Sexual Deprivation, Emotion, and Longevity:

Neuropeptidergic Regulation of Aging in *Drosophila*

By

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Dedication

This work is dedicated to my wife Amanda and son Bennett, whose love motivates me to press onward.

Acknowledgements

I arrived in Michigan knowing that I wanted to study aging, and little else about research. I like to think I'm eager to learn and willing to work hard to do it, but without good mentors that wouldn't amount to much even supposing it is true. I should start by thanking Dr. Ron Koenig, our MSTP director, first for being a terrific mentor himself, and second for encouraging me to rotate in Dr. Scott Pletcher's lab for his mentorship. While it seems obvious in hindsight, as a naïve graduate student I was thinking more about research interests than mentorship. Without the push from Ron and others who preached the importance of a good mentor, I might have shied away from a fruit fly lab in favor of something "closer to human relevence". My rotation in Scott's lab set me up for as incredible of a graduate experience as I could have hoped for.

When I started my rotation, I was a bit taken aback by the social atmosphere of the Pletcher lab. My undergraduate labs, while full of helpful students and post-docs, were generally quiet other than the sound of NPR on the radio, but in the Pletcher lab there were always lively discussions on sports, politics, Game of Thrones, movies, and occasionally even science. Between these discussions and board game nights, weekly trivia at Conor O'Neill's (and again in lab for the no-shows), tailgating, both sketchy and fancy conference dinners, and seeing the latest Star Wars movie, I've come to see the people in the Pletcher lab not just as colleagues, but as friends. I look forward to coming into lab every day not necessarily for the science, but for the people. Many people

have told me to make your work something you love to do, but my experience in the Pletcher lab has convinced me it's just as important to work with people you love being around.

I'm grateful to have gotten the chance to work with the entire lab, but some special thanks are in order. (Now Dr.) Mike Waterson took me on as a student despite the fact that I had never worked with flies and had no idea what I was doing, and was always helpful and encouraging, even while I was (accidentally) ruining his experiments. He even tolerated my Duke fandom, which is saying quite a bit for someone as gung-ho about Michigan sports as Mike. Soon-to-be Dr. Jenny Ro is perhaps the best CEO I could have asked for: her motivation and ability to push both herself and others out of their comfort zones can only lead to success. I look forward to many more years of working together in Flidea, LLC. I'm lucky to have worked with Emily Feuka and Sabine Hutter, two of the brightest and most driven undergraduates I've met here at Michigan. Both have been amazingly productive and a huge boon for my work, and while I'm sure they'll both be successful in whatever career they choose, I know they could both excel in science. David Paris ensures that everything in the lab runs smoothly to the point where he would bring me coffee if I was there "early" (it wasn't early for him). The lab would be a mess without his organization. Jacob Johnson gave me my first chance to mentor a graduate student, and was more than happy to take all the work I could pile on him. Dr. Christi Gendron was gracious and always willing to help, even when she initially mistook my shyness for animosity (I promise it wasn't!). She has continued to be good natured despite constant dumb jokes, and has been the angel on my shoulder combating the bad influence that is Dr. Brian Chung. Brian also tolerated my fandom (of the

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Dr. Scott Pletcher was a bit hesitant to accept me as a student at first, but I am truly thankful that he gave me the chance to join the lab and learn under his tutelage. Scott gave me just the right amount of independence and support, and I'm not sure I could have found a mentor better matched to my interests (both in and out of lab) and learning style. Scott insists on excellence everywhere, whether it's writing, creating new toys for the lab, board game nights, or the volleyball court at the Geriatrics picnic. While I still have a way to go, this insistence has made me a better scientist (and dare I say even more competitive when the games come out). He was always available to talk about ideas experimental design, statistics, writing, figure creation, and anything else I wanted. I always felt as though I left those conversations with much better ideas than I had beforehand, and more importantly I think the process of coming to those ideas have made me into a better scientist. A running joke in the lab is that I'm a mini-Scott, oftentimes when I'm harassing Christi to "be more efficient" (even when it wasn't). But ultimately, if my time in the lab has molded me to be more like Scott, I'd consider that to be a highly successful training regimen.

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Lastly, and most importantly, I want to thank my wife, Amanda, and our son, Bennett. After getting married right out of college, Amanda followed me to Michigan and has always supported me even at her own expense. She is the strongest person I have ever met, and has dealt with waking up at ungodly hours just to commute to teach in Detroit, dinners at midnight because my work ran late, overnight time courses, fruit flies being brought home, jokes about fly sex, and countless other inconveniences in the name of love. She helps me stay grounded and motivated simultaneously, reminding me that a

real world exists outside of lab. Best of all, she's given me the gift of our son, Bennett. Watching him struggle, grow, learn, and just exist over the past year reminds me that all of the support others have given to me should be paid back, and I hope to provide Bennett all that I can.

To those who have supported me, thank you. I hope I can repay you either directly or by pushing forward and being the best person, scientist, and physician that I can be.

Preface

This dissertation is focused on the biology of aging for two reasons. The first reason this dissertation studies aging is completely selfish: it is a biologically fascinating question to me. Aging seems to be optional: organisms exist that show extremely limited signs of aging, such as “the immortal jellyfish”, lobsters, and aspen trees, among others. Yet despite this, the vast majority of life on earth grow old and die, and do so at vastly different rates. While this might make sense comparing a fly and a human, even between bird species maximal lifespans can vary by over an order of magnitude, from 3 years to over 50 years. These observations bring up fundamental questions, like why do organisms “choose” to age, and why do they do so at such different paces?

Second, aging leads to a whole host of medical issues that decrease our ability to function. These issues are costly to society, as the morbidity from aging leads to economic losses and burdens when individuals become too old to care for themselves. As traditional medicine improves and more and more developing countries modernize, the percentage of the population that is too elderly to work will continue to grow. Identifying the mechanisms underlying the decline in function that occurs with age can help fight this burden for society. Of course, you have individuals who worry about creating a bunch of 100 year old (or older) invalids, but that’s not the goal. The goal is to let people get to 100, and feel like they’re still 50, not to keep an entire generation in a half-dead, sickly state. Frankly, I find the possibility of aging to the point of no longer being able to enjoy life to be both terrifying and tragic. Unfortunately, I may have been

born a little too early to really benefit from anti-aging treatments, but that is not a reason to abandon the goal. Ultimately the question becomes how can we develop treatments to slow the aging process?

I believe the answer to these questions can be found by understanding the biological mechanisms through which organisms naturally age. If we know the molecules or signals that cause aging, we can manipulate them to (1) treat the aging process and (2) determine why animals age at such different paces. So, if I can fulfill a selfish fascination while potentially helping society, the question becomes why not?

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List of Abbreviations, Acronyms, and Symbols

GnRH – gonadotropin-releasing hormone
NPY – neuropeptide Y
CRH – corticotropin-releasing hormone
IGF-1 – insulin-like growth factor
DR – dietary restriction
NPF/*npf* – neuropeptide F (PROTEIN/*gene*)
TOR – target of Rapamycin
AKH/*akh* – adipokinetic hormone (PROTEIN/*gene*)
CNS – central nervous system
PPK23/*ppk23* – pickpocket 23 (PROTEIN/*gene*)
CRZ/*crz* – corazonin (PROTEIN/*gene*)
WT – wild-type
Fem. – feminized
TAG – triacylglycerides
SEM – standard error of the mean
Masc. – masculinized
ANOVA – analysis of variance
♂ - male
♀ - female
$y^{-}w^{-}$ – a specific yellow bodied/white eyed laboratory fly lineage
w^{1118} – a specific white eyed laboratory fly lineage
PC(A) – principle component (analysis)
(q)PCR – (quantitative) polymerase chain reaction
dILP/*dILP* – drosophila insulin-like peptide (PROTEIN/*gene*)
Cont. – control
UAS – upstream activating sequence
Tra – transformer
FC – fold change
FM – feminized males
WM – wild-type males
SM – sensory mutant males
P_{death} – probability of death
I – count of pheromone exposure
μ_t – mortality rate
α – intrinsic mortality rate
β – aging rate
t - time
ME – mating expectation
D – duration
Z – magnitude of pheromone effect
SY – sugar/yeast food
ρ^2 – McFadden's pseudo R^2
FLIC – Fly Liquid-food Interaction Counter
CAFE – capillary feeder method
DFM – *Drosophila* feeding monitor
MCU – master control unit
PCB – printed circuit board
PI – preference index/pars intercerebralis (context dependent)
TDPI – time-dependent preference index
Canton-S – a specific red eyed laboratory fly lineage
SOG – subesophageal ganglion
MB – mushroom bodies
DH44/*Dh44* – diuretic hormone 44 (PROTEIN/*gene*)
DH44R1/*Dh44R1* – diuretic hormone 44 receptor 1 (PROTEIN/*gene*)
DH44R2/*Dh44R2* – diuretic hormone 44 receptor 2 (PROTEIN/*gene*)
IPCs – insulin producing cells

Abstract

While researchers often focus on the brain as a victim of aging via neurodegenerative diseases, recent work has demonstrated that the aging process is regulated by neural mechanisms. Thus, we asked which mechanisms and inputs might be important for the brain to regulate aging. We found that in male *Drosophila melanogaster*, the costs of reproduction on survival are mediated entirely through perception of the opposite sex, and that mating itself is actually beneficial. These effects are mediated through distinct neural circuits, with *neuropeptide F* (*npf*, an NPY homolog) required for the negative effects of pheromones and *corazonin* (*crz*, a GnRH homolog) driving the beneficial effects of mating. *dFoxo*, a common mediator of aging, regulates these effects on aging through an insulin-independent mechanism. Investigation of the dynamics of the effects of pheromones on mortality revealed two hypotheses: either population mortality rates reverse as a result of heterogeneity in individual probabilities of death, or the effects of pheromones on mortality rates are reversible in individuals. By combining *in vivo* and *in silico* approaches, we revealed that both explanations are correct, with individual reversibility dominating dynamics early in life, and heterogeneity becoming important in middle-age. Using a more global approach, we examined the effects of manipulating 78 distinct subsets of neurons on lifespan, and identified specific brain structures that are of prime importance for modulating aging. One of these structures is home to neurons expressing *diuretic hormone 44* (*Dh44*, a CRH homolog). *Dh44* and one of its receptors, *Dh44R1*, modulate lifespan, likely through insulin-like signaling pathways.

Furthermore, this effect of *Dh44* on lifespan is independent of diet, a fact obtained in part using the Fly Liquid-food Interaction Counter (FLIC), a novel assay developed to continuously measure feeding behavior in individual flies. The evolutionarily conserved neural circuits identified herein link aging to neural states consistent with primitive emotions in *Drosophila,* and these mechanisms deserve further exploration for their potential to explain connections between stress, emotions, and health in humans.

Chapter I: Aging, Social Signals, and Emotion[1]

The brain is not only the origin of thought and emotion but also the master regulator for homeostasis throughout the body. To modulate homeostasis, the brain is constantly perceiving and responding to its surroundings, including highly complex inputs such as an individual's social environment. As aging could be considered to be a failure of homeostasis, it is reasonable to ask what role the brain plays in regulating longevity. Herein, we identify the temporal dynamics and mechanisms through which altered neural states influence the aging process in the fruit fly *Drosophila melanogaster*. These conserved neuropeptides (homologous to mammalian GnRH, NPY, and CRH) are often associated with stress or emotions in humans. Thus, this work suggests that the known effects of emotions on health and longevity in humans may be mediated through evolutionarily conserved signaling pathways.

[1] Zachary Harvanek (ZMH) wrote and edited this chapter. Dr. Scott Pletcher (SDP) provided comments. Lindy Jensen (LJ) assisted in drawing Figure 1.1.

