom for each source of variation. Sum of Squares: sum of squares (SS) for each source of variation, along with the total from all sources. F Ratio: the mean square of the factor divided by the mean square of the error. A p-value less than 0.05 means that the factor or interaction has a statistically significant effect.

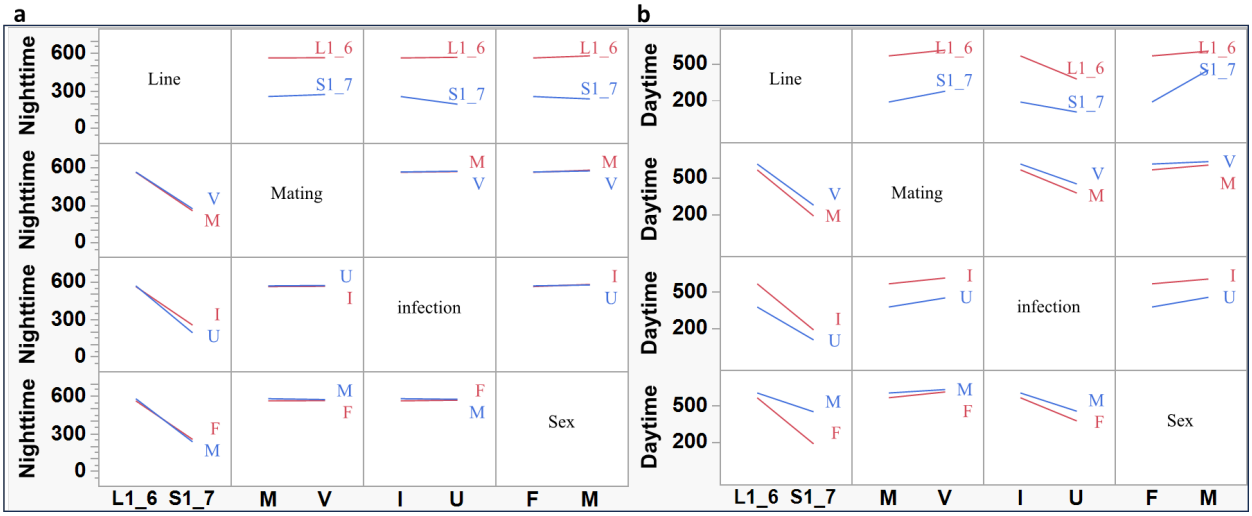
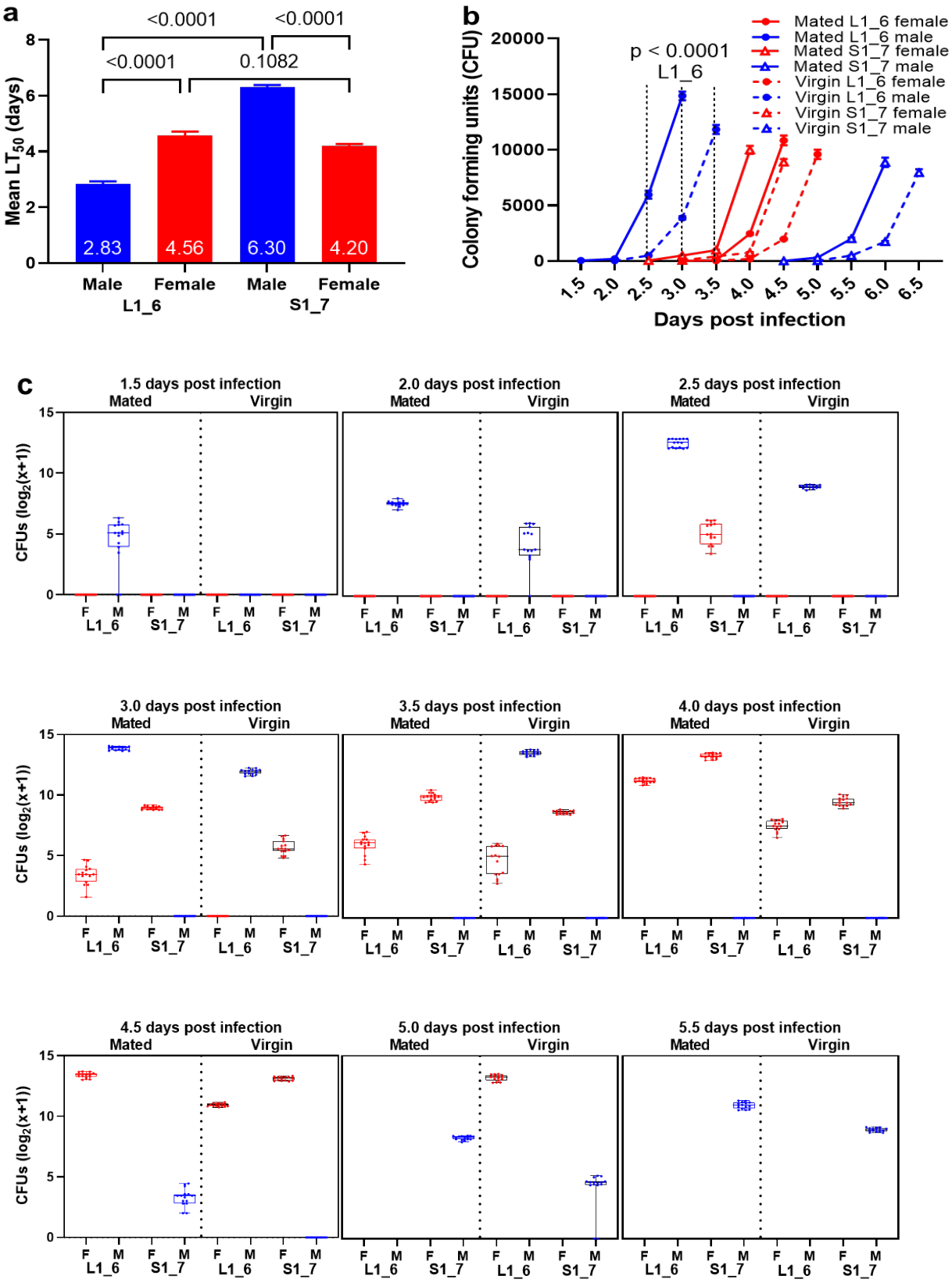


Figure 2.9 Interaction profiles of line, sex, mating status, and infection status on nighttime (**a**) and daytime (**b**) sleep (min/12h) across L1_6 and S1_7 females and males. These profiles were

derived from the sleep data of 512 individual flies, considering two genetic lines (L1_6, S1_7), both sexes (females and males), two mating statuses (mated and virgin), and two infection statuses (infected and uninfected). Abbreviations: sex (F = female, M = male), mating status (M = mated, V = virgin), and infection status (I = infected, U = uninfected).

2.3.6 The impact of host variation on fungal fitness

We analyzed the relationship between longevity and Ma549 colonization (measured as fungal load in colony forming units CFU) of L1_6 and S1_7. As previously noted, mated L1_6 females outlived males by 1.61-fold, while S1_7 males lived 1.50-fold longer than females (Figure 2.10a), and virgins outlived mated flies in both lines (Figure 2.7a). Mated L1_6 males were the least able to restrain fungal growth with CFUs appearing 1.5 days post infection and reaching 14,848.2 ± 391.16 CFUs/fly 3-days post infection, while virgin S1_7 males had the lowest counts (21.87 ± 2.60 CFUs/fly on day 5.0) (Figure 2.10b-c, S2.5 Table). Fungal loads increased rapidly near fly death in both lines, indicating a breakdown of host resistance. Virgin flies showed a 0.5-day delay in fungal proliferation compared to mated flies (Figure 2.10b-c), aligning with differences in their LT_{50} values (Figure 2.7a, Table 2.1 and S2.4c Table). LT_{50} values (Table 2.1 and S2.4c Table) were strongly correlated with both the onset (initial time point at which fungal growth becomes detectable) and peak times (time point maximum fungal growth achieved) of CFUs ($r = 0.9961$, $p < 0.0001$, Figure 2.10d-e) across mated and virgin L1_6 and S1_7 females and males. These findings suggest that selecting for long or short sleep is sufficient to influence fungal invasiveness, with proliferation coinciding with and likely causing death due to an inability to tolerate high fungal levels (Figure 2.10).


a
<0.0001
<0.0001
<0.0001
0.1082
Mean LT50 (days)
2.83
4.56
6.30
4.20
Male
Female
Male
Female
L1_6
S1_7
b
p < 0.0001
L1_6
Mated L1_6 female
Mated L1_6 male
Mated S1_7 female
Mated S1_7 male
Virgin L1_6 female
Virgin L1_6 male
Virgin S1_7 female
Virgin S1_7 male
Colony forming units (CFU)
Days post infection
c
1.5 days post infection
2.0 days post infection
2.5 days post infection
3.0 days post infection
3.5 days post infection
4.0 days post infection
4.5 days post infection
5.0 days post infection
5.5 days post infection
Mated
Virgin
CFUs (log2(x+1))
F M F M F M F M
L1_6 S1_7 L1_6 S1_7

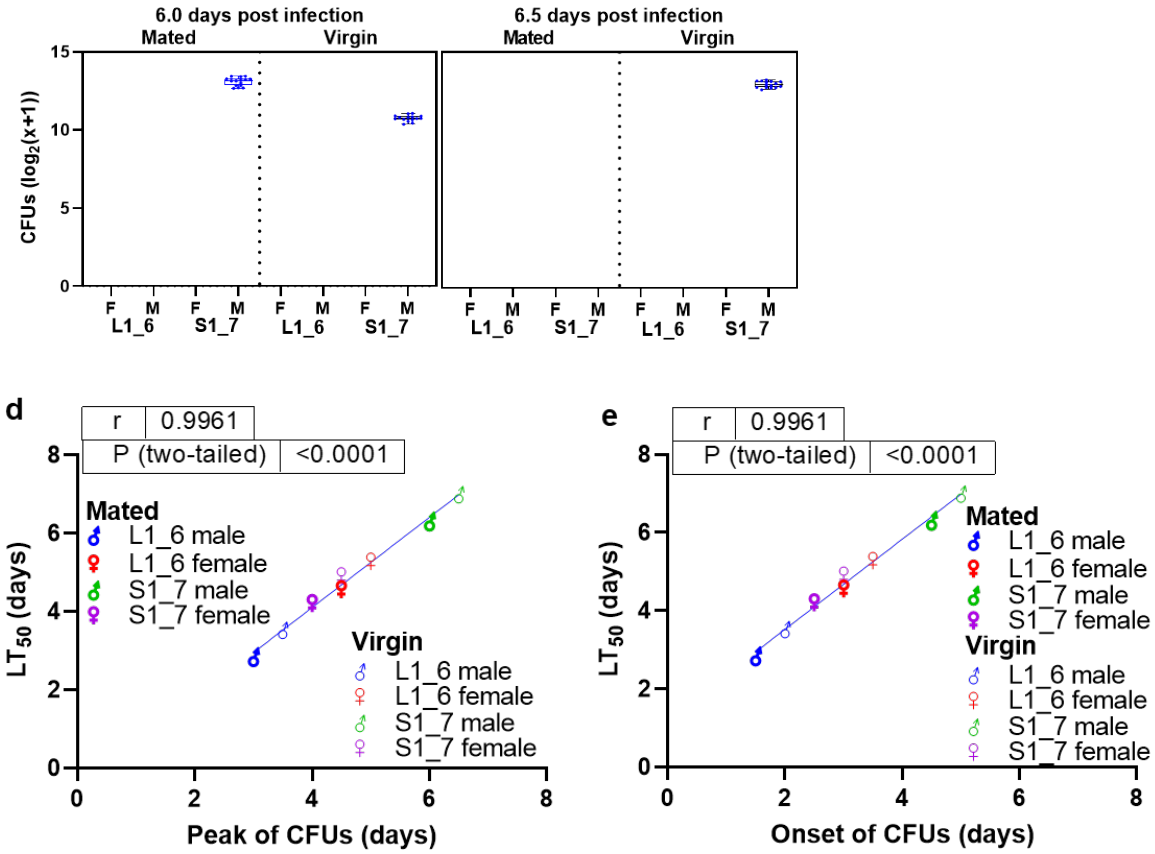


Figure 2.10 (**a**) Sexual dimorphism and longevity of infected L1_6 and S1_7 SIP flies. Separate bars were generated for L1_6 and S1_7 male (blue) and female (red) flies. The data display the mean ± SEM LT_{50} values from nine replicates, with 270 L1_6 females and 237 L1_6 males, and 297 S1_7 females and 290 S1_7 males across three experiments. Time course of CFU (colony forming units) production in mated and virgin (**b**) L1_6 and S1_7 SIP flies following Ma549 infection. Flies were homogenized and plated individually at 12-hour intervals until death. Each data point represents the average of CFU values from 15 flies, with five replicates each, per sex, mating status, line, and time point across three experiments. Means ± SEM and p-values were obtained from two-way ANOVA are indicated. Time course of log-transformed Ma549 fungal loads in the hemolymph of L1_6 and S1_7 SIP flies following Ma549 infection (**c**). Box plots depict each time point for each of the 15 individual replicates per sex, mating status, line, and time point across three repeated experiments, showing the mean ± SEM of CFUs. Correlation analyses between LT_{50} values and the onset (**d**) or peak (**e**) of CFUs for infected mated and virgin L1_6 and S1_7 females (red) and males (blue). Pearson correlation coefficients (r) and p-values are indicated in the figures.

2.4 *Discussion*

Understanding disease resistance in flies is relevant to human health due to the strong homology between insect and mammalian innate immune systems[130]. A sleeping animal is

vulnerable to predators and environmental perils, yet sleep is an ancient behavior that is observed throughout the animal kingdom and likely essential for survival[9, 131, 132]. The many proposed roles for sleep include it being an adaptive response to infection[133], as extra sleep and fatigue are common during illness in humans (sickness sleep), and observed in *Drosophila* post-bacterial infection[134-136].

Standing genetic variation in DGRP lines for disease resistance[49, 105] indicates significant differences in infection and stress responses within a localized *D. melanogaster* population, potentially aiding their survival in extreme environments with diverse pathogens. However, given *Drosophila's* relatively high migration rate[137], DGRP lines may not all originate from the same habitat or be adapted to the same local pathogens, which may contribute to their diversity. Long- and short-sleeping SIP lines were derived from ten extreme long or short DGRP night sleepers, with each of the 38 lines available to us having a distinct genomic haplotype blended from the 10 founders[125]. Predictably, the five long-sleeping progenitors (DGRP_379, _338, _335, _235, and _313) were major founders of L1 and L2, while the five short-sleeping progenitors (DGRP_808, _832, _38, _310, and _365) were major founders of S1 and S2 lines[125]. Reflecting the link between sleep and disease resistance, three of the short-sleeping progenitor lines (DGRP_38, _808, and _310) were previously identified as highly resistant to Ma549, with females (males) ranked 155 (156), 152 (155), and 149 (150) out of 188 DGRP lines, respectively[49]. Two long-sleeping progenitors (DGRP_335 and _313) were more susceptible to Ma549, with females (males) ranking 14 (6) and 55 (24), respectively[49].

Like the DGRP lines[49], our study showed that male and female survival post infection were positively correlated with each other, suggesting functional similarities in disease resistance. However, susceptibility to Ma549 within the 38 SIPs is sexually dimorphic with

males outliving females to a similar extent (1.05-fold) as DGRP lines[49]. L1 females were exceptional as they tended to outlive males, as did the LP progenitor DGRP_338, which suggests that DGRP_338 may be a particularly impactful founder of L1. More studies will be required to understand the underlying mechanisms, but dimorphism could be due to differences in sleep or activity prior to infection as roughly 68% of males that outlive females were from short-sleep groups.

Our study demonstrated that post-infection with Ma549, short-sleeping S1 and S2 flies of either sex live about 1.2-fold longer than long-sleeping L1 and L2 flies ($p < 0.0001$). Compared to the average of the entire DGRP lines[49], long-sleepers were more susceptible to Ma549 infection and short sleeper males more resistant[49]. The DGRP has not been subject to artificial selection for sleep, and as a more natural population probably balances many trade-offs between sleep and other physiological parameters, including mutually exclusive behaviors such as feeding, which influence sleep and disease resistance.

It was not axiomatic at the beginning of this study that short-sleepers would be more resistant to Ma549 as sleep deprivation usually correlates with decreased health. However, similar to DGRP lines[49], Ma549-resistant SIP flies, particularly males, exhibited fragmented sleep, i.e., they take more naps than susceptible flies at night. Insects might be able to anticipate infections by up-regulating immune genes at certain times of day when disease risk is high[138]. Fragmented sleep may result in heightened sensitivity to minor environmental perturbations, increasing arousal[139]. Previously, it was noted that startle-induced negative geotaxis (predator evasion) and oxidative stress positively correlate with disease resistance[49], suggesting that DGRP lines with an evolutionary history in particularly hazardous environments might have evolved

generalized resistance to multiple stresses, including pathogens so high awareness and fragmented sleep may itself be an adaptive response[64].

We examined the impact of infection on one long- and one short-sleep SIP line that we confirmed retained their original sleep and activity traits. Thus, long-sleeping L1_6 flies had significantly more 24-hour daily and nighttime sleep than short-sleeping S1_7 flies. Notably, Ma549 infection markedly increased daytime sleep, One possible explanation for increased post-infection sleep is that it is an early indicator of impending death. However, these changes in sleep are among the earliest signs of infection, preceding the expression of the antifungal peptide *Drosomycin*[19, 49]. Additionally, longevity differences between L1_6 and S1_7 align with their sickness sleep, suggesting that it is an adaptive response to fighting infection. Post-infection, males of the short-sleeper line slept much more during the day than at night, which may be a feature of artificial selection that would not be favored by natural selection if it disrupts vital activities. However, a survey of wild populations showed high individual variability, including flies that are nocturnal[140]. The genetic basis for diurnal preference in *Drosophila* is largely unknown but involves the circadian pacemaker[140]. Daytime temperature increases drive flies to nocturnality[141], and heat stress induces sickness sleep[142], suggesting overlapping mechanisms in phase shifts and sickness sleep. However, the SIP lines in our study were selected solely for extreme nighttime sleep traits, possibly reducing their ability to adjust night sleep post-infection but leaving daytime sleep more flexible. This explanation assumes that the mechanisms of healthy sleep and sickness sleep are also partly distinct. Consistent with this, infected short-sleeping males exhibited the largest increase in sickness sleep, mitigating their 'short-sleeper' traits and likely contributing to their longevity.

Our findings highlight a nuanced relationship between disease resistance and sleep shaped by sex-specific differences, especially in daytime sleep bout numbers. Strong correlations between ΔLT50 and Δdaytime sleep bout numbers across sexes underscore the role of sleep patterns in modulating disease resistance. Interestingly, virgin flies of both sexes exhibited prolonged sleep and increased resistance to infection, suggesting a complex interplay between sex, mating status, and sleep in determining resistance to pathogens. These results emphasize the importance of considering sexual dimorphism and behavioral traits like sleep in disease resistance research.

2.5 *Materials and Methods*

2.5.1 Fly stocks and virgin fly collection

All 38 available Sleep Inbred Panel (SIP) lines: SIP L1_1 (76271), SIP_L1_2 (76272), SIP_L1_3 (76273), SIP_L1_4 (76274), SIP_L1_5 (76275), SIP_L1_6 (76276), SIP_L1_7 (76277), SIP_L1_8 (76278), SIP_L1_9 (76279), SIP_L1_10 (76280), SIP_L2_1 (76291), SIP_L2_2 (76292), SIP_L2_3 (76293), SIP_L2_4 (76294), SIP_L2_5 (76295), SIP_L2_6 (76296), SIP_L2_7 (76297), SIP_L2_8 (76298), SIP_L2_10 (76299), SIP_S1_1 (76281), SIP_S1_2 (76282), SIP_S1_3 (76283), SIP_S1_4 (76284), SIP_S1_5 (76285), SIP_S1_6 (76286), SIP_S1_7 (76287), SIP_S1_9 (76289), SIP_S1_10 (76290), SIP_S2_1 (76300), SIP_S2_2 (76301), SIP_S2_3 (76302), SIP_S2_4 (76303), SIP_S2_5 (76304), SIP_S2_6 (76305), SIP_S2_7 (76306), SIP_S2_8 (76307), SIP_S2_9 (76308), SIP_S2_10 (76309), and five long-sleeping SIP progenitors (LP): DGRP-235 (28275), DGRP-313 (25180), DGRP-335 (25183), DGRP-338 (28173), DGRP-379 (25189), as well as five short-sleeping SIP progenitors (SP): DGRP-38 (28125), DGRP-310 (28276), DGRP-365 (25445), DGRP-808 (28238), DGRP-

832 (28245) were purchased from Bloomington *Drosophila* Stock Center (BDSC), Bloomington, Indiana. *Drosophila* were cultured on cornmeal-molasses media supplemented with yeast, agar, Tegosept, and propionic acid at 25°C and ~70% humidity under a 12-hour light:dark cycle. Virgin flies of each genotype were collected under light CO_2 anesthesia within 8 h of eclosion and housed in a separate vial for 2–3 days to mature.

2.5.2 Fungal strains and longevity bioassays

Metarhizium anisopliae 549 (Ma549) was acquired from the ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) (Ithaca, NY). Ma549 cultures were thawed from −80˚C stock vials and grown for 10 to 14 days at 27 °C on potato-dextrose-agar media plates.

Bioassays using *M. anisopliae* 549 transformed to express green fluorescent protein (Ma549-GFP) were performed according to Lu et al., 2015[19]. Briefly, fly populations were infected at the same time of day and survival was monitored after topical inoculation in groups of nine replicates (~30 flies each) per sex per line across three trials. To prepare inoculum, conidia were suspended in sterile distilled water, vortexed for 2 minutes and filtered through Miracloth (22-25μm) (Andwin Scientific). Flies (two to four days old) were vortexed with spore suspensions (20 ml, $2.5x10^4$ conidia/ml) for 30 seconds, collected by filtering through Miracloth, and transferred into vials containing fly stock food without Tegosept and propionic acid. Flies were cultured at 27°C, ~85% relative humidity. Male and female flies were housed in separate food vials to examine sexual dimorphism and to avoid an interaction between the effect of sex and age in response to infection. Less than 10% of flies vortexed with water alone (mock-infected), or conidial suspensions died within one day, with no significant differences between lines, so flies succumbing within one day post infection were deleted from the infection data. Flies were flipped into fresh vials of food every day. The number of dead flies was recorded

twice per day for 10-14 days. This method was highly reproducible with a mean LT_{50} value for control *Drs*-GFP flies of 4.682 ± 0.029, N = 126[19]. The inoculum load of spores per male and female *Drosophila* was approximately 200 colony forming units.

2.5.3 Fungal colonization of hosts measured by colony forming units (CFUs)

A time course bioassay of fungal growth in the hemolymph of mated and virgin L1_6 and S1_7 females and males was conducted using previously described protocols[19]. Fifteen flies per sex, mating status and line at each time point across three experiments were individually homogenized with 45 µl of 0.1% Tween 80. For resistant lines, the entire 45 µl homogenate was spread onto *Metarhizium* selective medium (Rose Bengal Agar plates supplemented with oxbile, CTAB, oxytetracycline, streptomycin, penicillin, chloramphenicol, and cycloheximide)[121]. For susceptible lines, 5 µl of a 10-fold dilution of the homogenate was spread onto each plate. Colony forming units (CFUs) were counted using the ImageJ Cell Counter up to 7-days incubation at 25°C.

2.5.4 Sleep assays and data analysis

The *Drosophila* Activity Monitor (DAM2) from Trikinetics, Waltham, MA, was used to monitor locomotor activity and analyze sleep patterns in L1_6 and S1_7 flies. Flies were individually housed in 5 x 65 mm monitor tubes containing 5% sucrose and 2% agar after CO_2 anesthesia, with any initial mortalities replaced within 12 hours to minimize acclimation effects. Monitoring occurred over seven days under a 12-hour light/dark (LD) cycle (lights on at 9:00 AM EDT, lights off at 9:00 PM EDT). Sleep data, defined as periods of 5 minutes or more of inactivity[82], were processed using DAMFileScan113 software (Trikinetics) and analyzed using custom software (Insomniac3, RP Metrix, Skillman, NJ; gift of Dr. Julie Williams, University of Pennsylvania). The DAM2 output was converted to total daily, nighttime, and daytime sleep

duration, represented by the number of minutes when a fly was asleep in 24 or 12 hours. Sleep patterns were examined from 12 to 60 hours post-infection with Ma549, spanning two light-dark cycles. This period precedes the onset of most visible symptoms, such as reduced feeding and reproductive behavior, and the upregulation of the immune gene *Drosomycin* in response to Ma549 infection[19, 97]. The sleep profiles of mated L1_6 and S1_7 flies were compared under normal conditions, and infected flies were compared with uninfected peers to assess the impact of infection within each line. Sex-specific differences in sleep and disease outcomes were also investigated along with the influence of mating status on disease resistance and sleep patterns. Sleep differences were quantified using the net minute or percentage differences between the experimental groups (S2.2 Table).

2.5.5 Statistical analyses on longevity and sleep parameters

Statistical analyses were performed using GraphPad Prism 10 software (GraphPad Software, Boston, MA, USA) and JMP® Pro, version 17.1 (SAS Institute Inc., Cary, NC, USA).

Host survival differences were measured as LT_{50} values (lethal time in days at which 50% of the flies had died) calculated using R 4.1.0 (R Core Team 2021). To assess the survival rates of flies following Ma549 infection, we performed Kaplan-Meier survival analysis. Survival curves were generated for each group (L1_6 and S1_7), including variations in sex (male and female) and mating status (mated and virgin). Kaplan-Meier plots were constructed using GraphPad Prism 10, and statistical significance between survival curves was determined using the log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test. Percentage survival and standard errors at all points were calculated and compared across different genotypes and conditions to assess the impact of genetic variation and infection on fly survival.

We assessed the normality of our data using the D'Agostino-Pearson normality test and the Shapiro–Wilk normality test, which have great power properties over a variety of different statistical distributions[122]. For data sets that passed at least one of the normality tests and had statistically similar standard deviations (SDs), we used the unpaired Student's t-test when comparing two groups and one-way ANOVA (Tukey's multiple comparisons) when comparing three or more groups. Otherwise, we applied Welch's correction (Welch's t-test) when comparing two groups and the one-way Welch's ANOVA tests (Dunnett's multiple comparisons) when standard deviations (SDs) were found to be significantly different ($p < 0.05$). For data sets that failed the normality tests, we used the Mann–Whitney U test when comparing two groups and the Kruskal-Wallis test with Dunn's post hoc test when comparing three or more groups. p-values, median ± IQR (interquartile range: the difference between the 75th and 25th percentile) from the Kruskal-Wallis test with Dunn's post hoc multiple comparisons test, and mean ± SEM from ordinary ANOVA or Welch's test were all reported. For all tests, statistical significance was set at $p < 0.05$.

For the ANOVA involving four factors and generating interaction profiles, JMP Pro software was utilized.

2.6 *Supplementary tables*

S2.1 Table: Activity and sleep data in Figure 2.1 and Figure 2.7b, c.

S2.2 Table: Average nighttime and daytime sleep in Figure 2.7d-i and Figure 2.8a-d.

S2.3 Table: Sleep comparisons in Figure 2.7d-i and Figure 2.8a-d.

S2.4 Table: Survival data following Ma549 infection for 38 SIP mated females and males, as well as virgin L1_6 and S1_7 females and males.

S2.5 Table: CFU data in Figure 2.10b-c

S2.6 Table: ANOVA summaries on main effects and interactions.

Table 2.5 Comparison of mean and variation in disease resistance between males and females

Line	t-test for equality of means			Levene's test for equality of variances		Longevity CV_E		
	t	p value	Δmean	F ratio	p value	Male	Female	ΔCV_E
SIP L1_1	12.37	<0.0001	1.08	31.65	<0.0001	24.16	19.75	4.41
SIP L1_2	4.11	<0.0001	0.28	0.63	0.4271	22.16	23.39	-1.22
SIP L1_3	-7.69	<0.0001	-0.79	7.73	0.0056	19.23	20.04	-0.81
SIP L1_4	-13.77	<0.0001	-1.28	4.60	0.0324	26.88	24.52	2.36
SIP L1_5	-7.14	<0.0001	-0.62	1.89	0.1695	21.59	18.82	2.77
SIP L1_6	-15.92	<0.0001	-1.61	28.35	<0.0001	25.23	28.68	-3.45
SIP L1_7	-1.79	0.0746	-0.18	4.85	0.0281	24.83	20.66	4.17
SIP L1_8	-0.27	0.7838	-0.03	0.04	0.8337	23.36	21.21	2.15
SIP L1_9	-10.06	<0.0001	-1.05	0.64	0.4252	23.33	25.11	-1.78
SIP L1_10	-17.54	<0.0001	-1.43	6.35	0.0121	29.40	19.42	9.98
SIP L2_1	-6.96	<0.0001	-0.61	15.06	0.0001	24.63	18.36	6.27
SIP L2_2	3.82	0.0001	0.38	0.37	0.5425	22.69	25.37	-2.67
SIP L2_3	-1.49	0.1371	-0.13	5.15	0.0236	20.61	17.38	3.23
SIP L2_4	10.75	<0.0001	1.28	3.76	0.0531	27.73	27.03	0.70
SIP L2_5	-0.18	0.8534	-0.02	8.91	0.0030	23.05	27.13	-4.07
SIP L2_6	-8.18	<0.0001	-0.72	0.78	0.3761	26.76	21.93	4.83
SIP L2_7	-4.33	<0.0001	-0.47	21.93	<0.0001	19.41	27.86	-8.45
SIP L2_8	2.49	0.0132	0.25	0.14	0.7107	26.30	28.19	-1.89
SIP L2_10	7.66	<0.0001	0.67	4.47	0.0350	23.28	24.04	-0.77
SIP S1_1	4.05	0.0001	0.44	0.51	0.4757	21.47	22.48	-1.01
SIP S1_2	0.67	0.5031	0.08	5.91	0.0154	23.59	21.39	2.20
SIP S1_3	-4.37	<0.0001	-0.44	6.19	0.0131	25.48	20.87	4.62
SIP S1_4	-3.11	0.0020	-0.31	0.20	0.6559	18.53	18.10	0.43
SIP S1_5	1.17	0.2442	0.15	10.25	0.0014	29.41	25.47	3.94
SIP S1_6	3.43	0.0007	0.49	0.17	0.6767	23.60	25.41	-1.82
SIP S1_7	16.67	<0.0001	2.14	8.03	0.0048	26.40	30.27	-3.87
SIP S1_9	-0.11	0.9121	-0.01	0.10	0.7477	24.41	22.84	1.57
SIP S1_10	9.41	<0.0001	1.18	98.51	<0.0001	28.52	18.09	10.43
SIP S2_1	11.70	<0.0001	1.81	7.47	0.0065	28.45	29.80	-1.35
SIP S2_2	10.29	<0.0001	1.10	23.24	<0.0001	25.10	20.53	4.56
SIP S2_3	16.01	<0.0001	2.04	70.08	<0.0001	25.51	19.52	5.99
SIP S2_4	10.11	<0.0001	1.57	6.58	0.0106	25.67	25.37	0.30
SIP S2_5	-4.95	<0.0001	-0.64	1.08	0.2984	26.37	23.67	2.70
SIP S2_6	3.67	0.0003	0.58	12.08	0.0006	36.50	30.88	5.62

SIP S2_7	15.85	<0.0001	2.31	21.37	<0.0001	27.59	27.07	0.52
SIP S2_8	1.75	<0.0001	0.26	16.63	0.0001	29.99	19.94	10.06
SIP S2_9	9.10	<0.0001	1.69	3.26	0.0716	30.26	31.07	-0.81
SIP S2_10	4.47	<0.0001	0.70	5.21	0.0228	29.00	28.49	0.51
Cross strain average CV_E						**25.28**	**23.69**	
				t-test:		**t = 1.822**		**p = 0.0725**

(Δmean = male mean -female mean and ΔCV_E =male CV_E -female CV_E)

Chapter 3: Sleep, sex, and mating status modulate fungal (*Metarhizium anisopliae*) infections in *Drosophila melanogaster*

(Manuscript under revision)

3.1 *Abstract*

Important biological functions, including host defense, are linked to circadian rhythm and sleep. Studies have also indicated that the sex of the host can alter disease processes. In this study, we used a *Drosophila* model to determine how sleep interacts with sex to influence the outcomes of infection with *Metarhizium anisopliae* strain Ma549. We found that, similar to other animals, *Drosophila* slept more after infection. To investigate the mechanisms and functions of this sickness sleep, we tested Ma549 against sleep and clock mutants. The sleep-deprived *Drosophila Shaker* mutant with intact circadian rhythms failed to undergo sickness sleep and succumbed quickly to infection. Mutants with disrupted PERIOD (PER) or CLOCK (CLK) affect circadian rhythms. The per^{01} mutant produced shifted day/night cycles with normal total sleep amounts, whereas the Clk^{Jrk} mutant produced reduced total sleep. We found that while mutations in *per* or *Clk* both impair sickness sleep, only *per* protects against disease, whereas active *Clk* reduces survival in infected *Drosophila,* attributable to it being pleiotropic. Independent of mutant status, males slept more than females, and virgins slept more than mated flies and survived infection longer, indicating that sex and mating status influence sleep and disease resistance.

3.2 *Introduction*

Understanding the interplay between host genetic variation, physiological state, and pathogen resistance is fundamental to understanding the dynamics of host-pathogen interactions.

The fruit fly *Drosophila melanogaster* is a widely used model organism for studying infection and host defense[123, 124]. Approximately 75% of genes responsible for human diseases have homologs in flies[16]. Many important disease-related genes in humans were first discovered in *Drosophila,* and the fly can also serve as a host for diverse human pathogens because of the remarkable conservation of pathogenesis and host defense mechanisms between insects and other animals[17]. Despite this conservation across phylogenetic distances, individual fruit flies and humans vary extensively from each other in their host responses to infectious diseases.

Fungi cause most insect diseases and infect insects by directly penetrating the cuticle using a combination of cuticle-degrading enzymes and mechanical pressure[55, 56]. Conversely, host defenses against specialized entomopathogenic fungi are comparatively understudied. Most studies on fly immunity have tested bacterial or viral pathogens against fly mutants or deployed opportunistic mammalian pathogens injected directly into flies, bypassing several initial host defense barriers[17]. The entomopathogenic fungus used in this study, *Metarhizium anisopliae* ARSEF549 (Ma549), is naturally pathogenic to *Drosophila*[19, 34, 49] and does not, like many other entomopathogen species, produce toxins that inhibit innate immune responses[143]. *Metarhizium spp*. have been used in host-defense studies to provide insights into emerging human pathogens[30, 51-53].

We previously demonstrated that approximately 9% of mutant *D. melanogaster* lines have altered disease resistance to Ma549[19], and that DGRP (*Drosophila* Genetic Reference Panel) flies differ greatly in longevity after Ma549 infection[49]. Our GWAS analysis of DGRP flies revealed a complicated network of immune and physiological genes, particularly sleep-related genes involved in *D. melanogaster* interactions with Ma549[49]. According to this GWAS analysis, a major predictor of how long a wild-caught fly will survive infections is its sleep

pattern[49]. Sleep is an ancient behavior observed throughout the animal kingdom and is essential for survival because prolonged deprivation frequently leads to serious physiological changes. This is true for *Drosophila* as well as higher animals[10, 11]. Despite extensive research, the mechanism by which sleep enhances survival remains unclear. However, one hypothesis is that sleep deprivation has deleterious effects on immune functions[12]. Thus, the level of illness increases in night shift workers who report less sleep than daytime workers[144]. The dominant model to explain natural or healthy sleep that occurs with an approximately 24 h (circadian) periodicity is called the two-process model, whereby sleep duration is regulated by the interaction of two physiological processes: a circadian process tracking time of day and a homeostatic process tracking time awake and time asleep[145]. However, in addition to natural sleep, which occurs daily, several studies have identified sleep as a component of host defense that increases during an acute host response to infection[146]. This sickness sleep is regarded as a cardinal manifestation of sickness behavior, although the mechanisms and functions of sickness sleep are poorly understood[83].

The conventional way to achieve sleep deprivation (SD) is by sensory stimulation[147], and several mechanical devices that achieve SD with shaking have been applied to *Drosophila*[148]. However, the results have been unpredictable and non-reproducible, which may be attributable to damage to the flies and compensatory sleep increases[132, 148-150], which overestimate the role of sleep[147]. An alternative promising approach is genetic SD, which leads to more specifically targeted and robust sleep loss[147]. The development of genetic engineering tools has already led to the use of *Drosophila* models to investigate the role of circadian variation in the ability to fight bacteria[20, 21, 151-153]. Here, we utilized well-characterized sleep mutant lines to examine the role of sickness sleep and circadian clock-controlled genes in fruit fly responses to Ma549. The *period*

(*per*) gene was first described in 1971[154, 155], and along with the equally conserved transcription factor CLOCK, is critical for maintaining the circadian cycle in insects and vertebrates[154, 156]. In flies, CLK, along with another transcription factor CYCLE (CYC) forms the positive branch of the feedback loop, whereas PER and TIMELESS (TIM) form the negative branch[157]. The *Shaker* gene is involved in clock-independent control of sleep and facilitates plasticity of sleep in response to environmental conditions[158]. It encodes several voltage-gated potassium channel α-subunits and plays a key role in controlling neuronal membrane repolarization and transmitter release[159].