Introduction

While many theories exist regarding the origin of aging, there is still no consensus amongst the scientific community about how or why organisms grow old and die. Generally, the question of "how" has fallen on molecular biologists working in model systems, whereas the "why" is more often addressed from an ecological or evolutionary perspective in a diverse range of species. Therefore, these groups each have a unique perspective, and would likely give completely different responses if asked to finish the sentence "aging results from…". A molecular biologist or geneticist might, depending on their beliefs, state aging results from accumulation of DNA damage, oxidative stress, senescence, hormonal signaling, or decreased immune function, just to name a few possibilities. The evolutionary biologist might instead answer that aging is caused by pleiotropic effects from developmental genes, a lack of natural selection for survival in old age, environmental influences, or trade-offs between longevity and reproduction. As is true with many complex sets of hypotheses, these theories can complement each other. For example, Insulin-like Growth Factor-1 (IGF-1) is a hormone known to regulate aging with ties to development, DNA damage, environmental influences, the immune system, resistance to oxidative stress, and reproductive function. The most likely scenario is that many (if not all) of these components are important for how aging comes about. Without at least considering these varied viewpoints, it will be difficult to understand the multifaceted nature of the regulation of aging.

The environment regulates aging through sensory and hormonal signaling pathways

While the field of biogerontology has ballooned in recent years with advances in biochemistry, genetics, and related techniques, the first and best-studied method for regulating the aging process is a simple environmental manipulation: dietary restriction (DR). Eighty years ago Clive McCay discovered that decreasing caloric intake (without causing malnutrition) increased both median and maximum lifespan in rats, which he interpreted to be a result of retarded growth slowing the maturation process[2]. While its importance to aging was not recognized at the time, over the past 30 years many studies have confirmed that the effects of DR on aging are conserved across taxa, including nematodes[3-5], flies[6-8], and mice[9-11], and potentially both humans[12] and non-human primates[13-15]. DR does not just influence longevity, but also has profound effects on many aging-related diseases and pathologies including immune function, cancer, and glucose sensitivity[10,12,13,15-17]. Research into the underlying mechanisms of DR have found that the effects are not primarily mediated by restriction of calories, but instead by decreasing intake of specific nutrients, with amino acids such as methionine being of particular importance[18-20]. These results have pushed researchers to consider that DR may regulate aging not through slowing metabolism by blocking access to energy but instead through signaling pathways that recognize the presence of these key nutrients.

One potential mechanism for how the "signal" from environmental manipulations such as DR could be transmitted throughout the body is by hormone signaling pathways. While correlative evidence had long suggested an interplay between aging and hormones[21,22], the first genetic study to demonstrate that a peptidergic signaling

pathway can affect aging came from Cynthia Kenyon's lab with the *daf-2* mutant[23]. *daf-2* is the receptor for the insulin-like signaling pathway in the nematode *Caenorhabditis elegans*, with deletion of *daf-2* leading to an extension of lifespan by up to 130%[23]. Furthermore, the Kenyon lab (and others) found that *daf-16*, a homolog of the FOXO transcription factors, is required for the effect of *daf-2* on aging[23,24]. The transcription factor *daf-16* regulates the cleanup of aberrant protein aggregates[25], resistance to multiple types of stresses[26-28], and works together with a number of other cellular signaling pathways[29-31]. Further work found that the effects of insulin-like signaling on aging is conserved in fruit flies[32] and rodents[33], while correlative studies have demonstrated a link between longevity and FOXO3 polymorphisms in humans[34]. Despite the common link to nutrient intake, multiple studies have in fact found that insulin-like signaling and DR modulate lifespan independently of each other[8,35].

These results increased interest in searching for other hormones and similar molecules that could be involved in the aging process. Beyond insulin, several other long-range signaling pathways have been found to regulate aging and lifespan, including several neuroendocrine signaling pathways. Growth Hormone (GH)[36], Neuropeptide F/Y (NPF/NPY)[37], and LIPL-4[38] have all been found to regulate longevity, and several other neuropeptides are correlated with aging-related conditions[39-41]. NPY in particular has recently been linked to the effects of DR on lifespan in mice, though the authors note that the genetic background was not controlled in that study[37]. However, the mechanisms through which these hormones speed or slow the aging process are still hotly debated.

From a molecular perspective a reasonable assumption is that hormonal regulators of aging must modify the ability of cells to respond to insults such as oxidative stress or DNA damage, which could result from alterations in cell-autonomous biochemical signaling pathways. Many such signaling pathways have been discovered to regulate longevity including FOXO, the heat shock response[29,42], Sirtuins[43,44] and the Target of Rapamycin (TOR)[45,46], and others[47]. Some studies even suggest that these pathways may regulate longevity through cell-non-autonomous mechanisms. For example, overexpression of *Foxo* specifically in the muscle of fruit flies extends lifespan and decreases protein aggregates not only in the muscle, but also in the brain, retina, and fat body[48]. There appears to be significant interaction between these pathways, but how this crosstalk influences aging is not entirely clear[30,31,49].

Of course, something must be sensing the environmental cues (such as DR) in order to modulate downstream pathways. There are two methods through which relevant environmental changes might be sensed. The first is through internal sensors that detect changes within the organism's composition or physiology. Such sensors monitor parameters such as blood glucose, core temperature, or carbon dioxide and elicit responses to maintain homeostasis by modifying behavior and/or physiology. Presumably, a restricted diet might be sensed by a change in the levels of sugars or amino acids in the organism. Alternatively, external sensory inputs, such as taste or olfaction, detect stimuli and invoke the proper response. These inputs provide information about the external (as opposed to internal) environment, providing information about potential rewards or hazards. In this case, a restricted diet might be identified by decreased gustatory signaling due to decreased food intake.

While it is still an area of active study, results suggest that internal nutrient perception is important for the effects of diet on aging. AMP-activated protein kinase (AMPK) responds to increases in AMP when energy reserves fall, and can regulate both transcriptional pathways, such as *foxo*[50], and hormonal signaling, such as the glucagon-like hormone in flies (Adipokinetic hormone or *Akh*)[51]. Mammals also take advantage of ATP as a signal for energy status to regulate insulin signaling, as insulin-producing beta cells in the pancreas sense glucose metabolism in the cells through an ATP-sensitive potassium channel, Kir6.2, leading to release of insulin into the blood[52]. Beyond general energy status, the TOR pathway is commonly thought to sense amino acid levels, and perhaps non-coincidently, is a well-known regulator of aging[53]. In flies, some neurons in the brain express sugar-sensitive gustatory receptors[54], as do mammalian adipocytes[55], suggesting that internal "sensory neurons" may exist and respond to food intake, though the potential interplay between these cells and aging has not been sufficiently explored. Recent studies have demonstrated that mice can recognize and pursue not just sweetness from sugar, but the nutritive value as well[56]. Furthermore, recent work within our lab suggests that internal nutrient receptors detect the protein content of consumed foods, allowing flies to make value judgments based on their nutritional needs at the time. These internal protein valuations, which are communicated through serotonin signaling, can significantly extend lifespan when flies can build their own diet(Jennifer Ro, unpublished data).

Perhaps more surprising is that traditional, external senses can profoundly influence aging. The ability of sensory input to alter physiology is not a new concept: perception can directly influence hormonal secretions in humans and other animals. We have

decades of research on phenomena such as the Cephalic Phase of digestion, where the sight, taste, or even thought of food can stimulate release of gastrin into the blood and secretion of gastric acid and pepsin into the stomach[57]. However, the first indication that sensory input could regulate aging did not arrive until the community developed improved genetic tools and model systems. Pioneering work again from Cynthia Kenyon's group described evidence that mutating sensory cilia on neurons extended lifespan in *C. elegans*[58]. Following this result a number of high-profile studies have identified a number of senses, including temperature, pain, and multiple types of gustatory and olfactory inputs can either positively or negatively impact lifespan in a diverse range of species ranging from nematodes to mice[59-64]. One of these studies from our laboratory demonstrated that olfactory input could regulate longevity and that the effects of smell on lifespan were intertwined with DR[64]. This study found that when flies subjected to a low-calorie diet were provided the smell of a high-calorie diet, the beneficial effects of DR were significantly limited. These results suggest that a significant component of the organisms' response to DR involves their *perception* of the food in their environment, not just the value of the food itself.

Social interaction and emotions influence health and aging

If perception of simple environmental cues such as olfaction is sufficient to alter the aging process, it begs the question as to whether more complex environments might also be able to influence health and longevity. Perhaps the most complex interactions organisms have with their environment revolve around socialization. In humans, social interaction is required for healthy aging. Elderly individuals with stronger social support benefit from a significantly decreased risk of death[65,66]. This decreased risk may result

from the diseases being less severe or from improved recovery from the illness, though for some diseases incidence is also slightly decreased[67,68]. Social interaction also improves quality of life and decreases mortality for stroke patients[69]. These health effects are not limited to the elderly: social isolation can also increase the risk of cardiovascular disease and mortality in working-age individuals[70]. Numerous other studies demonstrate the value of strong social circles for aging and general health[71]. It is worth noting that negative effects might result from just the *perception* of social isolation (i.e., loneliness), independently of any objective measurement, which supports the hypothesis that perception of the environment can regulate health and aging just as much as the actual, objective environmental conditions[71-75]. However, the reasons underlying these effects are generally not well understood from a mechanistic perspective. The two explanations typically provided are that social networks motivate individuals to maintain their health and/or social networks allow individuals to better cope with emotional and psychological stress[76].

While the former explanation does not easily lend itself to study from a biological perspective, the decreased ability of socially isolated individuals to cope with stress is likely the result of altered signaling pathways within the body that are amenable to scientific study. Social isolation can directly impact health through decreased immune function[77] and altered neuroendocrine[78] and cardiovascular activity[70]. Specifically, social isolation appears to affect the hypothalamic-pituitary-adrenocortical signaling axis, demonstrating that social and emotional experiences can alter concrete biochemical signaling pathways to alter human physiology[78,79]. The effects of social interactions could also fall under the umbrella of emotions, specifically loneliness.

While many intuit that emotions such as loneliness, happiness, and sadness could impact health, there are a range of scientific studies that generally (though not universally[80]) agree[81-84]. Many studies have found that happiness increases longevity, with one study even suggesting that unhappiness can be as harmful as smoking, at least in healthy individuals[85]. One suggested mechanism for this effect is increased vagal tone[86-88], though whether this is causative or simply an associated sign is unknown. On the other hand, anger and hostility seem to be risk factors of cardiovascular disease, stroke, and decreased longevity[89-92]. Anger may have even stronger negative effects if that anger is suppressed[89]. Those who anger easily often have increased cholesterol, blood pressure reactivity and platelet activation, which could explain the health consequences of anger[90,92-94]. Despite the numerous studies linking human health and emotions, the mechanistic underpinnings of these effects are largely correlative and unable to suggest causality.

Model organisms can accelerate mechanistic studies of emotions

The use of model systems will allow researchers to uncover the principle mechanisms underlying emotions' impact on health. Mammalian systems are already in use for studying the effects of social interaction on health. Social isolation increases liver metastases in mice, presumably by suppression of immune function[95,96]. Also in mice, social interaction following experimentally-induced stroke decreased both mortality and infarct size and enhanced behavioral recovery, which tracked with brain-derived neurotrophic factor (BDNF) and neurogenesis[97,98]. However, while implications about loneliness and health could potentially be inferred from these results, many animal researchers actively avoid using emotional language to discuss their observations. In

fact, there is considerable pressure to remove language that is deemed “unscientific”. As a result of the subjective nature through which humans experience emotions, discussions regarding anger, fear, frustration, and happiness are often edited out of scientific manuscripts to avoid the perception that the researcher is anthropomorphizing the animal subjects. Yet at the same time, evidence is mounting that emotions exist in animals[99-102], and objectively studying the mechanisms through which emotions occur and alter behavior and physiology could profoundly impact human health.

Thus, we should not be avoiding research on emotions, but instead developing a structure to objectively study emotions in model systems. In a recent review[1], David Anderson and Ralph Adolphs laid out a framework to use even the simplest animal models to study emotion-like states(See Figure 1.1 for an overview). From their viewpoint, emotions are internal states in the central nervous system that arise from specific stimuli. This internal state then causes the behaviors, physiologic responses, and subjective feelings we associate with a given emotion. As emotion causes human feelings (as opposed to emotion *being* feelings), it is reasonable that animals can have emotional states yet not be consciously aware of them. They lay out four building blocks, which they term “emotion primitives”, which emotions have in common across species: scalability, valence, persistence, and generalization. Scalability refers to gradations existing within an emotion, as someone might go from annoyed to angry to furious. Valence, in this context, denotes that an “opposite” emotion will exist, such as happiness and sadness. For persistence, the effects of the emotions (i.e., changes in heart rate, hormone signaling, etc.) must remain after the stimulus is removed. This period can be

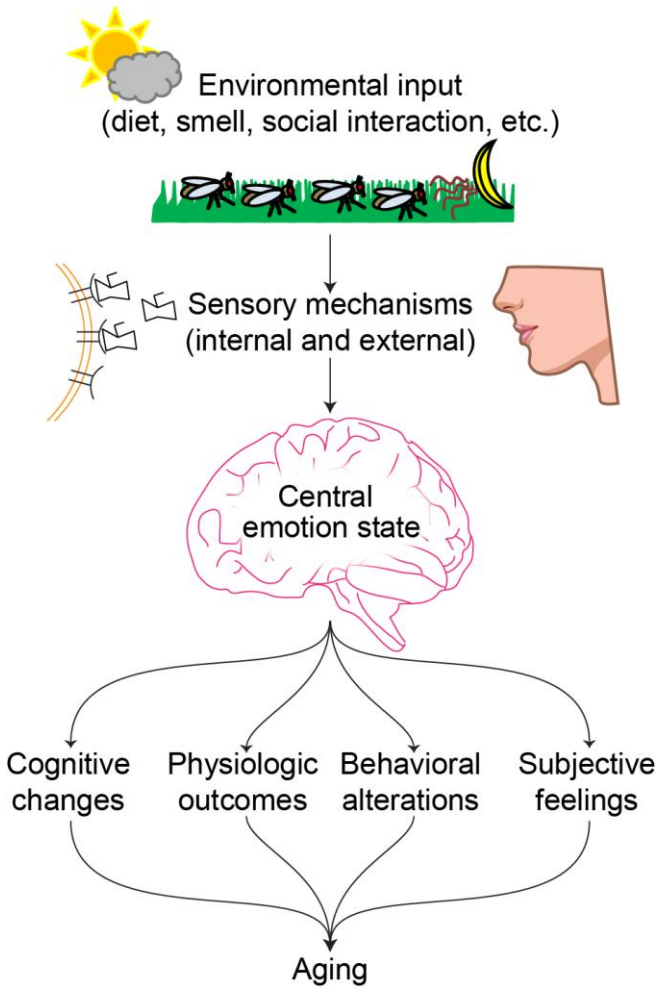

Figure 1.1: Model of the development of emotion states and how they could affect aging. This diagram is based in large part on Anderson and Adolph's guidelines for studying emotion in model systems. They propose "*…that an 'emotion' constitutes an internal, central (as in central nervous system) state, which is triggered by specific stimuli (extrinsic or intrinsic to the organism). This state is encoded by the activity of particular neural circuits that give rise, in a causal sense, to externally observable behaviors, as well as to associated cognitive, somatic, and physiological responses*"[1]. To this concept, we have made two slight additions to adapt this definition to studying the interaction between aging and emotions. First, we separated environmental inputs from the sensory mechanisms that recognize them. This was done to emphasize the importance of not just the stimulus, but also the perception of the stimulus in the aging process. Second, we placed aging downstream of the results of emotions. While it is possible to argue that aging should be included under the "physiologic outcomes" category, we feel this is inappropriate for two reasons. First, behavioral changes could also be responsible for changes in the aging process, such as a change in diet. Second, the direct physiologic effects of the central state are acute by nature, and unless the emotional state is maintained over time it is unlikely there are long-lasting effects on the aging process. These modifications allow for this pathway, along with the four required characteristics (generalization, scalability, persistence, and valence) to be adapted to study the mechanisms underlying how emotions regulate aging.

only a few minutes, or have longer-lasting repercussions. Finally, emotions must be generalizable, which simply means that emotions must have a range of pleiotropic effects. Thus, Anderson and Adolphs believe that when studying emotions researchers should search for behaviors that exhibit some or all of these "emotion primitives". Then researchers must prove that these behaviors result from changes in the internal state of the brain to conclude they are based on emotions.

Ideally, studies of these emotional states would be able to demonstrate that direct manipulation of the internal state of the brain can alter the output behaviors or physiology. The fruit fly *Drosophila melanogaster* is an ideal system for identifying potentially conserved circuits that can regulate emotions. As one of the most prolific model organisms, a wealth of methods and information exists regarding various fruit fly behaviors, many of which exhibit some or all of the emotion primitives discussed above[103-106]. While the fly brain is obviously simpler than mammalian brains, there is striking functional homology, with flies having brain regions similar to many mammalian structures including the hypothalamus, olfactory bulb, and basal ganglia[107-109]. The simpler brain may actually be advantageous, streamlining the process of identifying neuronal circuits while still having potential relevance for humans. Additionally, the vast fruit fly genetic toolbox allows for temporally- and geographically-specific activation or inhibition of neuronal subsets, providing the ability to directly examine both requirement and sufficiency with regards to emotions[110-113]. However, it is important to select "emotions" simple enough for the fly.

Reproductive drive may link primitive emotions and aging to a conserved mechanism

Perhaps one of the most fundamental emotions is the drive to reproduce, which we might term lust or attraction in humans[114]. However, from an insects' perspective, these can be filtered down to the drive to mate with *any* member of the species and the drive to select *the best* mate. Separate and specific neural circuits, hormones, physiologic changes, and behaviors have been characterized for lust and attraction in mammalian and insect literature[115-118]. In *Drosophila*, dopaminergic circuit activity correlates with changes in male libido with age[117], while visual detection of female size regulates male choice between and investment in females[118]. Many of the behaviors associated with courtship and mate selection are well-characterized and some even have well-mapped neural circuits, such as abdomen tapping and courtship songs[119-121]. Recent work has demonstrated that courtship in *Drosophila* leads to a distinct excitatory state within the central nervous system[122], similar to the emotional states discussed by Anderson and Adolphs[1]. There is also a plethora of evidence that correlates reproductive behavior to health and aging[123]. However, the mapping of the underlying signaling pathways has not progressed nearly to the point of the stereotyped behaviors discussed above.

These discrepancies can be chalked up to a difference in who is heading up the research in the field: evolutionary biologists are the primary researchers examining the interplay between reproduction and physiology, whereas neurobiologists focus on reproductive behaviors. Easily observed, stereotypic behaviors are ideal for mapping out brain circuits as they are immediately apparent upon initiation or ending and must directly result from neuronal pathways. Physiologic measures, on the other hand, are often time-

and labor-intensive and typically persist for longer periods after stimulus removal. From an evolutionary perspective sexual selection and reproductive drive have obvious implications, and while behaviors are important evolutionary scientists are more likely to be interested in the "end result". Reproductive success and survival are the key output parameters in the evolutionary field, and physiologic responses to reproduction have clear importance for both factors.

This interplay between reproduction and health has often been termed the "costs of reproduction"[123,124]. Mating and reproduction decrease health and longevity across a wide range of species, from nematodes to primates[125-130]. Even humans may not be exempt from these costs, with historical data suggesting that having more children may decrease lifespan[131,132], whereas eunuchs had significantly longer lifespans[133]. However, there is no agreement upon reproductive costs in humans, and a wide host of confounding factors make their study difficult[134]. The highly conserved nature of these costs suggests that the underlying mechanism is likely conserved as well. These costs can be either ecologic or physiologic in nature[135]. Ecologic costs of reproduction are commonly thought of as environmental dangers that result from reproductive behavior, such as increased risk of predation and disease. This can result from searching for and courting a mate, copulation itself, or caring for the offspring. In evolutionary or ecological contexts, these are clearly important drivers of reproductive costs. However, for researchers interested in using the costs of reproduction to study health and physiology, such costs would only serve as potential confounders. In laboratory settings protection from predators, disease, and parasites can diminish or even eliminate these effects and allow for the focus on the physiologic costs of reproduction.

The physiologic costs of reproduction are often explained as a result of the distribution of limited resources, with organisms "choosing" to direct energy toward maintenance of somatic tissue or investment in reproduction [123,135]. These costs have been best characterized in *Drosophila*, though the majority of research in the field focuses on females and reproductive behaviors such as egg-laying as opposed to survival and longevity[123]. Perhaps the discovery most closely resembling a molecular mechanism for these costs is Sex Peptide. Sex Peptide is transferred to females along with seminal fluid and leads to changes in physiology, feeding, and egg-laying behavior, which could explain how the "choice" between reproduction and survival is made [136-139]. Females that mate with males with reduced Sex Peptide have increased lifetime egg production and mate 12 times as frequently as females that mate with control males, yet have similar or even longer lifespans[136]. Sex Peptide acts on Sex Peptide Receptor in both in the reproductive tract as well as directly in the CNS, where it influences neural signaling[138,139]. These results suggest a potential role for perception (through internal sensors) in regulating these physiologic costs of reproduction. However, in males there has been little suggestion as to which pathways may be responsible. More recent work in the field suggests that these trade-offs (in both sexes) may be regulated through hormonal activity, with the most obvious candidate being insulin signaling[123]. However, as the ecological and evolutionary biologists that generally drive the field often focus on functional causes over molecular mechanisms, the signaling pathways underlying these costs remain understudied, though recent trends are beginning to correct this imbalance[123].

Costs of reproduction on survival may be separable from reproduction itself

If the costs of reproduction act through biochemical signaling pathways, then it is possible that the costs of decreased health and longevity may be separable from reproduction itself. However, as the concept of reproductive costs is rooted in life history theory it is often simply assumed to be true. Indeed, both common sense and life history theory suggest that maximizing the combined value of current and future reproduction is the optimal strategy, and therefore if current reproduction is separable from costs (which come in the form of declining future survival or fecundity) then such costs would cease to exist[124]. This inverse relationship between current and future reproduction has generally held true across an impressively wide range of experimental conditions and species, but potential exceptions have dotted the literature for years[140]. One set of studies found that predatory selection in flea larva can create a "super flea" that grows faster and reproduces quicker seemingly without cost[141]. Another experiment demonstrated that *Drosophila* selected for long-life can increase both lifespan and fecundity, though fecundity was only higher than controls under specific conditions[142]. While these experiments can (perhaps rightfully) be dismissed as a result of selecting for more fit organisms and not truly a violation of reproductive costs, other experiments provide evidence that specific disruptions can break the link between reproduction and longevity. For example, knockdown of *daf-2* in *C. elegans* adults can extend lifespan without a decrease in fecundity[143]. Conversely, ablating the germline and somatic gonad together blocks reproduction without extending longevity[23,144]. In light of these results, it is necessary to reconsider the fundamental nature of the costs of reproduction.

Justification for this project

With these experiments suggesting that the link between reproduction and survival is not as simple as the concept of reproductive costs would imply, our lab and others considered a potential role for sensory input in the costs of reproduction. When I joined the Pletcher laboratory, Drs. Christi Gendron and Tsung-Han Kuo had discovered that effects similar to the costs of reproduction could be induced by exposure to pheromones of the opposite sex, and that in males these effects are mediated through the pheromone receptor *pickpocket23* (*ppk23*) and neurons expressing the neuropeptide *npf*[145]. In flies, pheromones provide social signals including sexual identity, attractiveness, and availability to mate. The *ppk23* receptor has been demonstrated to be involved in courtship by perception of the attractive female pheromone 7,11- heptacosadiene (7,11-HD)[116,146,147]. Unbeknownst to us, other labs were investigating similar effects of pheromone perception in *C. elegans*[148,149]. These results suggested that the costs of reproduction could be stimulated without mating or reproduction, and brought to mind several questions that served as a jumping off point for this project.