The intricate interplay among host genetics, sleep patterns, and immune responses to pathogens is gaining recognition as a critical factor in the health of diverse organisms. Studies in *Drosophila* have shown that mating reduced daytime but not nighttime sleep in uninfected females[115, 160], and have documented sex-based differences or mating effects on immunity against various pathogens[49, 161-163]. Males of most *Drosophila* lineages are more resistant to Ma549 than are females, an effect mediated by immune responses[97]. However, the precise mechanisms underlying these effects, and their dependence on specific pathogens, remain unclear. Elucidating how host variation and sleep interact to influence immune defense against different pathogens, such as Ma549, is essential for developing a more holistic understanding of *Drosophila* immunity.

In the current study, we demonstrated that infected flies increased daytime sleep soon after Ma549 infection, even before other overt signs of disease. Sleep mutants with an inactive *period* or *Shaker* gene had less sickness sleep and died significantly faster after infection, while a mutant with an impaired *Clock* gene showed enhanced survival. We explored the interaction of sleep/circadian genes with sex and mating status. Virgin flies of both sexes exhibited a lower

fungal burden (within-host growth) than mated flies, which was surprising as any trade-offs produced by diverting energy and resources to egg production would not impact males. However, males had a lower fungal burden than females irrespective of mating and mutant status, indicating that sexual dimorphism in disease resistance is not causatively linked with differences in sleep patterns. These findings highlight the complex interplay between sleep, circadian genes, sex, mating, and fungal resistance in *Drosophila*.

3.3 *Results*

3.3.1 Ma549 infection promotes sickness sleep in the wildtype Canton-S line

We used *Drosophila* Activity Monitors (DAM2) following established protocols[82] to analyze daytime and nighttime sleep in various fly lines, including Canton Special (CS) flies, a commonly used laboratory "wild-type" strain, and three sleep-affecting mutant strains, all of which were entrained to 12h:12h light:dark (LD) cycles[82]. The first 12 hours of sleep data were discarded to mitigate the effects of CO_2 anesthesia and other acclimation effects. We monitored sleep-infection interactions from 12 to 60 hours post-infection, including two light-dark cycles. This period is before most flies showed overt symptoms such as reduced feeding and reproductive behavior, and before they up-regulated the immune gene *Drosomycin* in response to Ma549 infection[19, 97].

Consistent with a previous report[164], mated Canton-S males and females had similar amounts of nighttime sleep ($p = 0.7820$, Figure 3.1a-d and S3.1-S3.3a Tables), but females slept 63.8% less than males during the daytime (ZT24-36 and ZT48-60, $p < 0.0001$, Figure 3.1a-d and S3.1-S3.3a Tables). Our initial investigation focused on determining whether infection alters sleep, with the aim of understanding the impact of infection on sleep metrics. Compared to

uninfected CS controls, Ma549 infection significantly increased daytime sleep in mated or virgin females and males between 21.6% and 36.4% ($p \leq 0.0063$, Figure 3.1e-h and S3.2, S3.3c Tables). Following precedent in other animals, including humans, we refer to this increase in sleep following infection as sickness sleep. However, Ma549 infection did not significantly alter nighttime sleep in either sex, regardless of mating status ($p \geq 0.2378$, Figure 3.1e-h and S3.2, S3.3c Tables). These findings indicate that Ma549 infection promotes daytime sickness sleep in the CS line.

3.3.2 Sickness sleep is disrupted in short-sleeping *Shaker* line and clock gene mutants, with variation in survival to Ma549 infection

We investigated three well-characterized mutant fly lines: Sh^{mns}, per^{01}, and Clk^{Jrk}, carrying inactivated *Shaker (Sh)*, *period (per)*, and *Clock (Clk)* genes, respectively. Mated mutant flies were compared with wild-type CS controls to establish and verify baseline sleep profiles under normal conditions. When analyzing the sleep of infected mutants, we compared them with their uninfected peers to accurately assess the impact of infection on sleep within each mutant line. We examined male and female sickness sleep and survival after Ma549 infection to examine potential sex-specific differences in sleep, immune response, and disease outcomes. Additionally, we compared mated and virgin flies to determine whether mating status alters disease resistance and sleep. To quantify the sleep difference between groups within the same fly line, we calculated the net percentage change in sleep duration of group B relative to group A. This involved subtracting group A sleep from group B sleep and then normalizing by dividing by the group A value (S3.3 Table).

Sh^{mns} flies are characterized by a *Shaker* gene mutation that affects the amount of sleep but not circadian regulation[159]. Compared with uninfected CS flies, mated *Sh*^{mns} females (males) slept 74.5% (77.2%) less at night and 26.6% (59.1%) less during the day ($p \leq 0.0407$, Figure 3.1a-d and S3.1, S3.2, S3.3b Tables). This resulted in a significant two-thirds reduction in total sleep compared to wild-type CS flies, consistent with a previous report[159], confirming that the *Sh*^{mns} line had maintained its short sleep phenotype even after several years of lab captivity. Compared to uninfected *Sh*^{mns} flies, Ma549 infection significantly reduced daytime sleep in mated and virgin *Sh*^{mns} females and males by between 38.0% and 40.4% ($p \leq 0.0079$, Figure 3.1e-h and S3.2, S3.3c Tables). However, Ma549 infection did not significantly alter nighttime sleep in either sex, regardless of mating status ($p \geq 0.2599$, Figure 3.1e-h and S3.2, S3.3c Tables). Therefore, while Ma549 infection promoted daytime sleep of the CS line, both mated and virgin *Sh*^{mns} flies showed reduced daytime sleep post-infection compared to their uninfected peers, suggesting a heightened susceptibility to sleep disruption.

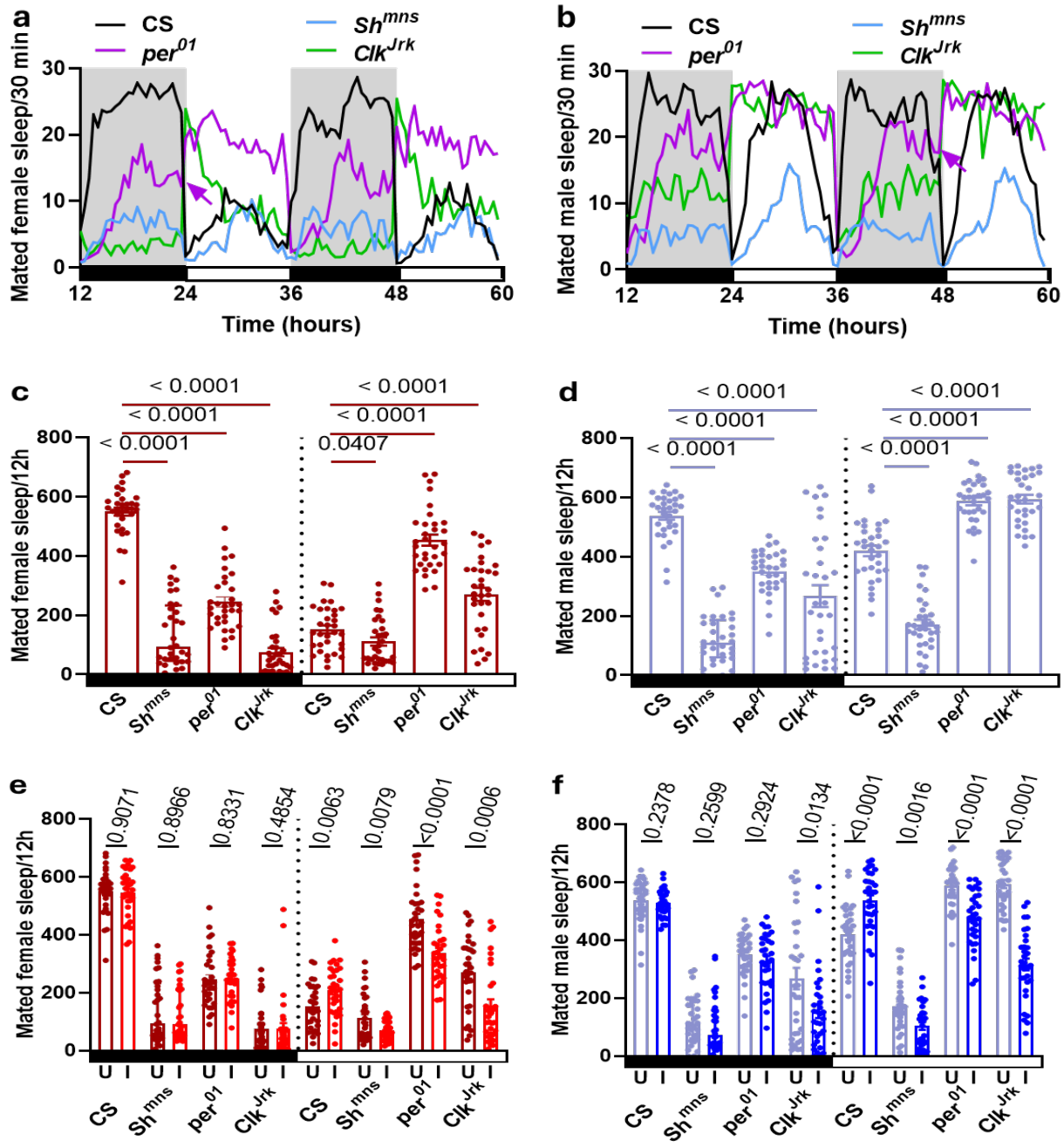
Given the central role of CLOCK and PERIOD in regulating circadian rhythms, using *per*^{01} and *Clk*^{Jrk} mutants provides a unique opportunity to further study the interaction between Ma549 infection and sleep. *Per*^{01} flies harbor a stop codon in *period* that disrupts the circadian cycle in both sexes[155] but they only display complete arrhythmicity in constant darkness[165]. Our results showed that uninfected mated *per*^{01} females (males) had a significant decrease of 55.5% (34.8%) in nighttime sleep and a significant increase of 198.1% (39.7%) in daytime sleep compared to CS flies ($p < 0.0001$, Figure 3.1a-d and S3.1, S3.2, S3.3b Tables). Reduced nighttime and increased daytime sleep evened out, such that total sleep per 24 h was not significantly reduced in the *per*^{01} mutants of either sex. Similar to previous reports[155, 165], we found that *per*^{01} flies exhibited fluctuating sleep patterns because of the loss of the light-off to

light-on transition (arrows shown in Figure 3.1a-b), albeit with periodic behaviors in LD cycles. Compared to uninfected per^{01} flies, Ma549 infection did not significantly alter nighttime sleep of per^{01} ($p \geq 0.2325$, Figure 3.1e-h and S3.2, S3.3c Tables). However, it reduced daytime sleep by between 14.1% and 25.5% in mated and virgin females and males ($p \leq 0.0001$, Figure 3.1e-h and S3.2, S3.3c Tables). Thus, following Ma549 infection, the nighttime sleep of the per^{01} mutants remained unaffected, while daytime sleep was significantly reduced compared to that of uninfected peers, similar to the pattern observed in Sh^{mns} flies.

The Clk^{Jrk} arrhythmic mutant carries a premature stop codon that eliminates much of the activation domain of this transcription factor, and it only expresses low levels of PERIOD and TIMELESS[166]. Similar to a previous report[166], we found that Clk^{Jrk} flies did not react to the lights-on transition (Figure 3.1a-b). Compared to uninfected CS flies, uninfected Clk^{Jrk} females (males) showed an 86.2% (50.3%) reduction in nighttime sleep, but a 77.7% (41.6%) increase in daytime sleep ($p < 0.0001$, Figure 3.1a-d and S3.1, S3.2, S3.3b Tables). Thus, Clk^{Jrk} mutants sleep much less at night and much more during the day, indicating a shift in the day-night sleep cycle. Compared to uninfected Clk^{Jrk} flies, daytime sleep decreased following infection between 36.6% and 46.8% in mated or virgin females and males ($p \leq 0.0006$, Figure 3.1e-h and S3.2, S3.3c Tables). However, nighttime sleep remained unaffected in females ($p \geq 0.4854$, Figure 3.1e-h and S3.2, S3.3c Tables) but showed a significant reduction of approximately 42% in both mated and virgin males ($p \geq 0.0134$, Figure 3.1e-h and S3.2, S3.3c Tables), suggesting a sex-specific effect of disrupting the day-night cycle. Thus, while Ma549 infection primarily affects daytime sleep in Clk^{Jrk} mutants, nighttime sleep is significantly reduced only in males.

Longevity following Ma549 infection varied significantly between the mutant flies and wild-type CS. Compared to infected CS flies, mated (virgin) Sh^{mns} flies of both sexes died

approximately 21.4% (16.3%) faster, and similarly *per*^{01} flies died approximately 22.3% (14.5%) faster ($p < 0.0001$, Figure 3.1i-l and S3.4 Table), showing that active *Shaker* and *per* genes both protect against fungal infection. In contrast, mated (virgin) *Clk*^{Jrk} flies of both sexes outlived wild-type CS flies by approximately 15.8% (13.0%) ($p < 0.0001$, Figure 3.1i-l and S3.4 Table), indicating that a functioning *Clk* gene reduces longevity.



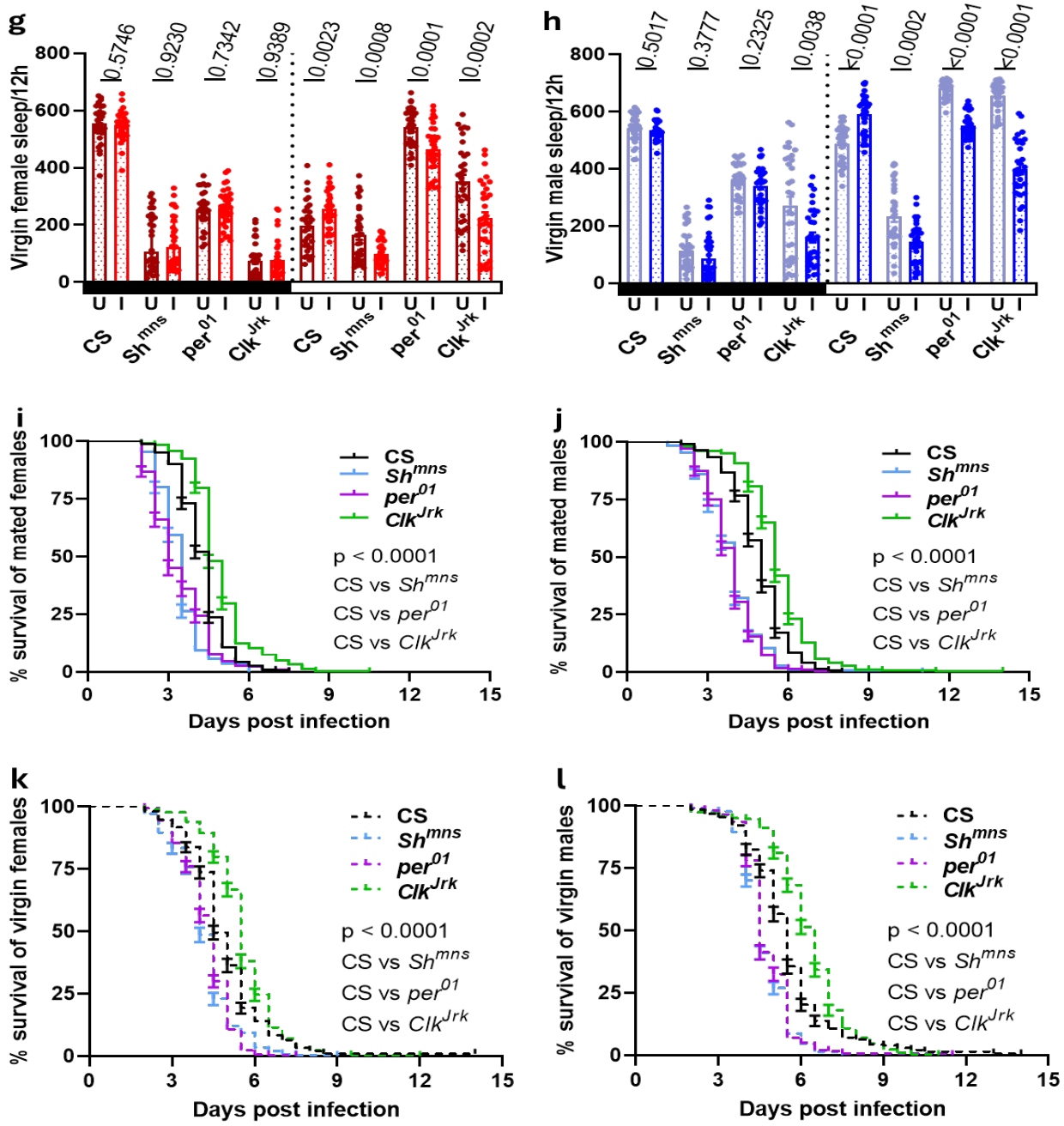


Figure 3.1 Ma549 infection affects sleep and survival. Sleep profiles show sleep in min per 30 min from 12-60 h for 32 flies pooled from three trials for uninfected mated CS/Sh^{mns}/per^{01}/Clk^{Jrk} females (**a**) and males (**b**). Gray shading indicates nighttime. Sleep comparisons included uninfected mated CS versus mutant females (**c**) or males (**d**), as well as uninfected (U) versus infected (I) mated females (**e**), mated males (**f**), virgin females (**g**), and virgin males (**h**) across the CS/Sh^{mns}/per^{01}/Clk^{Jrk} lines. Bars depict nighttime (black) and daytime (white) sleep for uninfected (dark red for females, sky blue for males) and infected (scarlet red for females, navy blue for males) mated and virgin (shaded) flies pooled from three trials. Each data point represents the average sleep/12h of a single fly (n = 32) from 12-60 h post infection. In panels c-d, statistical analyses included the Mann-Whitney U test for nighttime sleep in per^{01} males,

Welch's t-tests for nighttime sleep in Sh^{mns} females and Clk^{Jrk} males, and for daytime sleep in Clk^{Jrk} females. Unpaired Student's t-tests for all other comparisons. In panels e-h, Mann-Whitney U tests for nighttime sleep in mated CS, Sh^{mns} flies, virgin Sh^{mns} females, and Clk^{Jrk} females, and Welch's t-tests for nighttime sleep in virgin CS males, Clk^{Jrk} males, and for daytime sleep in mated Sh^{mns} females, virgin Sh^{mns} flies, and virgin Clk^{Jrk} males. Unpaired Student's t-tests for all other comparisons. The mean ± SEM and p-values are indicated. Differences in survival after Ma549 infection. Kaplan-Meier plots illustrate survival disparities between CS and Sh^{mns}, or per^{01}, or Clk^{Jrk} in mated females (n = 230-321) (**i**), mated males (n = 268-329) (**j**), virgin females (n = 288-346) (**k**), and virgin males (n = 281-345) (**l**). Statistical significance was determined using the log-rank and Wilcoxon tests for intergroup comparisons.

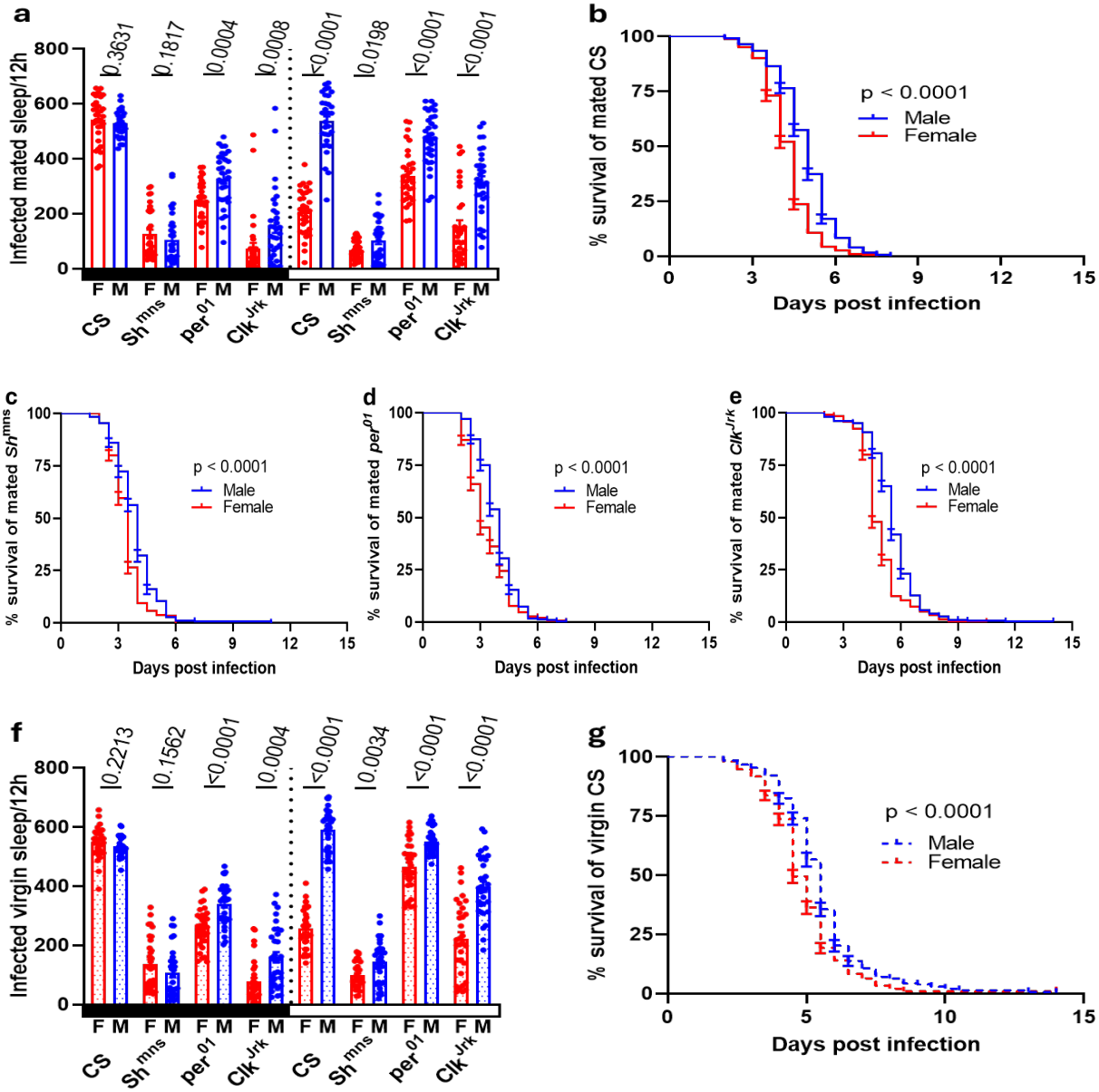
3.3.3 Sexual dimorphism in sleep and longevity in response to Ma549 infection

Next, we specifically examined the effects of sexual dimorphism on sleep and longevity after Ma549 infection. Only males with clock-disrupted per^{01} and Clk^{Jrk} mutations exhibited significantly ($p \leq 0.0008$) more nighttime sleep following infection, increasing their overall sleep compared to that of infected females (Figure 3.2a & f and S3.2, S3.3d Tables). Neither mated nor virgin CS or Sh^{mns} males differed significantly ($p \geq 0.1562$) from females in nighttime sleep (Figure 3.2a & f and S3.2, S3.3d Tables). However, daytime sickness sleep varied according to sex in all lines. Specifically, compared to infected mated (virgin) females, infection increased daytime sleep in mated (virgin) males by 158.8% (130.4%) in CS, 49.4% (46.3%) in Sh^{mns}, 40.9% (18.5%) in per^{01}, and 105.1% (78.6%) in Clk^{Jrk} ($p \leq 0.0198$, Figure 3.2a & f and S3.2, S3.3d Tables). Thus, as previously stated, Ma549 infection reduced daytime sleep in mutant strains but enhanced it in the CS line (Figure 3.1e-h and S3.2, S3.3c Tables). However, these results also show that infected male flies sleep significantly more than female flies, highlighting sex-specific differences in sleep responses to infection and variations across different genetic strains.

Interestingly, differences in sleep patterns between males and females translated into differences in longevity. Mated (virgin) males in the CS, Sh^{mns} per^{01}, and Clk^{Jrk} lines outlived

mated (virgin) females by 15.0% (13.9%), 15.8% (12.7%), 16.6% (13.2%), and 14.3% (12.6%), respectively ($p < 0.0001$, Figure 3.2b-e, Figure 3.2g-j, and S3.4 Table).

Taken together, our findings suggest that males, irrespective of mating status and mutant line, exhibit longer sickness sleep and greater disease resistance than females following Ma549 infection.



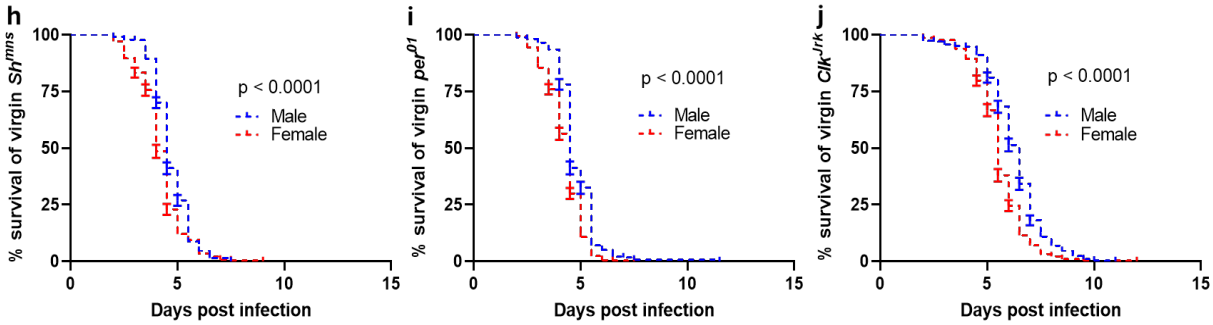
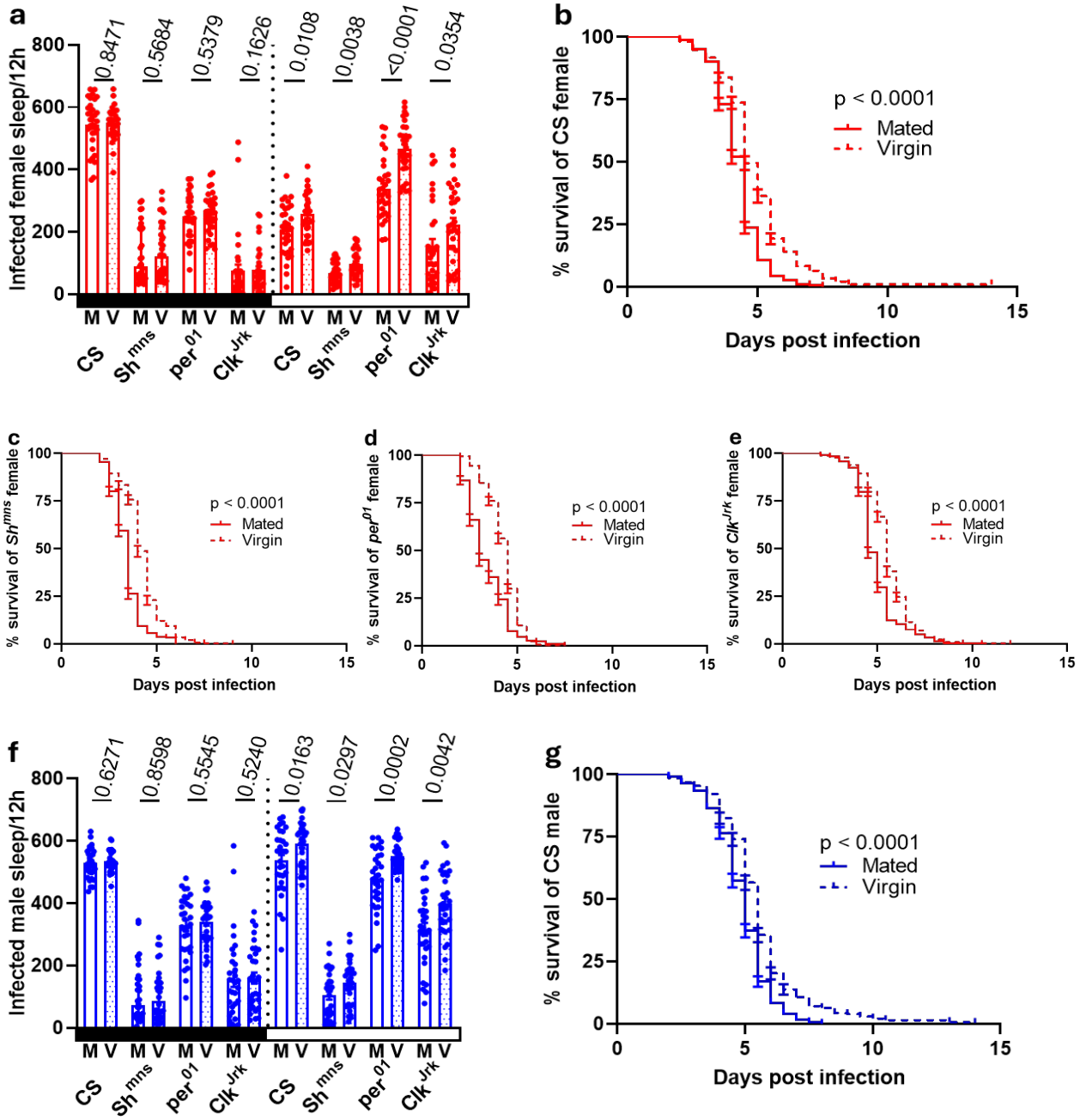


Figure 3.2 Sexual dimorphism in sleep and longevity against Ma549 infection in CS, Sh^{mns}, per^{01}, and Clk^{Jrk} flies. Comparison of sickness sleep between females and males in mated (**a**) and virgin (**f**) groups. Each data point represents the average sleep/12 h of a single fly (n = 32 flies) during nighttime and daytime from 12-60 h post infection pooled from three trials. Statistical significance was assessed for comparisons between the female and male groups within CS, Sh^{mns}, per^{01}, and Clk^{Jrk} lines. For nighttime sleep, Welch's t-test was used for mated and virgin CS, and virgin Clk^{Jrk}, with the Mann-Whitney U test for mated Sh^{mns} and Clk^{Jrk}. For daytime sleep, Welch's t-test was used for mated and virgin Sh^{mns}, and virgin per^{01}. The unpaired Student's t-test was used for all other comparison groups. The mean ± SEM and p-values are indicated. Black (white) rectangles on the x-axis indicate nighttime (daytime), whereas shaded bars denote virgin flies. Line Abbreviations: F for females and M for males. Survival differences between males and females after Ma549 infection. Kaplan-Meier plots illustrate the survival disparities between females (n = 230-346) and males (n = 268-345) of mated CS (**b**), Sh^{mns} (**c**), per^{01} (**d**), Clk^{Jrk} (**e**), and virgin CS (**g**), Sh^{mns} (**h**), per^{01} (**i**), Clk^{Jrk} (**j**). Statistical significance at $p < 0.0001$ was determined using both the log-rank test and Wilcoxon test for comparisons between two groups.

3.3.4 Impact of mating status on sleep and survival in flies following Ma549 infection

Next, we analyzed the data with the goal of disentangling the effect of mating status on fly sleep and survival following Ma549 infection. Mating status did not affect the nighttime sleep of infected CS, Sh^{mns}, per^{01}, and Clk^{Jrk} females or males ($p \geq 0.1626$, Figure 3.3a & f and S3.2, S3.3e Tables). However, mating significantly reduced daytime sleep across all strains irrespective of sex and infection status. Specifically, infection reduced daytime sleep in mated females (males) by 19.2% (9.2%) in the CS line, 29.5% (28.0%) in Sh^{mns}, 27.5% (13.8%) in per^{01}, and 30.8% (20.6%) in Clk^{Jrk} ($p \leq 0.0354$, Figure 3.3a & f and S3.2, S3.3e Tables).

After infection, virgin females (males) outlived their mated peers by 16.7% (15.6%) in CS, 25.4% (22.0%) in *Sh*^{mns}, 29.7% (25.9%) in *per*^{01}, and 14.2% (12.5%) in *Clk*^{Jrk} ($p < 0.0001$, Figure 3.3b-e & 3g-j and S3.4 Table). These findings reveal a notable impact of mating status on the regulation of sleep and immunity in flies, although these effects are independent of mutant status.



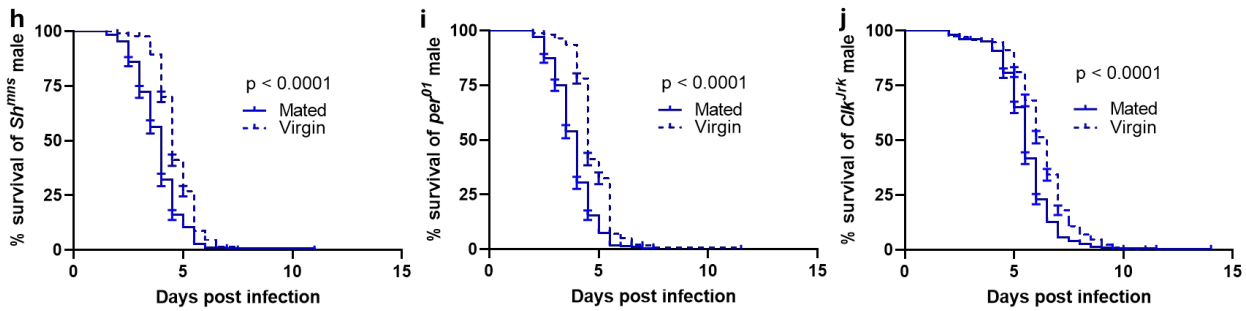


Figure 3.3 Mating status affects sleep and longevity following Ma549 infection in CS, Sh^{mns}, per^{01}, and Clk^{Jrk} flies. Differences in nighttime and daytime sleep between mated and virgin flies in the infected female (**a**) and male (**f**) groups. Each data point represents the average sleep per 12 h of a single fly (n = 32 flies) during the nighttime and daytime from 12-60 h post infection. Statistical significance was assessed for comparisons between mated and virgin females or males within the CS, Sh^{mns}, per^{01}, and Clk^{Jrk} fly lines. For nighttime sleep, the unpaired Student's t-test was used for infected per^{01} females and males, with Welch's t-test for infected CS females and males. The Mann-Whitney U test was used for all other comparison groups. For daytime sleep, Welch's t-test was used for infected CS and per^{01} males, and the unpaired Student's t-test was used for all other comparison groups. The mean ± SEM and p-values are indicated. Black (white) rectangles on the x-axis indicate nighttime (daytime), whereas shaded bars denote virgin flies. Line Abbreviations: V for virgin and M for mated. Survival differences between mated and virgin flies after Ma549 infection. Kaplan-Meier plots illustrate the survival disparities between mated (n = 230-329) and virgin (n = 281-346) flies of CS (**b**), Sh^{mns} (**c**), per^{01} (**d**), and Clk^{Jrk} females (**e**), as well as CS (**g**), Sh^{mns} (**h**), per^{01} (**i**), and Clk^{Jrk} (**j**) males. Statistical significance at $p < 0.0001$ was determined using both the log-rank test and Wilcoxon test for comparisons between two groups.

3.3.5 Effects of line, sex, mating status, and infection on sleep

Next, we used analysis of variance (ANOVA) to investigate the interplay of genetic line, sex, mating status, and infection on nighttime (Figure 3.4a) and daytime (Figure 3.4b) sleep (Table 3.1 generated from data in S3.2 Table). Both night and daytime sleep patterns exhibited highly significant main effects of genetic line ($p < 0.0001$, Table 3.1 and Figure 3.4), confirming that CS, Sh^{mns}, per^{01} and Clk^{Jrk} showed distinct sleep behaviors. Males and females differed significantly ($p < 0.0001$) in both night and daytime sleep durations (Table 3.1 and Figure 3.4), consistent with sexual dimorphism in sleep patterns. Infection status significantly affected both night and daytime sleep ($p \leq 0.0003$, Table 3.1 and Figure 3.4), underscoring the role of infection

in altering sleep across different times of the day. However, the effect of mating status on sleep patterns diverged between the night and daytime. Although mating status had no significant effect on nighttime sleep ($p = 0.3739$, Table 3.1 and Figure 3.4), it significantly affected daytime sleep ($p < 0.0001$, Table 3.1 and Figure 3.4). Both night and daytime sleep exhibited significant ($p \leq 0.0072$) interactions between line and sex, line and infection, and sex and infection, although the interactions, reflected in F-values, were much greater during the daytime (Table 3.1 and Figure 3.4). A three-way interaction among line, sex, and infection was also significant during both day and nighttime sleep ($p \leq 0.0019$, Table 3.1 and Figure 3.4), demonstrating that the influence of one of these factors on sleep patterns can be significantly modified by the presence of the other factors.