Are these effects long-lasting or quickly reversible? Initial experiments by both myself and Drs. Gendron and Kuo suggested that the effects of pheromones on lifespan in fruit flies are reversible. However, when studying mortality rates (a population measure), care must be taken to ensure that a reversal in the population measures is indicative of actual decreases in individual probabilities of death[150]. Furthermore, due to a low sampling rate these initial experiments could not differentiate whether the timing of reversibility in mortality rates is similar to or distinct from the reversibility of other observed physiologic phenotypes. This is an important question, as a large variation in

the timing of effects could suggest that these phenotypes are the result of distinct molecular mechanisms. Beyond specific mechanisms, understanding the dynamics of pheromonal effects on longevity could inform the dynamics of reproductive costs as well, assuming the effects of pheromones on aging are actually the result of the costs of reproduction.

If pheromone exposure is sufficient for the costs of reproduction, what role does mating play? This is a two-part question: we must determine whether the effects of pheromone exposure are equivalent to the costs of reproduction, and if so then how mating impacts these costs. While data discussed previously in this chapter show both sensory perception and social signals can regulate health and aging, to our knowledge *ppk23* is the first "social receptor" known to influence longevity in any species. While we obviously do not expect a pheromone receptor to be conserved in humans, it is not unreasonable to think these effects might work through similar downstream mechanisms. This is especially true if we can demonstrate that the effects of pheromones, which could be considered as perception of the flies' social environment, regulate aging through the highly conserved "costs of reproduction". Human data does indicate that *perception* of the social environment has a stronger effect on health than the *actual* social environment. Perhaps the effects observed in these human studies are more subtle, complex manifestations of the same underlying mechanisms that drive the costs of reproduction.

Assuming that pheromones are sufficient to drive the costs of reproduction, it leaves three potential roles for mating. Mating could provoke further costs, decreasing lifespan even more than pheromone exposure alone. This would resemble the relationship

between DR and olfaction, where perception of high-calorie food reduces lifespan, and consuming that food decreases lifespan even further. Mating could also have no effect, which would suggest that the costs of reproduction are entirely due to perception. The final possibility is for mating to be beneficial. This would fly in the face of the concept of costs of reproduction, and an explanation that does not rely on direct energetic trade-offs would be necessary.

Could the effects of pheromones on aging be considered from the perspective of emotions? A beneficial effect of mating could potentially be explained by a change in the internal state of the brain (i.e. emotions) of the fly. Negative emotions have been shown to lead to health problems, and it is reasonable to assume that perception of the opposite sex without the ability to mate could be a stressor on flies, as it decreases resistance to stress[145]. Thus mating may decrease that stress by fulfilling the need of the organism, providing the reward that would be expected after exposure to the motivating force of pheromone perception. This could benefit the organism through either a unique pathway or the same pathway as the costs of reproduction. Differentiating these possibilities requires the ability to separate mating from pheromone perception, which is possible through either manipulation of pheromone profiles or blocking pheromone perception pathways. If primitive emotions can regulate aging in flies, the mechanism underlying both the emotions themselves and their effects on aging can be studied and perhaps translated to more complex organisms.

What are the downstream pathways through which pheromones alter longevity? To understand how emotions, pheromones, and the costs of reproduction could regulate aging, it is necessary to identify downstream pathways through which they enact these

effects. There are a handful of downstream pathways that most aging manipulations converge upon, including insulin-like signaling, Foxo, TOR, and Sirtuins. These mechanisms have generally been shown to regulate aging across a wide range of taxa, and thus if pheromone perception alters aging through one of these pathways it would support the possibility that the underlying mechanisms of how emotions or the costs of reproduction influence longevity may be conserved as well. Of course, finding novel mediators of the aging process would be exciting for a completely different reason: the opportunity to study a pathway that has not been explored in an aging context is a potential well of new discoveries. These new mediators of aging could be either at the same level as Foxo/Tor/Sirtuins, or alternatively could be upstream of these well-studied aging regulators.

On a more general level, does the internal state of the brain regulate the aging process? While the costs of reproduction are a highly conserved phenomenon that regulates health and longevity across taxa, ultimately we would like to understand how the brain as a whole modulates aging. The growing number of studies linking sensory inputs and aging indicates that perception of the environment impacts how organisms age. As the target of sensory signals the brain must also be involved in the aging process, but its role is far more mysterious. Though still a significant endeavor, the relatively simple brain and large genetic toolbox of *Drosophila* provides an ideal system for a thorough investigation of how the brain regulates aging. If direct manipulations of the brain's internal state can alter the aging process, it would suggest similar mechanisms for emotions (which can be considered brain states) to potentially regulate aging as well. Thus, these investigations could reveal "aging centers" within the brain, just as there are

centers for learning and memory, proprioception, and homeostasis, many of which have functionally conserved counterparts in the mammalian brain.

Emotions could regulate aging either directly, through changes in physiology, or indirectly, through changes in behaviors that impact health. While genetic tools and physiologic measurements are quite advanced in flies, behavioral assays often lag behind[151-153]. In the context of aging, feeding and dietary restriction is arguably the best-studied behavioral regulator, save perhaps reproduction. However, analysis of feeding behavior still relies on either labor-intensive, low-throughput assays or trinary colorimetric observations[154,155]. By improving both automation and resolution of feeding measurement in *Drosophila*, the interplay between behavior and neuronal signaling can be more accurately characterized. Furthermore, the development of new paradigms that allow for the analysis of complex behaviors throughout life will improve our understanding of how behaviors and internal states ultimately impact the aging process.

Summary of this project

Herein, we have demonstrated that an animal's internal neuronal state (i.e., primitive emotion) is capable of regulating aging in response to a dynamic, changing environment. Manipulation of the socio-sexual environment of male fruit flies leads to measureable changes in not only lifespan and physiology, but also a demonstrably altered neurometabolome. The effects of these manipulations are reversible upon removal of pheromone exposure, though the increased mortality rates can persist for weeks before returning to control levels. We also were able to regulate aging through direct manipulation of neuropeptidergic signaling. These effects on lifespan are independent of changes in behavior and diet, which we demonstrated through both an

innovative lifelong choice diet paradigm and the development of an entirely new device to accurately measure feeding preference over time. It is our hope that these studies will spur researchers to consider aging as a process that is dynamically regulated by the brain in response to environmental inputs, and not simply as the random accumulation of damage over time.

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Chapter II: Male Costs of Reproduction are Self-Imposed and Mediated by the Neural State[2]

The near-universal nature of costs of reproduction on longevity suggests that the mechanisms underlying these costs may be highly conserved across taxa and thus an ideal model for researching the biology of aging. Recent data demonstrating that perception of the opposite sex can alter lifespan suggested that such costs may be regulated, and brought into question the role of mating itself in precipitating these costs. The fact that perception of the opposite sex is sufficient for altering longevity also suggests that these effects on longevity may be regulated by the brain. Thus, we set out to examine both the role of mating in the costs of reproduction as well as the mechanisms underlying these costs, while using primitive emotions as a context for guiding and interpreting our data.

[2] Zachary Harvanek (ZMH) wrote and edited this chapter. Dr. Scott Pletcher (SDP) provided comments. Christi Gendron (CMG) and Tsung-Han Kuo (THK) performed the preliminary work discussed in the introduction. Joanne Yew (JYY) analyzed pheromone extracts sent by ZMH (data not shown). Daniel Promislow (DEP) analyzed neurometabolites sent by ZMH and created the plots in figure 2.3. CMG performed the experiment in figure 2.4 A. Brian Chung (BYC) performed the experiment in figure 2.4 B. Jacob Johnson assisted with the experiment in figure 2.4 D-F. SDP assisted with data analysis and visualization of the RNA sequencing experiment. The entire Pletcher laboratory, and CMG and SDP in particular, provided helpful comments regarding experimental design. All other experiments and analysis were performed by ZMH, with input from SDP.

Abstract

The aging process is actively regulated in response to environmental cues. The effects of social interactions on lifespan are commonly studied as a component of the idea of "costs of reproduction". We hypothesized that these reproductive costs are induced not be reproduction itself, but instead are regulated in response to perception of the social environment. Herein, we demonstrate that, at least for male *Drosophila melanogaster*, perception of pheromones of the opposite sex drive the "costs of reproduction" on fat stores, stress resistance, and lifespan. Mating itself is beneficial in the context of these pheromones, and has no effect in their absence. The short- and long-term effects are separable, with the fat storage and starvation phenotypes being dependent on AKH signaling, while the lifespan effects are not. All three phenotypes are mediated through the pheromone receptor *ppk23* and the transcription factor *dFoxo*. Furthermore, we demonstrate that the beneficial effects of mating require and are potentiated by CRZ signaling. These results provide a mechanistic basis for how social interaction may impact health and longevity over a lifetime. Exploration of the neural circuits underlying these phenotypes may reveal evolutionarily conserved circuits that connect CNS states similar to primitive emotions with downstream physiologic outcomes.

Introduction

Fitness trade-offs are a fundamental principle of evolutionary biology. Organisms with distinct mating strategies will invest time, resources, and effort in vastly different ways. In some spider species, male spiders may sacrifice themselves to be eaten by their mate, which appears to be favored by sexual selection as being eaten increases egg fertilization and decreases subsequent female re-mating[1,2]. On the other end of the scale, elephants

can be reproductively active for over 50 years, caring for their young for up to a decade after birth[3]. Even within species, individuals may vary their reproductive strategies to adapt to their environments. In some animals, these strategies have been linked to changes in neural signaling states. For example, in male wolf spiders increased octopamine signaling correlates with aggressive direct-mount mating tactics as opposed to courtship of the female[4]. In humans, there is evidence that traumatic experiences during development can lead to accelerated reproductive maturity[5], and in turn, decreased health and longevity.

One prominent trade-off posits that enhanced reproductive effort compromises future survival and limits lifespan. Such a fitness cost of reproduction is a major component of nearly all models of life history evolution[6-8]. Putative costs of reproduction have been characterized as either ecologic or physiologic in nature[8], and have been described in nearly all taxa, from brine shrimp[9], nematodes[10] and fruit flies[11] to rodents[12], primates[13,14] and even humans[15-17]. Ecologic costs of reproduction are commonly thought of as environmental dangers that result from reproductive behavior, such as increased risk of predation and disease. While clearly important drivers of reproductive costs in nature, ecologic costs are not relevant to an investigation using the costs of reproduction as an avenue to study how environmental conditions affect the aging process. Thus, we decided to focus on the physiologic costs of reproduction.

The physiologic costs of reproduction are often explained as a result of the distribution of limited resources, but relatively few studies have been designed to identify the underlying mechanisms of the costs[6,8]. Recently, our lab found that perception of pheromones of the opposite sex, without mating, was sufficient to decrease lifespan, fat

stores, and stress resistance[18]. These effects are mediated through the pheromone receptor *pickpocket23* (*ppk23*)[18], which recognizes the female pheromone 7,11-heptacosadiene (7,11-HD), and may recognize other molecules as well[19-21]. Inhibition of neurons expressing *neuropeptide F* (*npf*, homolog of mammalian NPY)[22] prevented the effects of pheromones on starvation resistance and fat stores, and activation of these neurons appeared to mimic these phenotypes[18]. These results suggested that the social environment may regulate aging through specific neural circuits.