Table 3.1 Effects of line, sex, mating status, and infection as well as their interactions on night and daytime sleep

Source	Nparm	DF	Nighttime			Daytime		
			Sum of squares	F ratio	p values	Sum of squares	F ratio	p values
line	3	3	28548460	1114.806	<0.0001	18415088	782.8967	<0.0001
sex	1	1	608595	71.2962	<0.0001	8357700	1065.955	<0.0001
mating	1	1	6755	0.7913	0.3739	1158987	147.8191	<0.0001
Infection	1	1	112770	13.2109	0.0003	1298460	165.6077	<0.0001
line*sex	3	3	1199024	46.8214	<0.0001	2495510	106.0938	<0.0001
line*mating	3	3	2677	0.1045	0.9574	98815	4.2010	0.0058
line*Infection	3	3	103486	4.0411	0.0072	2619158	111.3505	<0.0001
sex*mating	1	1	29	0.0034	0.9536	48	0.0061	0.9378
sex*Infection	1	1	87357	10.2338	0.0014	77719	9.9124	0.0017
mating*Infection	1	1	191	0.0224	0.8812	484	0.0617	0.8038
line*sex*mating	3	3	842	0.0329	0.9920	13549	0.5760	0.6309
line*sex*Infection	3	3	128490	5.0175	0.0019	320912	13.6432	<0.0001

line*mating*Infection	3	3	138	0.0054	0.9995	10194	0.4334	0.7291
sex*mating*Infection	1	1	5.0625	0.0006	0.9806	1418	0.1809	0.6707
line*sex*mating*Infection	3	3	401	0.0157	0.9973	17772	0.7556	0.5192

Source: This is a list of the tested factors and interactions. Nparm: Number of parameters associated with the effect. DF: degrees of freedom for each source of variation. Sum of Squares: sum of squares (SS) for each source of variation, along with the total from all sources. F Ratio: the mean square of the factor divided by the mean square of the error. A p-value less than 0.05 means that the factor or interaction has a statistically significant effect.

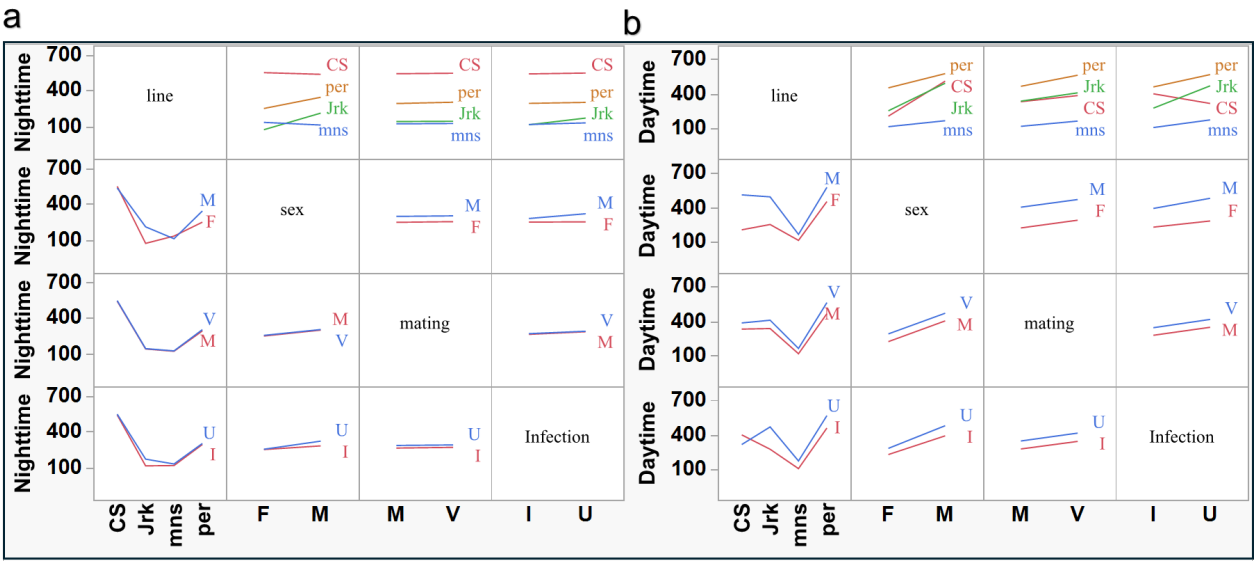


Figure 3.4 Interaction profiles of line, sex, mating status, and infection status on nighttime (**a**) and daytime (**b**) sleep, covering four genetic lines (CS, Sh^{mns}, per^{01} and Clk^{Jrk}), both sexes (males and females), two mating statuses (mated and virgin), and two infection statuses (infected and uninfected). These profiles were generated from the sleep data of 1,024 individual flies. Abbreviations: sex (F = female, M = male), mating status (M = mated, V = virgin), and infection status (I = infected, U = uninfected).

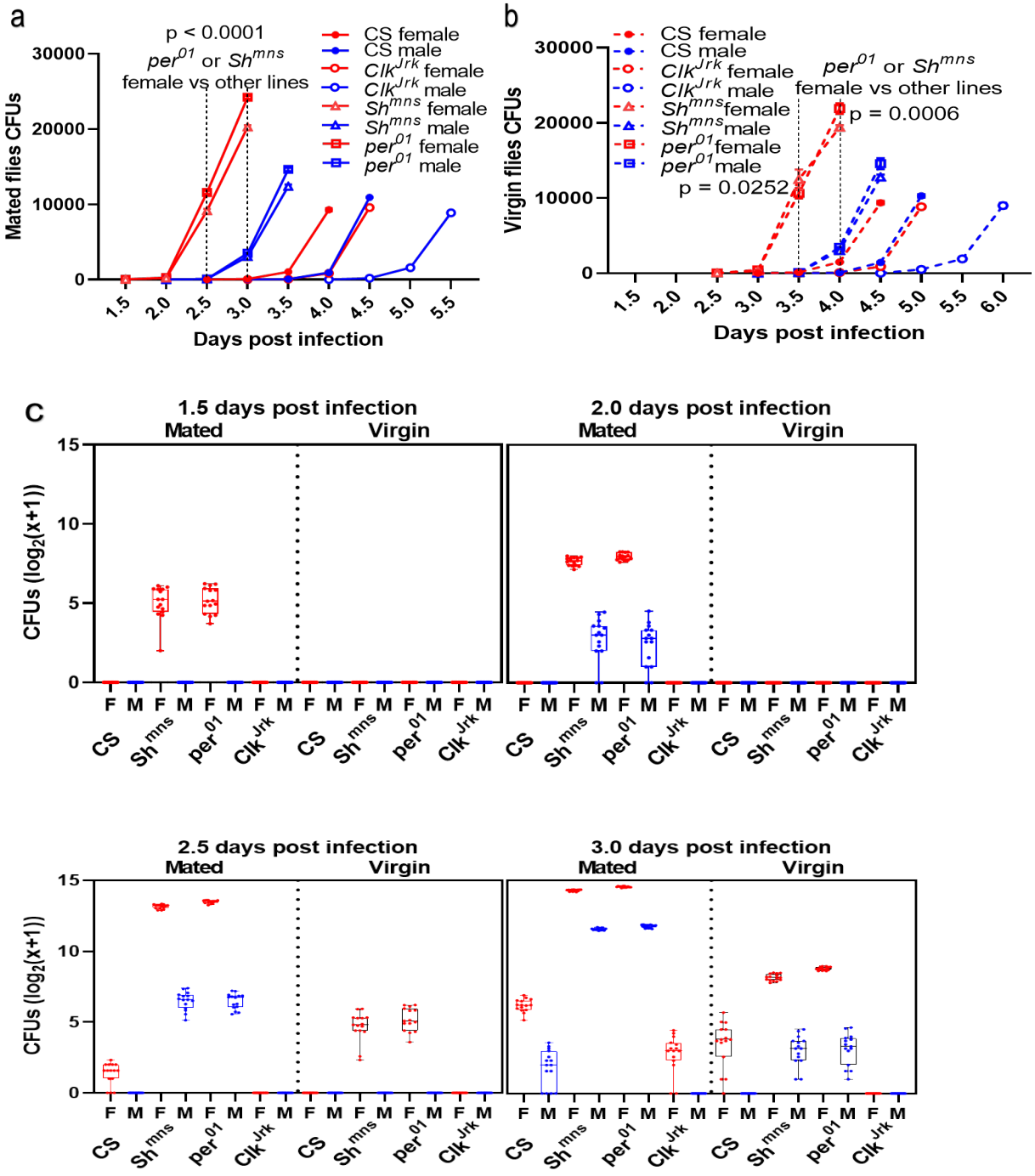
3.3.6 The impact of natural host variation and sleep mutants on fungal fitness

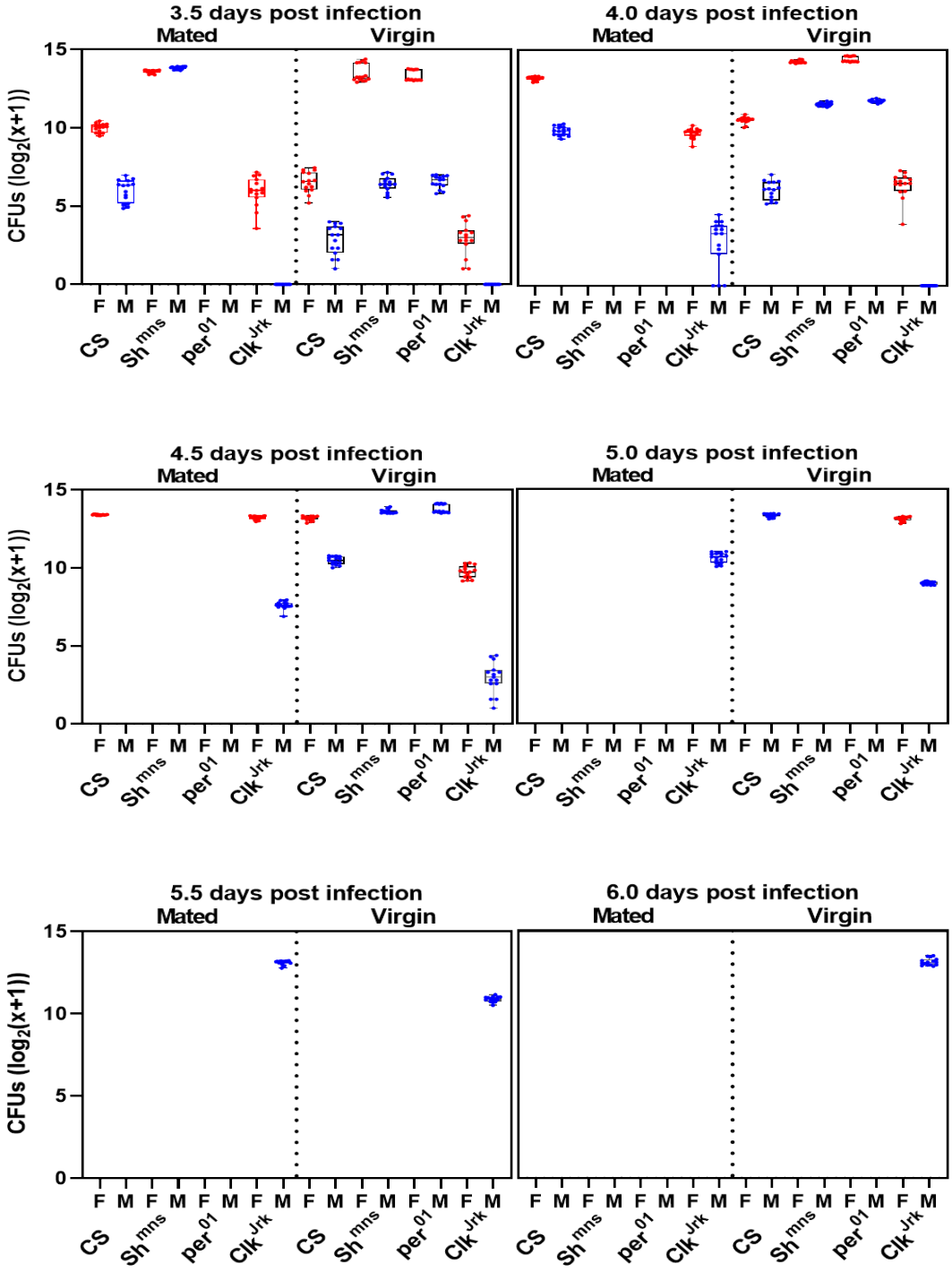
To investigate the impact of host variation on fungal fitness, we compared within-host growth [fungal load, measured as colony forming units (CFU)] between the CS and sleep mutant lines. The time course of CFU counts showed that irrespective of mating status, CS and Clk^{Jrk} flies delayed fungal growth compared to the more susceptible Sh^{mns} and per^{01} flies, although CS flies exhibited higher fungal loads than the more resistant Clk^{Jrk} flies (Figure 3.5a-c generated

from data in S3.5 Table). CFUs/fly ranged from ≤ 8.4 ± 1.5 in virgin male Clk^{Jrk} flies at day 4.5 to ≥ 9149.0 ± 244.7 in the susceptible lines at day 2.5, showing that the susceptible sleep mutant lines, particularly if they were female and mated, were much less able to restrain fungal growth. CFUs appeared 1.5 days post-infection in the particularly susceptible mated per^{01} and Sh^{mns} females, with an average of 40.5 ± 5.6 and 38.2 ± 5.2 CFUs/fly, respectively. In contrast, CFUs appeared 2.5 days post-infection in wild-type CS females, but remained at low levels (< 5 per fly) until day 3. CFUs appeared 4.5 days post infection in the most resistant virgin Clk^{Jrk} males, but remained ≤ 20 per fly until day 5. In all four lines, fungal loads only climbed rapidly when flies were close to death, indicative of host resistance breaking down.

Irrespective of mating status, males exhibited a lower fungal load than females, with proliferation delayed approximately 0.5-1 day (Figure 3.5a-c), aligned with LT_{50} values (Figure 3.2b-e, g-j). Likewise, proliferation was delayed in virgin flies by approximately 0.5-1 day relative to mated flies (Figure 3.5b, c), also aligned with LT_{50} values (Figure 3.3b-e, g-j). ANOVA revealed significant differences in fungal loads on days 2.5 and 3.0 between mated per^{01} or Sh^{mns} females and mated females from other lines, as well as all males across the four lines studied ($p < 0.0001$, Figure 3.5a). Additionally, significant differences were found on days 3.5 and 4.0 between virgin per^{01} or Sh^{mns} females and virgin females from other lines, along with all males across the four lines studied ($p \leq 0.0252$, Figure 3.5b). In addition, Pearson correlation analyses demonstrated that the LT_{50} values (S3.4b Table) positively correlated with both the onset (initial time point at which fungal growth becomes detectable) and peak time (time point maximum fungal growth achieved) of CFUs ($r = 0.9871$, $p < 0.0001$, Figure 3.5d-e) across mated and virgin CS, Sh^{mns}, per^{01}, and Clk^{Jrk} females and males. Thus, flies with delayed onset or peak times of fungal load survived longer.

These results indicate that the fungal load in the hemocoel is affected by the fly's genetically determined ability to regulate sleep, and proliferation is coincident with and presumably causes death; that is, the flies cannot tolerate high levels of fungi (Figure 3.5).




3.5 days post infection
4.0 days post infection
4.5 days post infection
5.0 days post infection
5.5 days post infection
6.0 days post infection
Mated
Virgin
CFUs (log2(x+1))
0
5
10
15
F M F M F M F M F M F M F M F M
CS
Sh mns
per 01
Clk Jrk

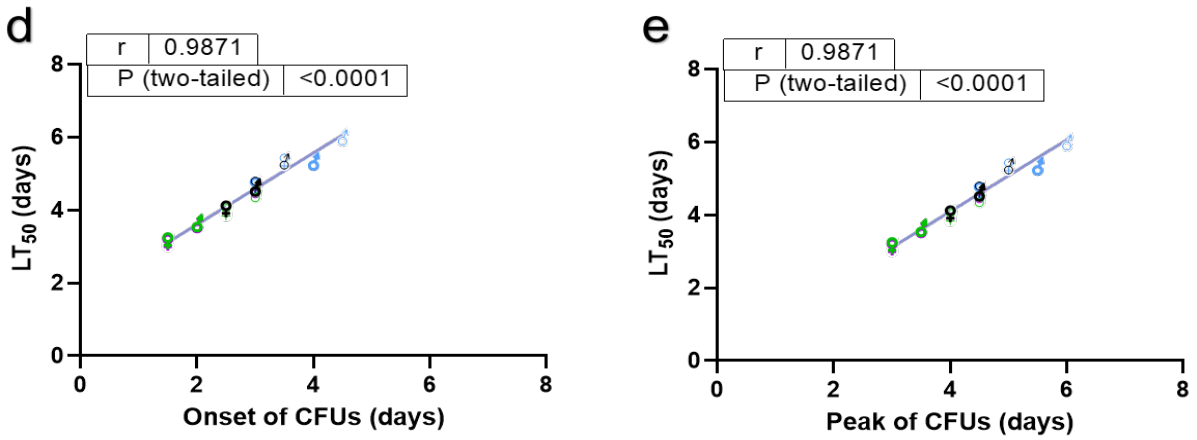


Figure 3.5 Time course of CFU (colony forming units) production in mated (**a**) and virgin (**b**) CS and mutant flies following Ma549 infection. Flies were individually homogenized and plated at 12-hour intervals until death. Each data point represents the average of CFU values of one of the CS, *Sh*^{mns}, *per*^{01}, and *Clk*^{Jrk} lines from 15 flies, with five replicates each, per sex, mating status, line, and time point across three experiments. Means ± SEM and p-values were obtained from two-way ANOVA and are indicated. Time course of log-transformed Ma549 fungal loads in the hemolymph of sleep mutants and their corresponding isogenic WT backgrounds (**c**). Results are depicted as box plots for each time point, with the mean ± SEM of CFUs presented for 15 individual replicates per sex, mating status, line, and time point across three repeated experiments. Correlations between LT_{50} values and CFU onset (**d**) or peak (**e**) were analyzed for infected mated and virgin CS (black symbols), *Sh*^{mns} (bright green symbols), *per*^{01} (electric purple symbols), and *Clk*^{Jrk} (dodger blue symbols) females (♀) and males (♂). Pearson correlation coefficients (r) and p-values indicated.

3.4 *Discussion*

Infection, mating, and sex are critical factors that intricately influence sleep and survival in various species. Infection often triggers an immune response that can significantly alter sleep patterns. For instance, an infection can induce sleep to conserve energy and prioritize the body's resources toward fighting off pathogens[83]. This adaptive change in sleep architecture serves as a vital mechanism for enhancing survival during periods of illness[167]. On the other hand, mating and sexual behaviors are energy-intensive activities that can disrupt regular sleep schedules and immune functions[161]. During mating seasons, many species exhibit reduced sleep duration and altered sleep quality, as they prioritize reproductive efforts over rest[115, 160]. However, the balance

between sleep and reproductive activities is crucial, as insufficient sleep can impair cognitive and physical performance, thus reducing survival prospects. The interplay of these factors highlights a complex evolutionary trade-off in which organisms must navigate the demands of immune function, reproductive success, and restorative sleep to optimize their overall fitness and survival.