Coinciding with this research and demonstrating its potential applicability across taxa, recent work in *C. elegans* identified reproductive and social signals that mediate longevity. In nematodes, simple perception of the opposite sex, without actual mating, was sufficient to induce effects similar to the costs of reproduction[23]. Other findings demonstrated that in nematode hermaphrodites sperm and seminal fluid regulated health and longevity through specific signaling pathways[24]. These findings confirm that perception of the sexual environment can regulate reproductive costs, and these costs of reproduction may not be solely the result of an expenditure of limited resources. However, the mechanism underlying this response as well as the role of mating itself remain largely unknown.

Based on these initial results, I asked two questions. First, if pheromones are sufficient to induce reproductive costs, what role does mating play in the costs of reproduction? Based on previous observations of the interaction between perception of nutrients and dietary restriction, a naïve hypothesis would be that mating further increases the negative effects of pheromones, but this has not been studied[25]. Second, what signaling pathways are responsible for regulating the effects of pheromones on aging? As

reproductive costs are commonly observed across numerous species, it is possible these mechanisms could represent highly conserved, physiologic regulators of the aging process.

As our initial results suggest *npf*-expressing neurons may be required for the effects of pheromones, a reasonable hypothesis is that the effects of pheromones on aging are mediated through a change in the neural state. Anderson and Adolphs have argued that such changes in the state of the central nervous system (CNS) can resemble primitive emotions, given that they fulfill specific conditions[26]. In their definition, primitive emotions are CNS states triggered by specific stimuli inducing phenotypes that are scalable, persistent, valent, and generalizable. The effects of pheromones could fit this description: they are triggered by a specific stimuli (pheromones) and trigger clear, general changes in behavior (e.g., courtship, aversion/attraction) and physiology (e.g., lifespan, fat stores, stress resistance).

Thus, we hypothesized that the male costs of reproduction result primarily from perception of the social environment, and not mating itself. Herein, we demonstrate that pheromone exposure triggers increased reproductive drive and persistent, scalable changes in physiologic outcomes. These effects on aging are opposed by mating, demonstrating opposite valences between pheromone exposure with sexual deprivation and pheromone exposure with mating. The beneficial effects of mating are specific to the context of pheromone exposure, reinforcing this opposition while simultaneously suggesting that the costs of reproduction are not inevitable but instead are a regulated response to perception of the opposite sex. This opposition between pheromones and mating extends to a general measure of the neural state, the neurometabolome.

Furthermore, we demonstrate that *corazonin* (*crz*)-expressing neurons are required for and potentiate the beneficial effects of mating, while providing evidence that supports the role of *npf* in the negative effects of pheromones. Finally, through transcriptome analysis and subsequent genetic manipulations we ascertain that the effects of pheromones are regulated through an insulin-independent *dFoxo* mechanism, and that the long- and short-term effects are separable. Based on these results, we conclude that the costs of reproduction are not the result of mating, but instead due to perception of the social environment inducing changes in the neural state consistent with a primitive emotion that in turn alters dFOXO signaling and thus aging and physiology.

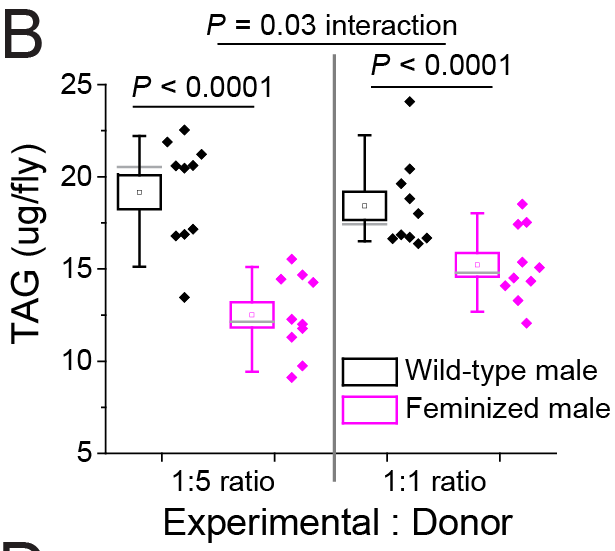
Results

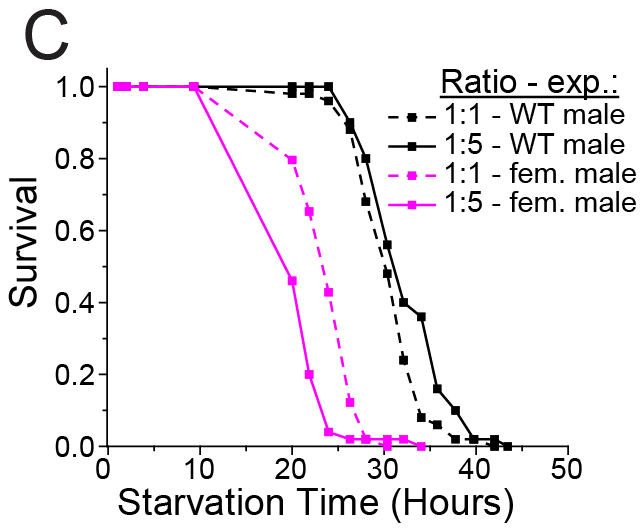
To test this hypothesis, we expanded upon previously used methods in Gendron et al. to independently manipulate both the perceived environment (i.e. pheromone exposure) and mating status of experimental males[18]. By exposing experimental males to donor males expressing female pheromones ("feminized males"), the effects of perceived mate availability without the ability to mate can be observed. Conversely, exposing experimental males to donor females expressing male pheromones ("masculinized females") allows observation of the effects of mating in the absence of mate perception (Figure 2.1 A). The pheromone profile of these feminized males and masculinized females is similar to the profile of wild-type female and wild-type males respectively, with the exception of cVA which is produced in the genitals and thus is not affected by our manipulation.

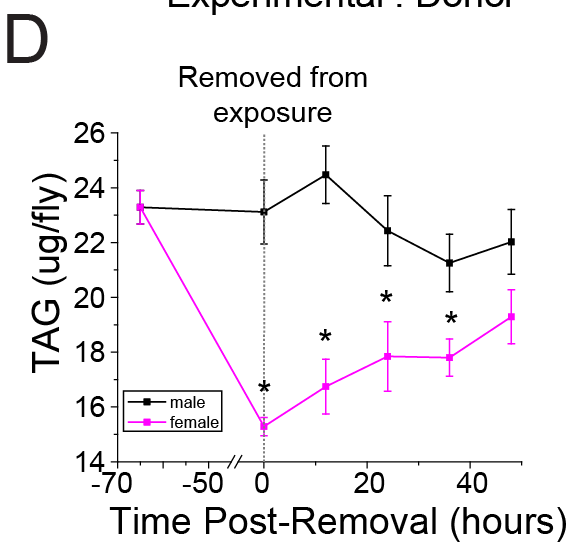
Pheromones drive persistent, scalable changes in physiology and increased reproductive drive. As the social environment likely effects aging through neural

A

	Male Pheromones	Female Pheromones
No Mating (Donors Male)	Wild-type Males	Feminized Males
Allows Mating (Donors Female)	Masculinized Females	Wild-type Females







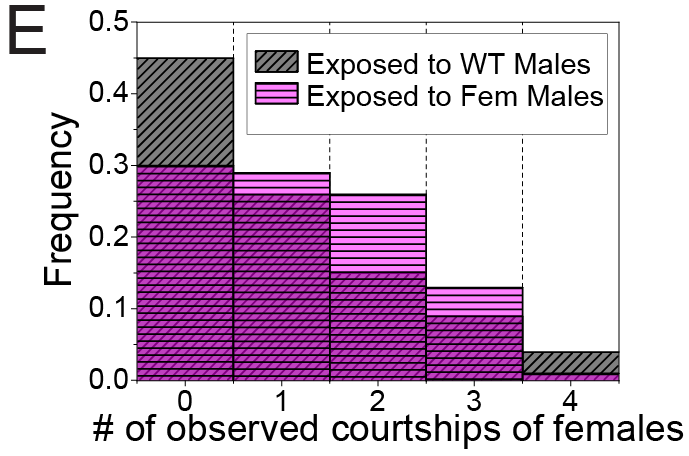


Figure 2.1: Pheromones drive scalable, persistent changes in physiology and increased reproductive drive. (A) Our method to isolate mating and pheromone perception. (B) Decreasing the ratio of experimental to donor flies from 5:25 to 15:15 per vial decreases the effect of pheromones on fat stores (interaction by two-factor ANOVA). (C) Decreasing the ratio of experimental to donor flies from 5:25 to 15:15 per vial decreases the effect of pheromones on starvation resistance (interaction: $P = 0.00019$ by cox regression). For both ratios, feminized male exposure decreases stress resistance ($P << 0.0001$). (D) Fat stores are progressively restored after removal (at t = 0) from feminized male exposure (N = 10 samples of 5 flies each for every time point). * = $P \leq 0.01$, no stars = $P \geq 0.05$ by t test. (E) 48 hours of exposure to feminized males increases subsequent courtship attempts toward females compared to wild-type male exposure ($P = 0.0456$ by two-tailed Mann-Whitney test).

circuits, we sought a framework to generate specific hypotheses to help characterize and identify these mechanisms. The concept of primitive emotions by Anderson and Adolph provides such a structure. Their theory posits that neural states similar to primitive emotions must be scalable, persistent, general, and valent[26], and we examined each of these requirements individually. Decreasing the dose of pheromone exposure leads to smaller changes in fat stores (Figure 2.1 B) and starvation resistance (Figure 2.1 C), demonstrating that the effects of pheromones scale with the ratio of donor to experimental flies. All measured phenotypes also persist for a period of time after removal of pheromone exposure. Removal from feminized male exposure leads to reversion of fat stores to control levels after 48 hours (Figure 2.1 D), and Drs. Gendron and Kuo observed similar results of pheromone removal on starvation resistance[18]. We have thoroughly investigated the dynamics of the reversal of mortality rates, which as shown in chapter 3 takes approximately five weeks. The multiple physiologic effects of pheromone exposure (e.g., changes in mortality, fat stores, and stress resistance) suggests a general response. Beyond that, pheromones alter behavior following exposure, as males exposed to feminized males court females more than males exposed to other wild-type males, suggesting pheromones may increase the drive to reproduce (Figure 2.1 E).

Mating partially reverses the effects of female pheromones, and has negligible effect in their absence. The requirement for valence suggests another neural state must oppose the effects of pheromones, and we hypothesized that mating could oppose the negative consequences of pheromone exposure. If true, this would suggest that mating would reverse the negative consequences of pheromones on physiologic measures. This hypothesis might also suggest that mating in the absence of female pheromones to be either beneficial or have no effect, a clear contrast to an energetic hypothesis of reproductive costs.

Thus, we asked how mating affects the lifespan of males in the presence of either male or female pheromones. As previously observed, exposure to female pheromones (without mating) significantly decreases lifespan of males (Figure 2.2 A). When mating is allowed in the presence of female pheromones, the negative effect of pheromone exposure is partially (though not completely) reversed, suggesting mating may be the valent opposite of pheromone exposure. Supporting this notion is the fact that in the presence of only male pheromones, mating has a negligible effect on overall lifespan. Thus, in the absence of female pheromones, there is essentially no effect of mating on longevity, demonstrating that mating is neither required nor sufficient to drive the costs of reproduction.

Next, we asked whether mating showed similar effects on other phenotypes. Indeed, mating partially rescues the negative effects of female pheromone exposure on both starvation resistance (Figure 2.2 B) and fat stores (Figure 2.2 C and Supplementary Figure 2.1 A). In the absence of female pheromones, mating had no effect on either phenotype, again suggesting that pheromones, and not mating itself, drive the costs of

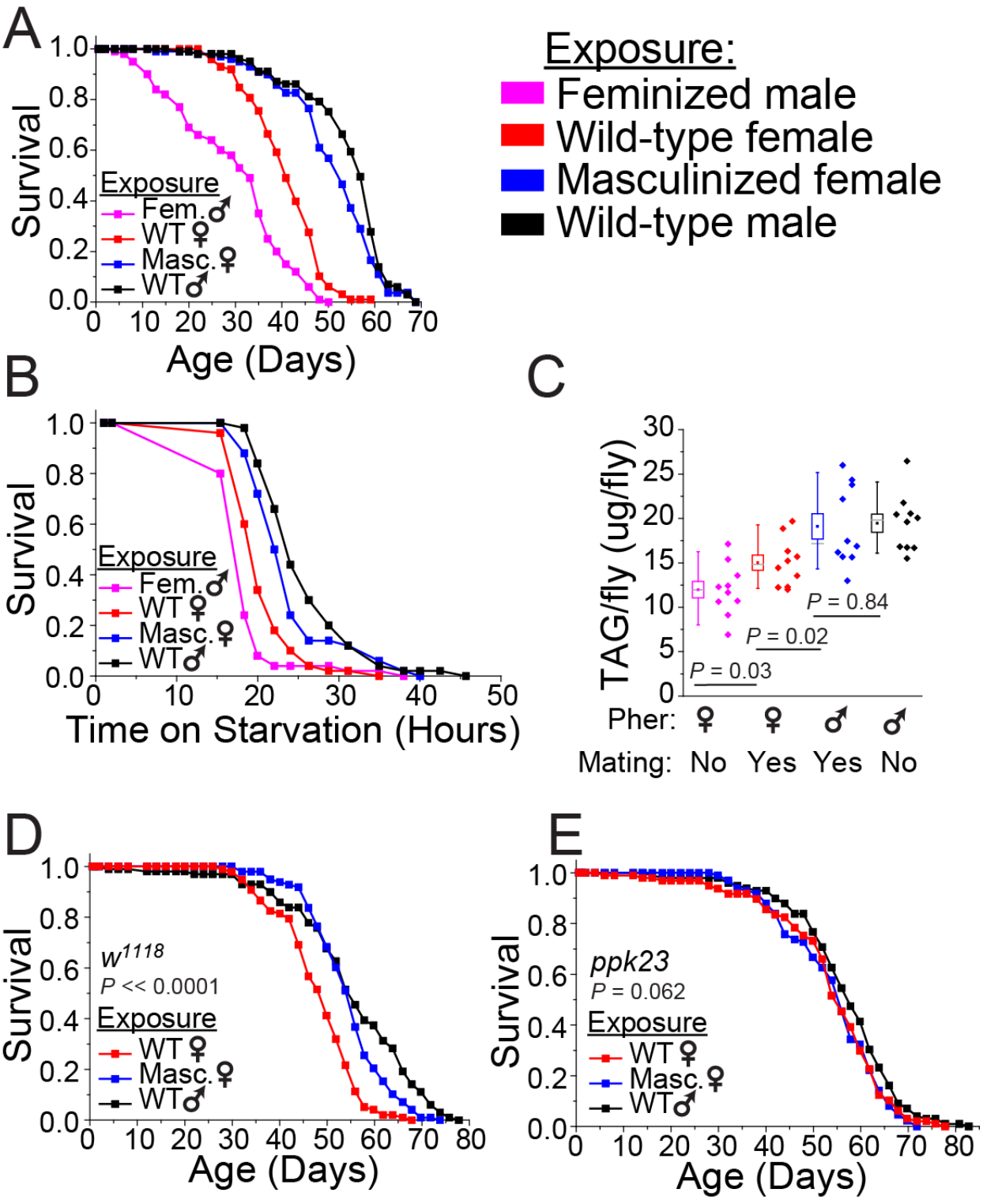

A
Survival
Age (Days)
Exposure
Fem.♂
WT♀
Masc.♀
WT♂
Exposure:
Feminized male
Wild-type female
Masculinized female
Wild-type male
B
Survival
Time on Starvation (Hours)
C
TAG/fly (ug/fly)
P = 0.03
P = 0.02
P = 0.84
Pher: ♀ ♀ ♂ ♂
Mating: No Yes Yes No
D
w1118
P << 0.0001
Survival
Age (Days)
E
ppk23
P = 0.062
Survival
Age (Days)

Figure 2.2: Pheromone exposure, and not mating, drives a change in the internal state of the CNS and the physiologic costs of reproduction. (A) In the presence of female pheromones, mating increases lifespan (comparing feminized male and wild-type female exposures $P << 0.00001$). When female pheromones are removed but mating is still allowed (i.e., masculinized female exposure), lifespan is further increased ($P << 0.00001$ comparing wild-type female and masculinized female exposures). In the absence of female pheromones, the effects of mating on lifespan are negligible ($P = 0.020$ comparing masculinized female and wild-type male exposures). (B) This same pattern is observed in starvation resistance, with mating increasing starvation resistance in the presence of female pheromones ($P = 0.0022$), removal of those pheromones further increasing resistance ($P = 0.00028$), and in the absence of female pheromones, the effects of mating are again negligible ($P = 0.16$). (C) Again, a similar pattern is observed on fat stores, with mating being beneficial in the presence of female pheromones, and having no effect in their absence (statistics are two-tailed t-tests). (D) w^{1118} controls exposed to wild-type females live significantly shorter than masculinized female ($P < 0.00001$) or wild-type male exposures ($P << 0.00001$). (E) *ppk23* mutants backcrossed into w^{1118} and exposed to wild-type females demonstrate no significant difference when exposed to either masculinized females ($P = 0.72$) or wild-type males ($P = 0.076$). *P* on plots (D) and (E) are from cox regressions testing the null hypothesis all three groups have equivalent survival curves.

reproduction in *Drosophila*. Both our short- and long-term phenotypes support our original hypothesis that the costs of reproduction are driven by perception of the social environment, and not simply energetics.

The most parsimonious explanation for the lack of negative effects in the masculinized female exposure is that the males are not mating with the masculinized females, and thus not incurring any costs of reproduction. There are several lines of evidence that establish this is not the case. We observed that, in both short-term and life-long exposure, fertilized eggs were produced in both wild-type and masculinized female vials, suggesting mating was occurring during the experiments. We also measured mating behavior of males with both types of females, and unsurprisingly found that, when given a choice, males will typically mate with a wild-type female before a masculinized female (Supplementary Figure 2.1 B). However, when only provided with one female male behavior toward masculinized females is not significantly different from that toward wild-type females in terms of both mating latency (Supplementary Figure 2.1 C) and

fertilization rates (Supplementary Figure 2.1 D). Thus, the negligible effect of mating in the absence of female pheromones is unlikely to be the result in changes in mating behavior.

Another alternative hypothesis to explain the difference between wild-type female and masculinized female exposures is that there may be unknown differences in the females besides their pheromone profiles. To address this possibility, we examined the hypothesis that pheromones drive the costs of reproduction from a genetic viewpoint using a loss of function mutant for the pheromone receptor *ppk23* in experimental males[21]. As expected, exposure of control males to wild-type females decreased lifespan compared to both masculinized female and wild-type male exposure (Figure 2.2 D). However, in *ppk23* mutant males, exposure to wild-type females does not significantly affect lifespan when compared to masculinized female or wild-type male exposures (Figure 2.2 E). In terms of mean lifespan, only control males exposed to wild-type females showed a significant change from the rest of the experimental groups (Supplementary Figure 2.2 A). Thus, pheromone perception, through the *ppk23* pheromone receptor, is required for the costs of reproduction to be incurred, and blocking pheromone perception by mutation of *ppk23* eliminates the differences in lifespan between wild-type female and masculinized female exposures.

These data can also be used to ask whether mutation of *ppk23* can extend lifespan in mixed sex populations. When comparing across experimental genotypes, we find that *ppk23* mutation does not affect lifespan upon exposure to wild-type males (Supplementary Figure 2.2 B) or masculinized females (Supplementary Figure 2.2 C), but extends lifespan upon exposure to wild-type females (Supplementary Figure 2.2 D).

These results demonstrate that mutation of *ppk23* is sufficient to extend lifespan specifically in males housed with wild-type females. Reproductive output from the *ppk23* males (as measured by offspring/vial) was surprisingly higher than that of control males (Supplementary Figure 2.2 E), providing further evidence that reproduction itself is not costly. Similar effects on lifespan were observed in a repeat experiment performed with non-virgin females (Supplementary Figure 2.2 F).

Beyond lifespan, it is possible that other reproductive costs more directly related to energetics might not be regulated by *ppk23*. We found that mutation of *ppk23* prevents pheromone effects on starvation resistance (Supplementary Figure 2.2 G) and fat store phenotypes (Supplementary Figure 2.2 H) in both the presence and absence of mating, with mating having a potentially beneficial effect. In combination, these results reinforce the finding that mating is not sufficient for the induction of these costs, and suggest that the negative effects of exposure to feminized males work through the same pathways as the negative effects wild-type female exposure.

Pheromones and mating have opposing effects on the neurometabolome. The opposing effects of pheromones and mating almost certainly alter the neural state, and based on the framework proposed by Anderson and Adolph[26], mating and pheromones might create general, antithetical changes in the brain. To answer this question, we first asked how female pheromone exposure alters the neural state as measured by the neurometabolome. We measured metabolite quantities present in heads of males exposed to wild-type males, wild-type females, or feminized males as a measure of the state of the brain. Principle component analysis (PCA) of the neurometabolome revealed that exposure to wild-type females and feminized males induces similar changes in the

neurometabolome. The neurometabolomes of these groups were found to be distinct from that of males exposed to other wild-type males (Figure 2.3 A). Thus, pheromone perception induces a change in the state of the brain. Additionally, the similarity between feminized male and wild-type female exposure have similar effects provides further evidence that pheromone perception is the driving force behind the physiologic costs of reproduction that are incurred by males, even when mating is available.

Because pheromone exposure results in detectable changes in the neurometabolome, we next asked whether mating can reverse some of the effects of pheromones on the neural state. Thus, we performed a second experiment, replicating the original experiment with the addition of a group of males exposed to masculinized females to allow a balanced analysis of the effects of mating and pheromones. Analysis of the repeat experiment without the masculinized female group provides similar results to the previous experiment (Figure 2.3 B). When the masculinized female exposure is included in the analysis, mating in the presence of female pheromones (i.e. wild-type female exposure) to drive the neurometabolome toward wild-type male exposure and away from feminized male exposure (Figure 2.3 C). However, mating in the presence of male pheromones (i.e. masculinized female exposure) has little effect on the neurometabolome, causing a shift in the opposite direction if causing any effect at all. These results are highly reminiscent of the effects of the exposures on lifespan, and suggest that female pheromone exposure causes alterations in the male neural state and at least some of these alterations are opposed by mating.