Our study provides a comprehensive analysis of the interplay between sleep-regulating genes, mating status, and pathogen resistance in *Drosophila melanogaster*. We observed significant differences in fungal loads and survival rates among sleep and circadian rhythm mutants compared to wild-type CS flies. Notably, Clk^{Jrk} mutants were the most able to repress fungal proliferation and survived the longest, whereas the Sh^{mns} and per^{01} mutants showed increased susceptibility. Additionally, virgin flies survived the infection longer than mated flies, which aligned with delayed fungal proliferation. Males also exhibited lower fungal loads and outlived females, highlighting sex-specific differences in the immune responses.

Drosophila exhibits a circadian rest-activity cycle, and continuous sleep deprivation results in death[9-11]. The mechanism(s) by which sleep enhances survival is not well understood, but one plausible explanation is that sickness sleep expedites organismal recovery by switching resources from waking activities to fighting off the infection and repairing cells[83]. Most studies of the impact of sleep on illness have examined the effects of sleep deprivation[12, 136]. The impact of Ma549 infection on enhancing sleep in wild-type flies may provide an alternative approach that examines the active function of sleep, rather than the effect of a lack of sleep.

A striking feature of our mutant study was that per^{01} flies succumbed rapidly to infection, whereas Clk^{Jrk} flies outlived WT flies. This is despite the fact that both the mutants block

sickness sleep. Similar results were obtained by stab-inoculating *Pseudomonas aeruginosa* into the same per^{01} and Clk^{Jrk} mutants[20, 135, 151]. This suggests that PERIOD function has a protective role in *Drosophila* against both pathogenic bacteria and fungi, while CLK undermines this role. We previously found that resistance to Ma549 in the DGRP was correlated with defense against *P. aeruginosa*, although in contrast to Kuo et al., 2010[135], we fed the bacteria to flies to mimic natural infections[49]. Bacteria and fungi evoke the IMD and Toll defense pathways, respectively, so we postulated that the correlation was due to a generalized multipurpose defense response to multiple pathogens[49]. The conservation of the response to *per* and *Clk* by bacteria and fungi suggests that this generalized defense includes circadian regulation. Kuo et al., 2010[135] suggested that clock mutants may differ in their effects on survival due to CLK having additional roles besides timekeeping in fighting microbial infections. In addition to promoting *per* and *tim* expression, CLK drives the rhythmic expression of approximately 150 downstream genes[168] that regulate other physiological processes[169]. The fact that Clk^{Jrk} mutants were the most able to repress fungal proliferation suggests that these processes are involved in immune responses. Previous studies have shown that repression of clock-controlled genes (CCGs) can enhance defense; for example, the CCG *Achilles* represses the expression of antifungal peptides[153], whereas the antimicrobial peptide NEMURI prolongs sleep and promotes survival after bacterial infection[167]. Other factors may also contribute to immune gene activation and deleterious effects. For example, excessive or inappropriate immune activation against *Metarhizium* spp. can shorten the lifespan of insects[170].

The Sh^{mns} mutation deployed in our study knocks out the evolutionarily conserved voltage-gated potassium channel *Shaker (Sh)*[159], and promotes sleep through GABA transmission[158]. Our data confirmed that uninfected Sh^{mns} mutants had significantly reduced

sleep compared to WT while maintaining circadian rhythms. However, infected Sh^{mns} flies did not sustain daytime sickness sleep and succumbed faster than WT flies, supporting our overall hypothesis that sickness sleep enhances survival.

Furthermore, our results underscore the influence of physiological state on immune competence. Males had a lower fungal burden (within-host growth) than females, indicating sex-specific variation in immune investment strategies. Virgin flies slept longer than mated flies, and were better able to delay fungal proliferation, suggesting that they were more resistant. Trade-offs produced by diverting energy and resources to egg production are known to affect disease resistance and sleep[115-117]. However, mating status also affected males. When male and female *Drosophila* are in close proximity, they are highly active and lose significant sleep[118]. This may explain our data, particularly because increased sexual activity in male *Drosophila* reduces their ability to clear bacterial infections[161].

In conclusion, our study highlights the critical roles of sleep and circadian rhythm genes in modulating the immune responses in *Drosophila*. We demonstrated that fungal infection in WT flies increases sickness sleep; however, knocking out the pivotal *Shaker* gene with normal circadian rhythms eliminates this effect. The circadian-disrupted clock gene per^{01} has an effect similar to that of Sh^{mns} on survival; however, Clk^{Jrk} undercuts this role possibly because it is pleiotropic[168]. Our findings also emphasize the importance of considering sex dimorphism and mating status in host-pathogen interactions. We previously demonstrated that some mutant *Drosophila* lines with disrupted components of the Toll and Imd immune pathways lose sex dimorphism or even reverse dimorphism, with females becoming more disease resistant[97]. Mutants with disrupted sleep and circadian rhythm genes did not reproduce this, but daytime

sleep in particular exhibited significant interactions with genetic lines, sex, and disease resistance. We conclude that the interplay between circadian regulation, sleep patterns, and immune function shapes the *Drosophila* resistance to fungal pathogens, along with genetic variation and physiological state.

3.5 *Materials and Methods*

3.5.1 Fly stocks and virgin fly collection

per^{01}(80928), *Clk*^{Jrk} (24515), *Sh*^{mns} (24149), and Canton-S (64349) flies were purchased from Bloomington *Drosophila* Stock Center (BDSC), Bloomington, Indiana. *Drosophila* were cultured on cornmeal-molasses media supplemented with yeast, agar, Tegosept, and propionic acid at 25°C and ~70% humidity under a 12-hour light:dark cycle. Virgin flies of each genotype were collected under light CO_2 anesthesia within 8 h of eclosion and housed in a separate vial for 2–3 days to mature.

3.5.2 Fungal strains and longevity bioassays

Metarhizium anisopliae 549 (Ma549) was acquired from the ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) (Ithaca, NY). Ma549 cultures were thawed from −80˚C stock vials and grown for 10 to 14 days at 27 °C on potato-dextrose-agar media plates.

Bioassays using *M. anisopliae* 549 transformed to express green fluorescent protein (Ma549-GFP) were performed according to Lu et al., 2015[19]. Briefly, fly populations were infected at the same time of day and survival was monitored after topical inoculation in groups of nine replicates (25-40 flies each) per sex per line across three trials. To prepare inoculum, conidia were suspended in sterile distilled water, vortexed for 2 minutes and filtered through

Miracloth (22-25µm) (Andwin Scientific). Flies (two to four days old) were vortexed with spore suspensions (20 ml, $2.5x10^4$ conidia/ml) for 30 seconds, collected by filtering through Miracloth, and transferred into vials containing fly stock food without Tegosept and propionic acid. Flies were cultured at 27°C, ~85% relative humidity. Male and female flies were housed in separate food vials to examine sexual dimorphism and to avoid an interaction between the effect of sex and age in response to infection. Less than 10% of flies vortexed with water alone (mock-infected), or conidial suspensions died within one day, with no significant differences between lines, so flies succumbing within one day post infection were deleted from the infection data. Flies were flipped into fresh vials of food every day. The number of dead flies was recorded twice per day for 10-14 days. This method was highly reproducible with a mean LT_{50} value for control *Drs*-GFP flies of 4.682 ± 0.029, N = 126[19]. The inoculum load of spores per male and female *Drosophila* was approximately 200 colony forming units.

3.5.3 Fungal colonization of hosts measured by colony forming units (CFUs)

A time course bioassay of fungal growth in the hemolymph of mated and virgin CS and sleep-mutant females and males was conducted using previously described protocols[19]. Fifteen flies per sex, mating status and line at each time point across three experiments were individually homogenized with 45 µl of 0.1% Tween 80. For resistant lines, the entire 45 µl homogenate was spread onto *Metarhizium* selective medium (Rose Bengal Agar plates supplemented with oxbile, CTAB, oxytetracycline, streptomycin, penicillin, chloramphenicol, and cycloheximide)[121]. For susceptible lines, 5 µl of a 10-fold dilution of the homogenate was spread onto each plate. Colony forming units (CFUs) were counted using the ImageJ Cell Counter up to 7-days incubation at 25°C.

3.5.4 Sleep assays and data analysis

Locomotor activity was monitored using *Drosophila* Activity Monitors (DAM2 Trikinetics, Waltham MA). Following CO_2 anesthesia, 32 flies per sex, mating status and line per uninfected control or treatment group were individually loaded into 5 x 65 mm monitor tubes (Trikinetics, Waltham MA) containing 5% sucrose and 2% agar. To mitigate the effects of CO_2 anesthesia, or any other potential acclimation effects, any dead flies within the first 12 hours were discarded and replaced prior to data collection. Flies were observed for seven days in DAM2 monitors synchronized to a 12 hr light: 12 hr dark (LD) cycle with lights on at 9:00 AM EDT (Zeitgeber time = 0 h) and lights off at 9:00 PM EDT (Zeitgeber time = 12 h). Sleep data was obtained from 32 flies pooled from three experiments. Locomotor activity is recorded as infrared beam crossings in one-minute bins in DAM2 monitors. *Drosophila* sleep is defined as periods of 5 min or more of inactivity[82]. Sleep and activity parameters were collected with the DAM308 system and pre-processed by DAMFileScan113 softwares (Trikinetics), and further analyzed using custom software (Insomniac3, RP Metrix, Skillman, NJ; gift of Dr. Julie Williams, University of Pennsylvania). The DAM2 output was converted to total daily, nighttime, and daytime sleep duration represented by the number of minutes when a fly is asleep in 24 or 12 hours.

3.5.5 Statistical analyses on longevity and sleep parameters

Statistical analyses were performed using GraphPad Prism 10 software (GraphPad Software, Boston, MA, USA) and JMP® Pro, version 17.1 (SAS Institute Inc., Cary, NC, USA).

Host survival differences were measured as LT_{50} values (lethal time in days at which 50% of the flies had died) calculated using R 4.1.0 (R Core Team 2021). To assess the survival

rates of flies following Ma549 infection, we performed Kaplan-Meier survival analysis. Survival curves were generated for each group (CS, Sh^{mns}, per^{01}, and Clk^{Jrk}), including variations in sex (male and female) and mating status (mated and virgin). Kaplan-Meier plots were constructed using GraphPad Prism 10, and statistical significance between survival curves was determined using the log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test. Percentage survival and standard errors at all points were calculated and compared across different genotypes and conditions to assess the impact of genetic variation and infection on fly survival.

We assessed the normality of our sleep data using the D'Agostino-Pearson normality test and Shapiro–Wilk normality test, which have great power properties over a variety of different statistical distributions[122]. For data sets that passed at least one of the normality tests and had statistically similar standard deviations (SDs), we used the unpaired Student's t-test when comparing two groups and one-way ANOVA (Tukey's multiple comparisons) when comparing three or more groups. Otherwise, we applied Welch's correction (Welch's t-test) when comparing two groups and the one-way Welch's ANOVA tests (Dunnett's multiple comparisons) when standard deviations (SDs) were found to be significantly different ($p < 0.05$). For data sets that failed the normality tests, we used the Mann–Whitney U test when comparing two groups and the Kruskal-Wallis test with Dunn's post hoc test when comparing three or more groups. We report p-values, median ± IQR (interquartile range: the difference between the 75th and 25th percentiles) from the Kruskal-Wallis test with Dunn's post hoc multiple comparisons test, and means ± SEM from ordinary ANOVA or Welch's test. For all tests, statistical significance was set at $p < 0.05$.

For the ANOVA involving four factors and generating interaction profiles, JMP Pro software was utilized.

3.6 *Supplementary tables*

S3.1 Table: Sleep profiles in Figure 3.1a-b.

S3.2 Table: Average nighttime and daytime sleep in Figure 3.1c-h, Figure 3.2a and f, Figure 3.3a and f, Figure 4, and Table 3.1.

S3.3 Table: Sleep analyses in Figure 3.2a and f, Figure 3.3a and f.

S3.4 Table: Survival data following Ma549 infection for mutant females and males in Figure 3.1i-l, Figure 3.2b-e, g-j, and Figure 3.3b-e, g-j, and Figure 3.5d-e.

S3.5 Table: CFU data in Figure 3.5a-e.

Conclusion

Over one billion people globally are infected by fungal diseases with more than 150 million severe cases, and an estimated 1.7 million deaths each year[26]. The fruit fly, *Drosophila melanogaster,* serves as a model for numerous infections or human diseases due to its considerable biological similarity to mammals and an abundance of available genetic tools[51, 52, 171-174]. Unlike acute infections caused by some other pathogens, *Metarhizium anisopliae* Ma549 does not employ toxins and instead kills *Drosophila* slowly, principally by invasive growth and depleting the host of nutrients[19]. This provides a window of time to observe sleep and host-pathogen interactions before severe sickness ensues. Consequently, the Ma549 and fruit fly combination provides a valuable model system for studying the relationship between fungal infection and sleep.

In the first chapter, we investigated the impact of naturally occurring variables on disease resistance by examining the heterogeneity (differential response) hypothesis in globally collected *Drosophila melanogaster* lines. We aimed to understand how latitudinal clines, biomes, and environmental factors such as temperature and humidity affect susceptibility to Ma549 infection. Our data revealed that fruit fly susceptibility to Ma549 infection varied by gender and regions where the flies were collected. Male and female LT_{50} values are strongly correlated, indicating shared genetic factors influencing infection resistance in both sexes globally. However, females generally succumbed faster than males to infection irrespective of collection site, and males exhibited a broader range of LT_{50} values in both the worldwide and DGRP collection of 188 lines from N. Carolina. This likely reflects sexual dimorphisms in innate immunity and host defense; such sexual dimorphism affects disease transmission throughout the

animal kingdom[175], and the Ma549-*Drosophila* system thus provides a useful model for further investigation.

African lines, representing the ancestral population of *Drosophila melanogaster*, are generally more resistant to Ma549 infection than European, American, and Asian lines, with the Ghana lines, in particular, displaying higher resistance across both sexes and more plasticity than the American DGRP. Some alleles previously linked to disease resistance in the DGRP[49] were more abundant in African populations. Our data indicated that environmental factors such as temperature, precipitation, and latitudinal and altitudinal gradients correlate with fly longevity following Ma549 infection. African and other aseasonal (tropical) populations, such as the Monkey Hill population (MH), from uniform climates generally exhibit more disease-resistance than seasonal (temperate) populations that endure intermittent thermal and humidity stress. These findings support studies showing that stable climates bolster vector populations, such as *Anopheles gambiae*, and influence malaria transmission patterns[92], underscoring the role of aseasonal regions in disease dynamics.

We utilized three fly lines from Monkey Hill, St. Kitts (MH), Ica, Peru (IP), and Crete, Greece (CG) to explore the mechanisms behind variations in longevity and sexual dimorphism. MH flies from the TSMF biome, originating from a stable, warm, and near-optimal environment, show high disease resistance, likely due to their aseasonal habitat fostering strong host defenses. MH males demonstrated significant plasticity in disease resistance, suggesting adaptive strategies for coping with environmental variability. However, a trade-off was noted between desiccation and disease resistance, with MH males being highly disease-resistant but more susceptible to desiccation. Many animals, including humans sleep more when infected, so called

sickness sleep[83]. Increased "sickness sleep" in MH flies, especially males, was linked to slower disease progression, indicating that sleep may enhance disease resistance. These findings suggest that in the absence of abiotic stressors MH flies have evolved strong immune defenses potentially aligned with changes in sleep patterns. CG flies, representing the global average, showed moderate disease resistance, reflecting their adaptation to a range of environmental conditions. Their resistance levels were lower than MH flies but higher than IP flies that derive from an environment that is arid and generally unfavorable to *D. melanogaster* and probably fungal diseases. These results suggest a balance between general robustness versus abiotic stresses in varying climates and disease resistance, reflecting the adaptability of *D. melanogaster* to diverse ecological contexts.

The study found that CG flies exhibited intermediate plasticity in disease resistance, indicating some capacity to cope with changes in biotic stresses, albeit less pronounced than in MH flies. The correlation between "sickness sleep" and disease resistance was also observed in CG flies, with increased sleep during infection contributing to better disease outcomes. The CG line has LT_{50} values for males and females close to the average of all lines. Our within-host fungal growth data demonstrated that the CG line had less ability to restrain *Metarhizium* growth than MH flies, especially males. Daytime sickness sleep increased within the first two days post infection with Ma549, particularly in males, correlating with higher disease resistance in MH compared to CG and IP. IP flies, adapted to the DXS biome with frequent thermal and humidity stress, may prioritize desiccation resistance over disease resistance, suggesting a trade-off between resistance to abiotic and biotic stressors. The study also found that IP males exhibited high nocturnal activity (less sleep), which may be an adaptation to their arid environment but not

necessarily linked to enhanced disease resistance. The reduced "sickness sleep" response in IP flies and high fungal loads soon after infection further indicates their limited immune defense capability. This susceptibility underscores the complex interplay between environmental adaptation and disease resistance, where surviving harsh abiotic conditions may compromise the ability to resist pathogens. Virgin flies, which typically sleep more than mated ones, exhibited a greater ability to delay fungal growth, indicating trade-offs between reproduction and immunity. Males displayed a lower fungal burden than females, consistent with sex-specific immune defense strategies. These results highlight the intricate relationship between ecological context, sexual dimorphism, and disease resistance, with implications for understanding host-pathogen interactions in varying climates.

Overall, our findings indicate that geographical factors such as latitudinal clines and biomes significantly shape disease resistance in *Drosophila melanogaster*. The Ma549-*Drosophila* system serves as a valuable model for exploring the interactions between environmental variables, sleep, and host defense mechanisms. These studies emphasize the importance of considering both biotic and abiotic factors in understanding the evolution of disease resistance and illustrate potential trade-offs that may affect the distribution and fitness of species across different habitats. They particularly point to how sleep, an ancient and conserved behavior, intersects with disease resistance mechanisms.

In the second chapter, utilizing the extreme long- or short-sleepers from the Sleep Inbred Panel (SIP), we examined the impact of artificially selected sleep-length differences on fungal infection resistance. On average, males from the 38 SIP lines were about 5% more resistant than females, but females outlived males in 20 of the 48 SIP plus progenitor DGRP

lines. Short-sleepers and their progenitors of either sex outlived long-sleepers and their progenitors and the entire DGRP.

SIP lines amplified trends observed in DGRP lines where DGRPs that take frequent "naps" survive longest. Thus, the resistance of the 38 SIP lines to Ma549 was negatively correlated with the length of sleep bouts and sleep duration (nighttime and daytime), in males and to a lesser extent females in healthy flies, so that short sleepers, characterized by more fragmented sleep patterns before infection, demonstrated increased survival post-Ma549 infection compared to long-sleepers. There was a positive association between resistance and the number of nightly sleep bouts in both sexes, as well as with the number of daytime sleep bouts in females, but not in males. However, daytime sleep bout numbers were positively correlated with sexually dimorphic disease resistance, specifically which ever sex had the most daytime sleep bouts lived longer.

To study variation in longevity and sleep in SIP lines, we selected a long-sleeping and a short-sleeping line (L1_6 and S1_7), which exhibited notable sexual dimorphism in survival post-Ma549 infection. We confirmed their long and short sleeping patterns after several years of lab maintenance. We found that Ma549 infection does not disrupt the circadian clock, but it increases daytime sickness sleep in both genders of L1_6 and S1_7, with infected S1_7 males showing a particularly large increase in daytime sleep. The varying survival times of L1_6 and S1_7 females and males correlated with increased daytime sickness sleep, indicating its survival value.

Collectively from Chapters I and II, using 43 worldwide and 38 SIP lines, we found that the susceptibility to Ma549 infection varied by gender, although male and female LT_{50} values

were correlated. Chapter I linked longevity to consolidated sleep of healthy females. Chapter II showed that artificially selected short sleepers outlive long sleepers, and that longevity correlates with fragmented sleep (i.e., the number of naps) that SIP (and DGRP flies) take before infection. We hypothesize that selection for short sleepers in nature could produce flies that are more easily aroused to wakefulness to escape danger, resulting in a generalized increased resistance to multiple stresses, including pathogens[64]. Although the correlation trends between longevity and consolidated versus fragmented sleep in worldwide and SIP flies prior to infection were different in our study, MH and CG male flies increase sickness sleep after infection, similar to short sleeper S1_7 and long sleeper L1_6.

In the final chapter, we aimed to elucidate the interaction between host defense and sickness sleep after Ma549 infection, examining the role of certain sleep-related genes, and whether disrupted circadian rhythms can alter disease outcomes.

In chapters I and II, we observed that reduced daytime sleep before infection or increased sickness sleep duration post-infection correlates with greater longevity in worldwide and SIP flies. We tested these findings using genetic sleep deprivation (SD)[147]. Monitoring the *Drosophila* rest-activity cycle in *Shaker minisleep* (*mns*) mutant without disrupted circadian rhythms[159], we found that both females and males exhibited a two-thirds reduction in 24-hour daily sleep compared to wildtype controls, consistent with previous reports[159]. Infected Sh^{mns} mutants showed decreased daytime sickness sleep and reduced longevity relative to Canton-S (CS) WT, supporting the theory that increased daytime sickness sleep aids in combating infection.

We also investigated the role of the molecular clock in disease susceptibility by infecting well-characterized *period* (*per*^{01}) and *clock* (*Clk*^{Jrk}) mutants. Uninfected *per*^{01} have significantly reduced nighttime sleep compared to CS WT flies, and infected *per*^{01} flies showed decreased daytime and daily sickness sleep with shorter survival. Uninfected *Clk*^{Jrk} mutants also had reduced nighttime sleep, and infection further decreased daytime and daily sickness sleep, although survival was slightly enhanced. This suggests that PERIOD protects *Drosophila* against fungi, while CLK undermines this protection.

Our data from the *Shaker* mutant with normal circadian rhythms and the *per*^{01} mutant without them support our hypothesis that increased daytime sickness sleep enhances survival. These findings contrast with results from *Clk*^{Jrk} flies, potentially due to *Clock* also regulating about 150 downstream genes[168] that affect various physiological and behavioral pathways, such as *Achilles*, which inhibits antimicrobial peptide expression in the immune system[153].

Collectively, these studies advance our knowledge of the interplay between fungal infection and sleep parameters, geographic variables, and host defense, and our findings pave the way forward for better understanding of the mechanism of how individuals respond to disease.

Bibliography

1. Joiner, William J. Unraveling the Evolutionary Determinants of Sleep. *Current Biology* **26**, R1073-R1087 (2016).
2. Dillon, M.E., Wang, G., Garrity, P.A. & Huey, R.B. Review: Thermal preference in Drosophila. *J Therm Biol* **34**, 109-119 (2009).
3. Hamada, F.N. et al. An internal thermal sensor controlling temperature preference in Drosophila. *Nature* **454**, 217-220 (2008).
4. Yoshii, T., Hermann-Luibl, C. & Helfrich-Förster, C. Circadian light-input pathways in Drosophila. *Commun Integr Biol* **9**, e1102805 (2016).
5. Majercak, J., Sidote, D., Hardin, P.E. & Edery, I. How a circadian clock adapts to seasonal decreases in temperature and day length. *Neuron* **24**, 219-230 (1999).
6. Xu, K., Zheng, X. & Sehgal, A. Regulation of feeding and metabolism by neuronal and peripheral clocks in Drosophila. *Cell Metab* **8**, 289-300 (2008).
7. Eban-Rothschild, A., Giardino, W.J. & de Lecea, L. To sleep or not to sleep: neuronal and ecological insights. *Current Opinion in Neurobiology* **44**, 132-138 (2017).
8. Aulsebrook, A.E., Jones, T.M., Rattenborg, N.C., Roth, T.C., 2nd & Lesku, J.A. Sleep Ecophysiology: Integrating Neuroscience and Ecology. *Trends Ecol Evol* **31**, 590-599 (2016).
9. Allada, R. & Siegel, J.M. Unearthing the phylogenetic roots of sleep. *Curr Biol* **18**, R670-R679 (2008).
10. Rechtschaffen, A., Gilliland, M.A., Bergmann, B.M. & Winter, J.B. Physiological correlates of prolonged sleep deprivation in rats. *Science* **221**, 182-184 (1983).
11. Shaw, P.J., Tononi, G., Greenspan, R.J. & Robinson, D.F. Stress response genes protect against lethal effects of sleep deprivation in Drosophila. *Nature* **417**, 287-291 (2002).
12. Everson, C.A. Sustained sleep deprivation impairs host defense. *Am J Physiol* **265**, R1148-1154 (1993).

13. Curtis, A.M., Bellet, M.M., Sassone-Corsi, P. & O'Neill, L.A. Circadian clock proteins and immunity. *Immunity* **40**, 178-186 (2014).

14. Carter, S.J. et al. A matter of time: study of circadian clocks and their role in inflammation. *J Leukoc Biol* **99**, 549-560 (2016).

15. Ranjbaran, Z., Keefer, L., Stepanski, E., Farhadi, A. & Keshavarzian, A. The relevance of sleep abnormalities to chronic inflammatory conditions. *Inflamm Res* **56**, 51-57 (2007).

16. Lemaitre, B. & Hoffmann, J. The host defense of Drosophila melanogaster. *Annu Rev Immunol* **25**, 697-743 (2007).

17. Igboin, C.O., Griffen, A.L. & Leys, E.J. The Drosophila melanogaster host model. *J Oral Microbiol* **4** (2012).

18. Jennings, B.H. Drosophila – a versatile model in biology & medicine. *Materials Today* **14**, 190-195 (2011).

19. Lu, H.L., Wang, J.B., Brown, M.A., Euerle, C. & St Leger, R.J. Identification of Drosophila Mutants Affecting Defense to an Entomopathogenic Fungus. *Sci Rep* **5**, 12350 (2015).

20. Lee, J.-E. & Edery, I. Circadian Regulation in the Ability of Drosophila to Combat Pathogenic Infections. *Current Biology* **18**, 195-199 (2008).

21. Stone, E.F. et al. The circadian clock protein timeless regulates phagocytosis of bacteria in Drosophila. *PLoS Pathog* **8**, e1002445 (2012).

22. Doeschl-Wilson, A., Bishop, S., Kyriazakis, I. & Villanueva, B. Novel methods for quantifying individual host response to infectious pathogens for genetic analyses. *Frontiers in Genetics* **3** (2012).

23. Mathur, V. & Schmidt, P.S. Adaptive patterns of phenotypic plasticity in laboratory and field environments in Drosophila melanogaster. *Evolution* **71**, 465-474 (2017).

24. Chen, S., Fuller, K.K., Dunlap, J.C. & Loros, J.J. Circadian Clearance of a Fungal Pathogen from the Lung Is Not Based on Cell-intrinsic Macrophage Rhythms. *J Biol Rhythms* **33**, 99-105 (2018).

25. Stop neglecting fungi. *Nat Microbiol* **2**, 17120 (2017).

26. Kainz, K., Bauer, M.A., Madeo, F. & Carmona-Gutierrez, D. Fungal infections in humans: the silent crisis. *Microb Cell* **7**, 143-145 (2020).

27. Savary, S., Ficke, A., Aubertot, J.-N. & Hollier, C. Crop losses due to diseases and their implications for global food production losses and food security. *Food Security* **4**, 519-537 (2012).

28. St. Leger, R.J., Cooper, R.M. & Charnley, A.K. Cuticle-degrading enzymes of entomopathogenic fungi: Cuticle degradation in vitro by enzymes from entomopathogens. *Journal of Invertebrate Pathology* **47**, 167-177 (1986).

29. Ortiz-Urquiza, A. & Keyhani, N.O. Action on the Surface: Entomopathogenic Fungi versus the Insect Cuticle. *Insects* **4**, 357-374 (2013).

30. Butt, T.M., Coates, C.J., Dubovskiy, I.M. & Ratcliffe, N.A. Entomopathogenic Fungi: New Insights into Host-Pathogen Interactions. *Adv Genet* **94**, 307-364 (2016).

31. Lu, H.L. & St Leger, R.J. Insect Immunity to Entomopathogenic Fungi. *Adv Genet* **94**, 251-285 (2016).

32. Gottar, M. et al. Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. *Cell* **127**, 1425-1437 (2006).

33. St. Leger, R.J., Wang, C. & Fang, W. New perspectives on insect pathogens. *Fungal Biology Reviews* **25**, 84-88 (2011).

34. St Leger, R.J. & Wang, J.B. Metarhizium: jack of all trades, master of many. *Open Biol* **10**, 200307 (2020).

35. Lovett, B. et al. Transgenic Metarhizium rapidly kills mosquitoes in a malaria-endemic region of Burkina Faso. *Science* **364**, 894-897 (2019).

36. Pal, S., St Leger, R.J. & Wu, L.P. Fungal peptide Destruxin A plays a specific role in suppressing the innate immune response in Drosophila melanogaster. *J Biol Chem* **282**, 8969-8977 (2007).

37. Mateo, L., Rech, G.E. & González, J. Genome-wide patterns of local adaptation in Western European Drosophila melanogaster natural populations. *Scientific Reports* **8**, 16143 (2018).

38. Arguello, J.R., Laurent, S. & Clark, A.G. Demographic History of the Human Commensal Drosophila melanogaster. *Genome Biology and Evolution* **11**, 844-854 (2019).

39. Mansourian, S. et al. Wild African Drosophila melanogaster Are Seasonal Specialists on Marula Fruit. *Curr Biol* **28**, 3960-3968.e3963 (2018).

40. Sprengelmeyer, Q.D. et al. Recurrent Collection of Drosophila melanogaster from Wild African Environments and Genomic Insights into Species History. *Molecular Biology and Evolution* **37**, 627-638 (2019).

41. David, J.R. & Capy, P. Genetic variation of Drosophila melanogaster natural populations. *Trends Genet* **4**, 106-111 (1988).

42. Haudry, A., Laurent, S. & Kapun, M. in Statistical Population Genomics. (ed. J.Y. Dutheil) 357-396 (Springer US, New York, NY; 2020).

43. Blanford, S., Thomas, M.B., Pugh, C. & Pell, J.K. Temperature checks the Red Queen? Resistance and virulence in a fluctuating environment. *Ecology Letters* **6**, 2-5 (2003).

44. Brown, E.B. et al. Variation in sleep and metabolic function is associated with latitude and average temperature in Drosophila melanogaster. *Ecol Evol* **8**, 4084-4097 (2018).

45. Keene, A.C. & Duboue, E.R. The origins and evolution of sleep. *J Exp Biol* **221** (2018).

46. Dybdahl, M.F. & Lively, C.M. HOST-PARASITE COEVOLUTION: EVIDENCE FOR RARE ADVANTAGE AND TIME-LAGGED SELECTION IN A NATURAL POPULATION. *Evolution* **52**, 1057-1066 (1998).

47. Lively, C.M. & Dybdahl, M.F. Parasite adaptation to locally common host genotypes. *Nature* **405**, 679-681 (2000).

48. O'Malley, L., Wang, J., Nikzad, M., Sheng, H. & St Leger, R. Genetic variation in disease resistance in Drosophila spp. is mitigated in Drosophila sechellia by specialization to a toxic host. *Sci Rep* **13**, 7793 (2023).

49. Wang, J.B., Lu, H.L. & St Leger, R.J. The genetic basis for variation in resistance to infection in the Drosophila melanogaster genetic reference panel. *PLoS Pathog* **13**, e1006260 (2017).

50. Tinsley, M.C., Blanford, S. & Jiggins, F.M. Genetic variation in Drosophila melanogaster pathogen susceptibility. *Parasitology* **132**, 767-773 (2006).

51. Ugur, B., Chen, K. & Bellen, H.J. Drosophila tools and assays for the study of human diseases. *Dis Model Mech* **9**, 235-244 (2016).

52. Panayidou, S., Ioannidou, E. & Apidianakis, Y. Human pathogenic bacteria, fungi, and viruses in Drosophila: disease modeling, lessons, and shortcomings. *Virulence* **5**, 253-269 (2014).

53. Råberg, L., Sim, D. & Read, A.F. Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science* **318**, 812-814 (2007).

54. Duneau, D. et al. Stochastic variation in the initial phase of bacterial infection predicts the probability of survival in D. melanogaster. *Elife* **6** (2017).

55. St. Leger, R.J. The evolution of complex Metarhizium-insect-plant interactions. *Fungal Biology* (2024).

56. Wang, C. & St. Leger, R.J. A collagenous protective coat enables Metarhizium anisopliae to evade insect immune responses. *Proceedings of the National Academy of Sciences* **103**, 6647-6652 (2006).

57. Hutter, S., Li, H., Beisswanger, S., De Lorenzo, D. & Stephan, W. Distinctly different sex ratios in African and European populations of Drosophila melanogaster inferred from chromosomewide single nucleotide polymorphism data. *Genetics* **177**, 469-480 (2007).

58. Kreft, H. & Jetz, W. Global patterns and determinants of vascular plant diversity. *Proceedings of the National Academy of Sciences* **104**, 5925-5930 (2007).

59. Dinerstein, E. et al. An Ecoregion-Based Approach to Protecting Half the Terrestrial Realm. *BioScience* **67**, 534-545 (2017).

60. Vasar, M. et al. Global soil microbiomes: A new frontline of biome-ecology research. *Global Ecology and Biogeography* **31**, 1120-1132 (2022).

61. Tedersoo, L. et al. Global diversity and geography of soil fungi. *Science* **346**, 1256688 (2014).

62. Větrovský, T. et al. A meta-analysis of global fungal distribution reveals climate-driven patterns. *Nature Communications* **10**, 5142 (2019).

63. Athanassiou, C.G., Kavallieratos, N.G., Rumbos, C.I. & Kontodimas, D.C. Influence of Temperature and Relative Humidity on the Insecticidal Efficacy of Metarhizium anisopliae against Larvae of Ephestia kuehniella (Lepidoptera: Pyralidae) on Wheat. *J Insect Sci* **17** (2017).

64. St. Leger, R.J. Insects and their pathogens in a changing climate. *Journal of Invertebrate Pathology* **184**, 107644 (2021).

65. Kellermann, V., Hoffmann, A.A., Overgaard, J., Loeschcke, V. & Sgrò, C.M. Plasticity for desiccation tolerance across Drosophila species is affected by phylogeny and climate in complex ways. *Proceedings of the Royal Society B: Biological Sciences* **285**, 20180048 (2018).

66. Stancioff, C.E. et al. Local Perceptions of Climate Change Impacts in St. Kitts (Caribbean Sea) and Malé, Maldives (Indian Ocean). *Atmosphere* **9**, 459 (2018).

67. Salem, B. Arid zone forestry: a guide for field technicians. (1989).

68. Morgante, F., Sørensen, P., Sorensen, D.A., Maltecca, C. & Mackay, T.F.C. Genetic Architecture of Micro-Environmental Plasticity in Drosophila melanogaster. *Scientific Reports* **5**, 9785 (2015).

69. David, J.R. et al. Isofemale lines in Drosophila: an empirical approach to quantitative trait analysis in natural populations. *Heredity (Edinb)* **94**, 3-12 (2005).

70. Lotterhos, K.E., Yeaman, S., Degner, J., Aitken, S. & Hodgins, K.A. Modularity of genes involved in local adaptation to climate despite physical linkage. *Genome Biology* **19**, 157 (2018).

71. Bogaerts-Márquez, M., Guirao-Rico, S., Gautier, M. & González, J. Temperature, rainfall and wind variables underlie environmental adaptation in natural populations of Drosophila melanogaster. *Molecular Ecology* **30**, 938-954 (2021).

72. Peixoto, J. & Oort, A.H. The Climatology of Relative Humidity in the Atmosphere. *Journal of Climate* **9**, 3443-3463 (1996).

73. Woods, E.C., Hastings, A.P., Turley, N.E., Heard, S.B. & Agrawal, A.A. Adaptive geographical clines in the growth and defense of a native plant. *Ecological Monographs* **82**, 149-168 (2012).

74. Manenti, T. et al. Responses to Developmental Temperature Fluctuation in Life History Traits of Five Drosophila Species (Diptera: Drosophilidae) from Different Thermal Niches. *Insects* **12** (2021).

75. Enriquez, T., Ruel, D., Charrier, M. & Colinet, H. Effects of fluctuating thermal regimes on cold survival and life history traits of the spotted wing Drosophila (Drosophila suzukii). *Insect Sci* **27**, 317-335 (2020).

76. Chakir, M., Chafik, A., Moreteau, B., Gibert, P. & David, J.R. Male Sterility Thermal Thresholds in Drosophila: D. simulans Appears More Cold-adapted Than its Sibling D. melanogaster. *Genetica* **114**, 195-205 (2002).

77. Al-Saffar, Z.Y., Grainger, J.N.R. & Aldrich, J. Temperature and humidity affecting development, survival and weight loss of the pupal stage of Drosophila melanogaster, and the influence of alternating temperature on the larvae. *Journal of Thermal Biology* **21**, 389-396 (1996).

78. Hamada, F.N. et al. An internal thermal sensor controlling temperature preference in Drosophila. *Nature* **454**, 217-220 (2008).

79. Sayeed, O. & Benzer, S. Behavioral genetics of thermosensation and hygrosensation in Drosophila. *Proc Natl Acad Sci U S A* **93**, 6079-6084 (1996).