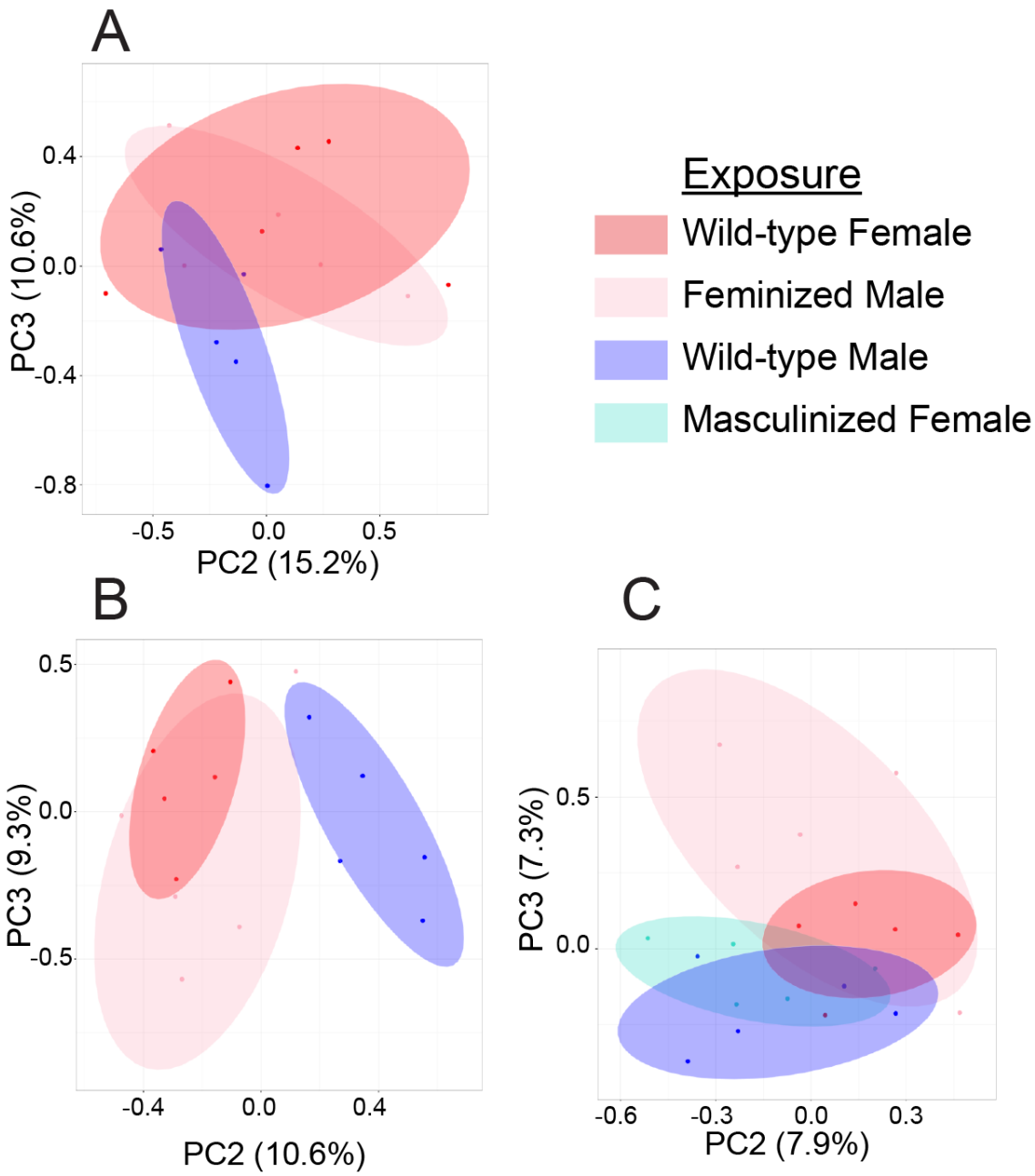


Figure 2.3: Pheromones induce a change in the neurometabolome of the brain. (A) When males are exposed to feminized males, their neurometabolome is more similar to that of males exposed to wild-type females than that of males exposed to other wild-type males (N = 508 features). (B) Analysis of a replicate experiment disregarding masculinized female exposure data largely replicates the pattern observed in 2.3A (N = 445 features). (C) When masculinized female data is considered, mating interacts with pheromone exposure to produce a pattern similar to that observed with longevity along the third principle component, with wild-type male and masculinized female exposures most similar, followed by wild-type female, and then feminized male exposure (N = 404 features). For all plots, positive mode data are presented, with shaded regions marking the 75% confidence intervals. K ≥ 3 features/treatment used as cutoff for each metabolite.

Neuropeptidergic signaling regulates the positive and negative effects of mating and pheromones on aging, respectively. In addition to measuring changes in the global neural state, we sought to identify specific subsets of neurons that are required for the effects of pheromones and mating. As previously mentioned, Drs. Gendron and Chung found that inhibition of *npf*-expressing neurons through expression of a temperature-sensitive dynamin gene mutant *shibire* (*shi*^{ts1}) prevents the effects of pheromones and activation of these neurons mimics their effects(Figure 2.4 A, B)[18,27]. Both I (Figure 2.4 C) and Dr. Gendron[18] have found *npf* transcript levels are increased upon pheromone exposure. Beyond these results, preliminary data utilizing a putative *npf* hypomorph to implicate *npf* in the effects of pheromones on lifespan are also included in the discussion chapter. These data suggest that *npf*-expressing neurons regulate the negative consequences of pheromone exposure.

Opposing neural states can be mediated by differing sets of neurons[28], and as mating and pheromones are distinct inputs, they may act through different neural circuits. We hypothesized that neurons expressing the GnRH homolog *corazonin* (*crz*) may mediate the beneficial effects of mating, as *crz* is linked to both stress resistance[29,30] and male copulation and ejaculation[31]. To examine this possibility, we independently manipulated pheromone and mating status of males while simultaneously manipulating CRZ signaling using temperature-based methods[32-34]. While control males responded generally as expected (Figure 2.4 D), we found that inhibition of *crz*-expressing neurons prevented the beneficial effects of mating on lifespan (Figure 2.4 E). Conversely, continuous activation of *crz*-expressing neurons led to a potentiation of the beneficial effects of mating (Figure 2.4 F). However, it is notable that these increased beneficial

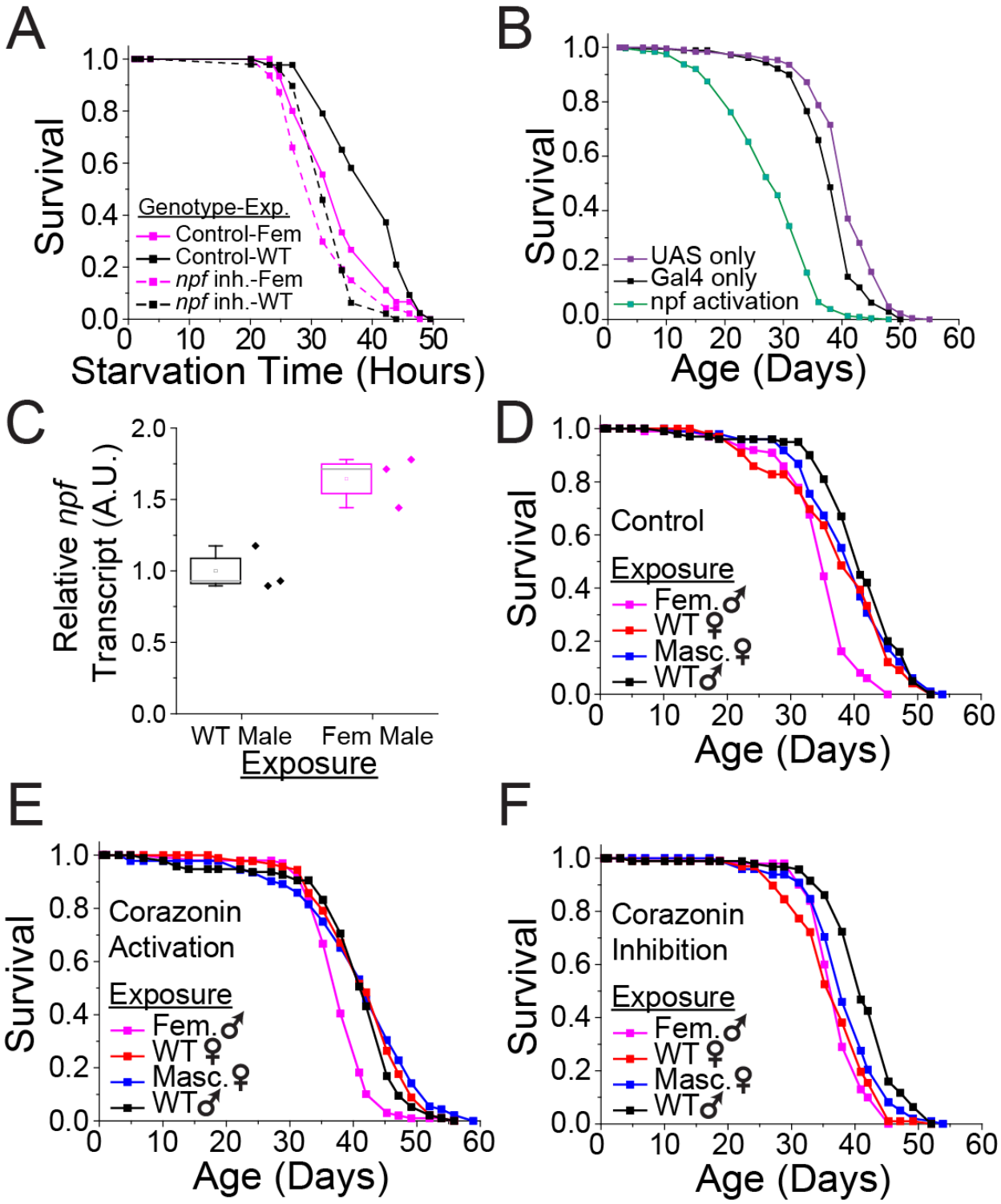

A
Survival
Starvation Time (Hours)
Genotype-Exp.
Control-Fem
Control-WT
npf inh.-Fem
npf inh.-WT
B
Survival
Age (Days)
UAS only
Gal4 only
npf activation
C
Relative npf Transcript (A.U.)
WT Male
Fem Male
Exposure
D
Survival
Age (Days)
Control
Exposure
Fem.♂
WT♀
Masc.♀
WT♂
E
Survival
Age (Days)
Corazonin Activation
Exposure
Fem.♂
WT♀
Masc.♀
WT♂
F
Survival
Age (Days)
Corazonin Inhibition
Exposure
Fem.♂
WT♀
Masc.♀
WT♂

Figure 2.4: *npf-* and *crz-* expressing neurons are required for negative and positive valences of pheromones and mating, respectively. (A) Inhibition of *npf*-expressing neurons using the temperature-sensitive dynamin mutant *shibire^{ts}* (npf-inh. = *npf-Gal4* x *UAS-shi^{ts1}*) prevents the effects of pheromones (P = 0.50), unlike in Gal4-only controls (*npf-Gal4* x *w^{1118}* P = 0.0050). (B) Activation of *npf*-expressing neurons using the temperature-sensitive cation channel *TrpA1* (*npf-Gal4* x *UAS-TrpA1*) decreases lifespan compared to Gal4-only and UAS-only controls (P << 0.00001). No difference was observed at non-activating temperatures. (C) Wild-type flies exposed to feminized males have significantly increased *npf* expression as measured by qPCR (P = 0.0090 by two-tailed t-test). (D) In the control genotype (*w^{1118}* x c*rz-Gal4*), we see responses similar to that seen in Figure 2 A, with feminized male exposure leading to the shortest lifespan (P < 0.00001), a marginally significant decrease in the wild-type female exposure compared to the wild-type male exposure (P = 0.064), and no change comparing masculinized female exposure to wild-type male exposure (P = 0.34). There is no significant difference between wild-type female and masculinized female exposure in this experiment (P = 0.38), likely because of temperature effects (these experiments were performed at 29C, and the high temperature compresses lifespan effects). (E) When *crz*-expressing neurons are inhibited (*UAS-kir2.1;tub5-Gal80^{ts} x crz-Gal4*), the beneficial effect of mating in the presence of female pheromones is eliminated (P = 0.48 comparing feminized male and wild-type female groups). There is also a decrease in lifespan of males exposed to masculinized females compared to wild-type male exposure (P = 0.0027). (F) When *crz*-expressing neurons are activated (*UAS-TrpA1 x crz-Gal4*), the beneficial effects of mating are potentiated, with mating now completely rescuing the negative effects of pheromone exposure (P = 0.31 comparing wild-type females to wild-type males). When comparing across genotypes, exposure to wild-type males leads to no differences (for each of the three comparisons, P > 0.86). However, exposure to wild-type females leads to a decrease in lifespan upon inhibition (P = 0.0044 compared to control) and an increase in lifespan upon activation (P = 0.0047 compared to control).

effects still require mating, as activation of *crz*-expressing neurons alone is not sufficient to cause this benefit. Thus, male costs of reproduction are modulated by changes in specific neuronal signaling pathways (*npf* and *crz*) which mediate the effects of pheromones and mating, respectively.

dFoxo regulates the physiologic effects of pheromone exposure. To identify how pheromone perception may alter physiological responses in peripheral tissues, we examined changes in gene expression using whole-genome RNA-sequencing technology in wild-type males exposed to feminized or other wild-type males, using *ppk23* mutants as a control. We found 195 genes with significantly different expression (using an experiment-wise error rate of 0.05) in wild-type male flies that were exposed to feminized or control donor males for 48 hours. Nearly all (188/195 = 96%) of the

changes appeared to be due to pheromone perception because they were not observed in identical experiments using *ppk23* mutant flies (Supplementary Figure 2.3, and Supplementary Table 2.1). Males exposed to female pheromones decreased transcription of genes encoding odorant-binding proteins and increased transcription of several genes with lipase activity. A significant enrichment was observed in secreted molecules, which includes genes encoding proteins mediating immune- and stress-responses. Many of these genes and pathways were highlighted in a recent meta-analysis of gene expression changes in response to stress and aging[35]. Interestingly, several of these genes such as *Pepck* and *ImpL2* are considered to be targets of *dFoxo*, a well-known regulator of aging[36,37].