80. Siddiqui, W.H. & Barlow, C.A. Population Growth of Drosophila melanogaster (Diptera: Drosophilidae) at Constant and Alternating Temperatures1. *Annals of the Entomological Society of America* **65**, 993-1001 (1972).

81. Rajpurohit, S. et al. Spatiotemporal dynamics and genome-wide association genome-wide association analysis of desiccation tolerance in Drosophila melanogaster. *Mol Ecol* **27**, 3525-3540 (2018).

82. Shaw, P.J., Cirelli, C., Greenspan, R.J. & Tononi, G. Correlates of Sleep and Waking in Drosophila melanogaster. *Science* **287**, 1834-1837 (2000).

83. Davis, K.C. & Raizen, D.M. A mechanism for sickness sleep: lessons from invertebrates. *J Physiol* **595**, 5415-5424 (2017).

84. Hervas, S., Sanz, E., Casillas, S., Pool, J.E. & Barbadilla, A. PopFly: the Drosophila population genomics browser. *Bioinformatics* **33**, 2779-2780 (2017).

85. Lack, J.B., Lange, J.D., Tang, A.D., Corbett-Detig, R.B. & Pool, J.E. A Thousand Fly Genomes: An Expanded Drosophila Genome Nexus. *Molecular Biology and Evolution* **33**, 3308-3313 (2016).

86. Pool, J.E. The Mosaic Ancestry of the Drosophila Genetic Reference Panel and the D. melanogaster Reference Genome Reveals a Network of Epistatic Fitness Interactions. *Molecular Biology and Evolution* **32**, 3236-3251 (2015).

87. Caracristi, G. & Schlötterer, C. Genetic Differentiation Between American and European Drosophila melanogaster Populations Could Be Attributed to Admixture of African Alleles. *Molecular Biology and Evolution* **20**, 792-799 (2003).

88. Maclean, H.J., Kristensen, T.N., Sørensen, J.G. & Overgaard, J. Laboratory maintenance does not alter ecological and physiological patterns among species: a Drosophila case study. *Journal of Evolutionary Biology* **31**, 530-542 (2018).

89. Souto-Maior, C., Serrano Negron, Y.L. & Harbison, S.T. Natural selection on sleep duration in Drosophila melanogaster. *Sci Rep* **10**, 20652 (2020).

90. Fabian, D.K. et al. Spatially varying selection shapes life history clines among populations of Drosophila melanogaster from sub-Saharan Africa. *J Evol Biol* **28**, 826-840 (2015).

91. Verspoor, R.L. & Haddrill, P.R. Genetic Diversity, Population Structure and Wolbachia Infection Status in a Worldwide Sample of Drosophila melanogaster and D. simulans Populations. *PLOS ONE* **6**, e26318 (2011).

92. Paaijmans, K.P., Read, A.F. & Thomas, M.B. Understanding the link between malaria risk and climate. *Proceedings of the National Academy of Sciences* **106**, 13844-13849 (2009).

93. Cheng, C. et al. Ecological genomics of Anopheles gambiae along a latitudinal cline: a population-resequencing approach. *Genetics* **190**, 1417-1432 (2012).

94. Tochen, S. et al. Humidity affects populations of Drosophila suzukii (Diptera: Drosophilidae) in blueberry. *Journal of Applied Entomology* **140**, 47-57 (2016).

95. Achumi, B., Hegde, S.N., Lal, P. & Yenisetti, S.C. Altitudinal and seasonal variation in Drosophila species on mount Japfu of Nagaland, a sub-Himalayan hilly state of India. *J Insect Sci* **13**, 117-117 (2013).

96. Bombin, A. & Reed, L.K. The changing biodiversity of Alabama Drosophila: important impacts of seasonal variation, urbanization, and invasive species. *Ecology and evolution* **6**, 7057-7069 (2016).

97. Wang, J.B., Lu, H.L., Sheng, H. & St Leger, R.J. A Drosophila melanogaster model shows that fast growing Metarhizium species are the deadliest despite eliciting a strong immune response. *Virulence* **14**, 2275493 (2023).

98. Brown, J.H. Why are there so many species in the tropics? *J Biogeogr* **41**, 8-22 (2014).

99. Salazar-Mendoza, P., Peralta-Aragón, I., Romero-Rivas, L., Salamanca, J. & Rodriguez-Saona, C. The abundance and diversity of fruit flies and their parasitoids change with elevation in guava

orchards in a tropical Andean forest of Peru, independent of seasonality. *PLOS ONE* **16**, e0250731 (2021).

100. Schemske, D.W., Mittelbach, G.G., Cornell, H.V., Sobel, J.M. & Roy, K. Is There a Latitudinal Gradient in the Importance of Biotic Interactions? *Annual Review of Ecology, Evolution, and Systematics* **40**, 245-269 (2009).

101. Pennings, S.C. et al. Latitudinal variation in herbivore pressure in Atlantic Coast salt marshes. *Ecology* **90**, 183-195 (2009).

102. Landis, G., Shen, J. & Tower, J. Gene expression changes in response to aging compared to heat stress, oxidative stress and ionizing radiation in Drosophila melanogaster. *Aging (Albany NY)* **4**, 768-789 (2012).

103. Hoffmann, A.A., Hallas, R., Anderson, A.R. & Telonis-Scott, M. Evidence for a robust sex-specific trade-off between cold resistance and starvation resistance in Drosophila melanogaster. *J Evol Biol* **18**, 804-810 (2005).

104. Lafuente, E., Duneau, D. & Beldade, P. Genetic basis of thermal plasticity variation in Drosophila melanogaster body size. *PLoS Genet* **14**, e1007686 (2018).

105. Wang, J.B., Elya, C. & St Leger, R.J. Genetic variation for resistance to the specific fly pathogen Entomophthora muscae. *Sci Rep* **10**, 14284 (2020).

106. Wiil, J., Sørensen, J.G. & Colinet, H. Exploring cross-protective effects between cold and immune stress in Drosophila melanogaster. *Parasite* **30**, 54 (2023).

107. Ramond, E. et al. Reactive Oxygen Species-Dependent Innate Immune Mechanisms Control Methicillin-Resistant Staphylococcus aureus Virulence in the Drosophila Larval Model. *mBio* **12**, 10.1128/mbio.00276-00221 (2021).

108. Hill, V.M. et al. A bidirectional relationship between sleep and oxidative stress in Drosophila. *PLOS Biology* **16**, e2005206 (2018).

109. Hoffmann, A.A. & Harshman, L.G. Desiccation and starvation resistance in Drosophila: patterns of variation at the species, population and intrapopulation levels. *Heredity* **83**, 637-643 (1999).

110. Lin, Y.-C., Zhang, M., Chang, Y.-J. & Kuo, T.-H. Comparisons of lifespan and stress resistance between sexes in Drosophila melanogaster. *Heliyon* **9**, e18178 (2023).

111. van den Heuvel, J. et al. The predictive adaptive response: modeling the life-history evolution of the butterfly Bicyclus anynana in seasonal environments. *Am Nat* **181**, E28-42 (2013).

112. Harts, A.M., Schwanz, L.E. & Kokko, H. Demography can favour female-advantageous alleles. *Proc Biol Sci* **281** (2014).

113. Howick, V.M. & Lazzaro, B.P. Genotype and diet shape resistance and tolerance across distinct phases of bacterial infection. *BMC Evol Biol* **14**, 56 (2014).

114. Toth, L.A., Tolley, E.A. & Krueger, J.M. Sleep as a prognostic indicator during infectious disease in rabbits. *Proc Soc Exp Biol Med* **203**, 179-192 (1993).

115. Garbe, D.S. et al. Changes in Female Drosophila Sleep following Mating Are Mediated by SPSN-SAG Neurons. *J Biol Rhythms* **31**, 551-567 (2016).

116. Rai, K.E. et al. Immune defense in Drosophila melanogaster depends on diet, sex, and mating status. *PLoS One* **18**, e0268415 (2023).

117. Gordon, K.E., Wolfner, M.F. & Lazzaro, B.P. A single mating is sufficient to induce persistent reduction of immune defense in mated female Drosophila melanogaster. *Journal of Insect Physiology* **140**, 104414 (2022).

118. Mishra, S., Sharma, N. & Lone, S.R. Understanding the impact of sociosexual interactions on sleep using Drosophila melanogaster as a model organism. *Frontiers in Physiology* **14** (2023).

119. Schneider, U., Becker, A., Finger, P., Rustemeier, E. & Ziese, M. (Research Data Archive at the National Center for Atmospheric Research, Computational and Information Systems Laboratory, Boulder, CO; 2020).

120. Gibbs, A.G., Chippindale, A.K. & Rose, M.R. Physiological Mechanisms of Evolved Desiccation Resistance in Drosophila Melanogaster. *Journal of Experimental Biology* **200**, 1821-1832 (1997).

121. Hu, G. & St Leger, R.J. Field studies using a recombinant mycoinsecticide (Metarhizium anisopliae) reveal that it is rhizosphere competent. *Appl Environ Microbiol* **68**, 6383-6387 (2002).

122. Yap, B.W. & Sim, C.H. Comparisons of various types of normality tests. *Journal of Statistical Computation and Simulation* **81**, 2141-2155 (2011).

123. Kounatidis, I. & Ligoxygakis, P. Drosophila as a model system to unravel the layers of innate immunity to infection. *Open Biol* **2**, 120075 (2012).

124. Mackay, T.F. et al. The Drosophila melanogaster Genetic Reference Panel. *Nature* **482**, 173-178 (2012).

125. Serrano Negron, Y.L., Hansen, N.F. & Harbison, S.T. The Sleep Inbred Panel, a Collection of Inbred Drosophila melanogaster with Extreme Long and Short Sleep Duration. *G3 (Bethesda)* **8**, 2865-2873 (2018).

126. Harbison, S.T., Serrano Negron, Y.L., Hansen, N.F. & Lobell, A.S. Selection for long and short sleep duration in Drosophila melanogaster reveals the complex genetic network underlying natural variation in sleep. *PLoS Genet* **13**, e1007098 (2017).

127. Thompson, J.B., Su, O.O., Yang, N. & Bauer, J.H. Sleep-length differences are associated with altered longevity in the fruit fly Drosophila melanogaster. *Biol Open* **9** (2020).

128. Li, W. et al. Chronic social isolation signals starvation and reduces sleep in Drosophila. *Nature* **597**, 239-244 (2021).

129. Harbison, S.T., McCoy, L.J. & Mackay, T.F. Genome-wide association study of sleep in Drosophila melanogaster. *BMC Genomics* **14**, 281 (2013).

130. Sheehan, G., Garvey, A., Croke, M. & Kavanagh, K. Innate humoral immune defences in mammals and insects: The same, with differences ? *Virulence* **9**, 1625-1639 (2018).

131. Campbell, S.S. & Tobler, I. Animal sleep: A review of sleep duration across phylogeny. *Neuroscience & Biobehavioral Reviews* **8**, 269-300 (1984).

132. Huber, R. et al. Sleep Homeostasis in Drosophila Melanogaster. *Sleep* **27**, 628-639 (2004).

133. Imeri, L. & Opp, M.R. How (and why) the immune system makes us sleep. *Nat Rev Neurosci* **10**, 199-210 (2009).

134. Kuo, T.H. & Williams, J.A. Increased sleep promotes survival during a bacterial infection in Drosophila. *Sleep* **37**, 1077-1086, 1086a-1086d (2014).

135. Kuo, T.-H., Pike, D.H., Beizaeipour, Z. & Williams, J.A. Sleep triggered by an immune response in Drosophila is regulated by the circadian clock and requires the NFκB Relish. *BMC Neuroscience* **11**, 17 (2010).

136. Besedovsky, L., Lange, T. & Haack, M. The Sleep-Immune Crosstalk in Health and Disease. *Physiol Rev* **99**, 1325-1380 (2019).

137. Lange, B.W., Langley, C.H. & Stephan, W. Molecular evolution of Drosophila metallothionein genes. *Genetics* **126**, 921-932 (1990).

138. Zhong, W. et al. Immune anticipation of mating in Drosophila: Turandot M promotes immunity against sexually transmitted fungal infections. *Proc Biol Sci* **280**, 20132018 (2013).

139. Wu, K.J., Kumar, S., Serrano Negron, Y.L. & Harbison, S.T. Genotype Influences Day-to-Day Variability in Sleep in Drosophila melanogaster. *Sleep* **41** (2018).

140. Pegoraro, M. et al. The genetic basis of diurnal preference in Drosophila melanogaster. *BMC Genomics* **21**, 596 (2020).

141. Yoshii, T., Rieger, D. & Helfrich-Förster, C. Two clocks in the brain: an update of the morning and evening oscillator model in Drosophila. *Prog Brain Res* **199**, 59-82 (2012).

142. Lenz, O., Xiong, J., Nelson, M.D., Raizen, D.M. & Williams, J.A. FMRFamide signaling promotes stress-induced sleep in Drosophila. *Brain Behav Immun* **47**, 141-148 (2015).

143. Samuels, R.I., Charnley, A.K. & Reynolds, S.E. The role of destruxins in the pathogenicity of 3 strains of Metarhizium anisopliae for the tobacco hornworm Manduca sexta. *Mycopathologia* **104**, 51-58 (1988).

144. Mohren, D.C.L. et al. Prevalence of common infections among employees in different work schedules. *J Occup Environ Med* **44**, 1003-1011 (2002).

145. Borbély, A.A. A two process model of sleep regulation. *Hum Neurobiol* **1**, 195-204 (1982).

146. Ibarra-Coronado, E.G. et al. The Bidirectional Relationship between Sleep and Immunity against Infections. *J Immunol Res* **2015**, 678164-678164 (2015).

147. Bringmann, H. Genetic sleep deprivation: using sleep mutants to study sleep functions. *EMBO Rep* **20** (2019).

148. Hendricks, J.C. et al. Rest in Drosophila is a sleep-like state. *Neuron* **25**, 129-138 (2000).

149. Harbison, S.T. & Sehgal, A. Energy stores are not altered by long-term partial sleep deprivation in Drosophila melanogaster. *PLoS One* **4**, e6211 (2009).

150. Vaccaro, A. et al. Sleep Loss Can Cause Death through Accumulation of Reactive Oxygen Species in the Gut. *Cell* **181**, 1307-1328.e1315 (2020).

151. Shirasu-Hiza, M.M., Dionne, M.S., Pham, L.N., Ayres, J.S. & Schneider, D.S. Interactions between circadian rhythm and immunity in Drosophila melanogaster. *Curr Biol* **17**, R353-355 (2007).

152. Allen, Victoria W. et al. period-Regulated Feeding Behavior and TOR Signaling Modulate Survival of Infection. *Current Biology* **26**, 184-194 (2016).

153. Li, J. et al. Achilles is a circadian clock-controlled gene that regulates immune function in Drosophila. *Brain, behavior, and immunity* **61**, 127-136 (2017).

154. Konopka, R.J. & Benzer, S. Clock mutants of Drosophila melanogaster. *Proc Natl Acad Sci U S A* **68**, 2112-2116 (1971).

155. Smith, R.F. & Konopka, R.J. Circadian clock phenotypes of chromosome aberrations with a breakpoint at the per locus. *Molecular and General Genetics MGG* **183**, 243-251 (1981).

156. Panda, S., Hogenesch, J.B. & Kay, S.A. Circadian rhythms from flies to human. *Nature* **417**, 329-335 (2002).

157. Dubowy, C. & Sehgal, A. Circadian Rhythms and Sleep in Drosophila melanogaster. *Genetics* **205**, 1373-1397 (2017).

158. Kim, J.-h. et al. The voltage-gated potassium channel Shaker promotes sleep via thermosensitive GABA transmission. *Communications Biology* **3**, 174 (2020).

159. Cirelli, C. et al. Reduced sleep in Drosophila Shaker mutants. *Nature* **434**, 1087-1092 (2005).

160. Isaac, R.E., Li, C., Leedale, A.E. & Shirras, A.D. Drosophila male sex peptide inhibits siesta sleep and promotes locomotor activity in the post-mated female. *Proc Biol Sci* **277**, 65-70 (2010).

161. McKean, K.A. & Nunney, L. Increased sexual activity reduces male immune function in Drosophila melanogaster. *Proceedings of the National Academy of Sciences* **98**, 7904-7909 (2001).

162. Belmonte, R.L., Corbally, M.K., Duneau, D.F. & Regan, J.C. Sexual Dimorphisms in Innate Immunity and Responses to Infection in Drosophila melanogaster. *Front Immunol* **10**, 3075 (2019).

163. Shahrestani, P. et al. Sexual dimorphism in Drosophila melanogaster survival of Beauveria bassiana infection depends on core immune signaling. *Scientific Reports* **8**, 12501 (2018).

164. Öztürk-Çolak, A. et al. Sleep Induction by Mechanosensory Stimulation in Drosophila. *Cell Rep* **33**, 108462 (2020).

165. Wheeler, D.A., Hamblen-Coyle, M.J., Dushay, M.S. & Hall, J.C. Behavior in light-dark cycles of Drosophila mutants that are arrhythmic, blind, or both. *J Biol Rhythms* **8**, 67-94 (1993).

166. Allada, R., White, N.E., So, W.V., Hall, J.C. & Rosbash, M. A mutant Drosophila homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. *Cell* **93**, 791-804 (1998).

167. Toda, H., Williams, J.A., Gulledge, M. & Sehgal, A. A sleep-inducing gene, nemuri, links sleep and immune function in Drosophila. *Science* **363**, 509-515 (2019).

168. Shafer, O.T. & Keene, A.C. The Regulation of Drosophila Sleep. *Curr Biol* **31**, R38-r49 (2021).

169. Hastings, M.H., Reddy, A.B. & Maywood, E.S. A clockwork web: circadian timing in brain and periphery, in health and disease. *Nature Reviews Neuroscience* **4**, 649-661 (2003).

170. St Leger, R., Joshi, L., Bidochka, M.J. & Roberts, D.W. Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 6349-6354 (1996).

171. Crivat, G. et al. Insulin Stimulates Translocation of Human GLUT4 to the Membrane in Fat Bodies of Transgenic Drosophila melanogaster. *PLOS ONE* **8**, e77953 (2013).

172. Chang, K.T. & Min, K.-T. Upregulation of three Drosophila homologs of human chromosome 21 genes alters synaptic function: Implications for Down syndrome. *Proceedings of the National Academy of Sciences* **106**, 17117-17122 (2009).

173. Lu, B. & Vogel, H. Drosophila models of neurodegenerative diseases. *Annu Rev Pathol* **4**, 315-342 (2009).

174. Mirzoyan, Z. et al. Drosophila melanogaster: A Model Organism to Study Cancer. *Frontiers in Genetics* **10** (2019).

175. Belmonte, R.L., Corbally, M.-K., Duneau, D.F. & Regan, J.C. Sexual Dimorphisms in Innate Immunity and Responses to Infection in Drosophila melanogaster. *Front Immunol* **10**, 3075-3075 (2020).