We used these data as well as common aging and reproductive pathways as a guide to selection of candidate genes that may be important for mediating the costs of reproduction throughout the body. Because of the large number of candidates, we screened for potential hits using the shorter-term phenotypes of starvation resistance and fat stores. Of the lines screened, 2 of 16 initially demonstrated no significant effect of pheromone exposure (Table 2.1). Repeating the potential hits demonstrated that two genes consistently prevented the effects of pheromones on either starvation resistance or fat stores: the transcription factor *dFoxo* (by insertion mutant $dFoxo^{w24}$ [38,39] Supplementary Figure 2.4 A) and the glucagon receptor homologue, *AkhR* (by deletion mutants $AkhR^{1}$ and $AkhR^{2}$ [40] Supplementary Figure 2.4 B). These results suggested that the effects of pheromones, and therefore the costs of reproduction, may be mediated through the insulin-glucagon axis.

Table 2.1: Targeted screen of potential downstream effectors for the costs of reproduction: We examined the effect of candidate genes/drugs on the pheromone response to either TAG or starvation resistance. In our screen, only two genes, AkhR and dFoxo, significantly reduced the effects of pheromones compared to controls. In the screen, genetic controls were used if available. If not, y^-w^- was used as the control to ensure a response occurred. For drug trials, y^-w^- flies were used and exposed to either vehicle + drug or vehicle alone (control).

Gene/Drug	*P* value	control *P* value
AkhR	0.0776	1E-14
Apoltp	7E-13	1E-09
CG10592	3E-09	1E-14
CG31272	5E-14	1E-14
CG6296	3E-09	2E-08
Diff	0	2E-16
Foxo	0.773	0.077
GstD2	2E-04	2E-08
ImpL2	6E-07	1E-09
Retinoic Acid	0	4E-10
Pepck	2E-11	1E-09
Relish	0.0004	0.1
Sodh2	1E-06	1E-14
sug	4E-06	2E-08
Rapamycin	2E-05	2E-06
UPD3	4E-07	2E-08

First, we asked whether *dFoxo* was required for the costs of reproduction. A second, more severe mutation of *dFoxo* (*dFoxo*$^{\Delta 94}$)[38] also results in no effect of pheromones on starvation resistance (Supplementary Figure 2.4 C), and a trans-mutant of the two *dFoxo* alleles also prevents the response (Figure 2.5 A). After backcrossing the *dFoxo*^{w24} mutation into a different (y^-w^-) background, feminized male exposure still does not alter starvation resistance (Figure 2.5 B) or fat stores (Figure 2.5 C) in *dFoxo* mutants. Additionally, pheromone exposure altered the expression of the *dFoxo* target gene *4EBP* (*Thor*)[36] (Figure 2.5 D). Furthermore, the *dFoxo*^{w24} mutation significantly reduced the effects of pheromones on lifespan (Figure 2.5 E), and the *dFoxo*$^{\Delta 94}$ mutation almost

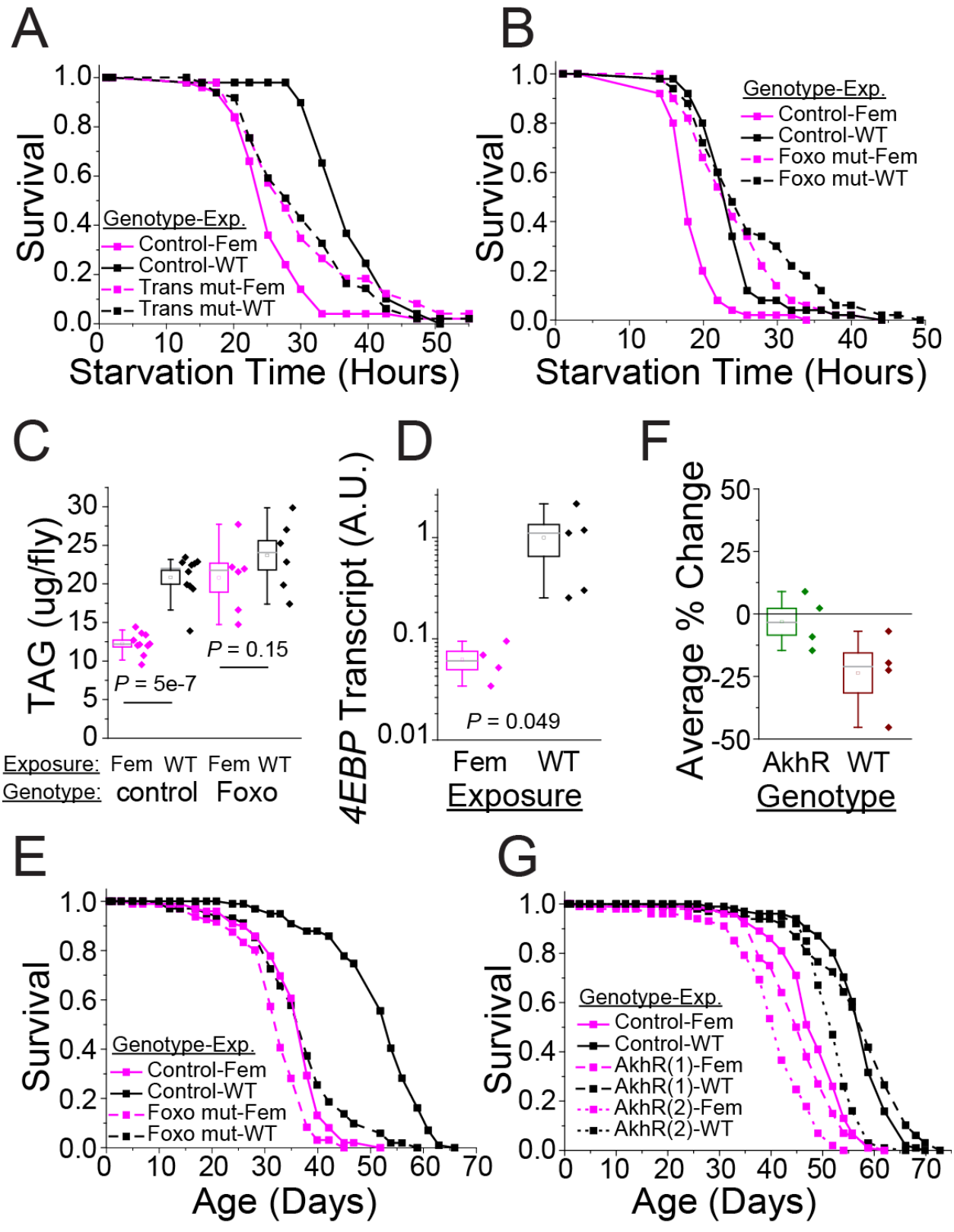

A
Survival
Starvation Time (Hours)
Genotype-Exp.
Control-Fem
Control-WT
Trans mut-Fem
Trans mut-WT
B
Survival
Starvation Time (Hours)
Genotype-Exp.
Control-Fem
Control-WT
Foxo mut-Fem
Foxo mut-WT
C
TAG (ug/fly)
P = 5e-7
P = 0.15
Exposure: Fem WT Fem WT
Genotype: control Foxo
D
4EBP Transcript (A.U.)
P = 0.049
Fem WT
Exposure
F
Average % Change
AkhR WT
Genotype
E
Survival
Age (Days)
Genotype-Exp.
Control-Fem
Control-WT
Foxo mut-Fem
Foxo mut-WT
G
Survival
Age (Days)
Genotype-Exp.
Control-Fem
Control-WT
AkhR(1)-Fem
AkhR(1)-WT
AkhR(2)-Fem
AkhR(2)-WT

Figure 2.5: The costs of reproduction are mediated through *dFoxo* and AKH signaling. (A) In trans-het mutants for *dFoxo* ($w^-;+;dFoxo^{w24}/dFoxo^{\Delta 94}$) the effects of pheromone exposure on starvation resistance are eliminated ($P = 0.82$). In control flies ($w^-;+;dFoxo^{w24}/+$) pheromone exposure still decreased starvation resistance ($P << 0.00001$). (B) Backcrossed $dFoxo^{w24}$ mutants are resistant to the effects of pheromones on starvation resistance ($P = 0.10$), while genetic controls (y^-w^-) respond as expected ($P = 0.0012$). (C) Backcrossed $dFoxo^{w24}$ mutants are resistant to the effects of pheromones on fat stores, while genetic controls (y^-w^-) respond as expected (one-tailed t-tests). (D) Wild-type males exposed to female pheromones have significantly lower Thor expression than males exposed to wild-type males (two-tailed t-test, confirmed with repeat experiment with $P = 0.026$). (E) $dFoxo^{w24}$ mutants are significantly more resistant to the effects of pheromones on lifespan than y^-w^- genetic controls ($P = 5.3 \times 10^{-7}$, interaction term from a cox regression). It is notable there is still a significant response in the $dFoxo^{w24}$ mutants ($P < 0.00001$), though a more severe mutation almost completely eliminates the effects of pheromones on lifespan (Supplementary Figure 2.4 D). (F) Summary of four experiments examining response of fat stores to pheromone exposure in *AkhR* mutant vs. revertant control response ($P = 0.038$, one-sided t-test). (G) Two separate AkhR mutants do not prevent, or even reduce, the effects of pheromones on lifespan ($P << 0.00001$ comparing within any genotype).

completely eliminates the effects (Supplementary Figure 2.4 D). These effects on lifespan are specific to *dFoxo* signaling and not found in other aging pathways, as manipulation of both the TOR and *sir2* pathways did not prevent the effects of pheromone perception on lifespan (Supplementary Figure 2.4 E, F). Combined, these results strongly suggest that *dFoxo* is required for the effects of pheromones on the costs of reproduction.

The effects of pheromones on lifespan are separable from the effects on fat stores.

Further examination of the other hit from the initial screen, *AkhR*, revealed that it is required for the effects of pheromones on fat stores (Figure 2.5 F), and partially required for the effects of pheromones on starvation resistance (Supplementary Figure 2.4 G). However, two independent mutations of *AkhR* demonstrate that *AkhR* is not required for the effects of pheromones on longevity (Figure 2.5 G). These results demonstrate that the short- and long-term effects of pheromone perception are separable, and that the decrease in fat stores do not cause the decrease in longevity.

The effects of pheromones are independent of insulin-like signaling. The most studied regulator of *dFoxo* is insulin-like signaling, which in *Drosophila* consists of several insulin-like-peptides (*dILPs*), and thus we asked whether pheromone exposure modulates the dILP pathway. Surprisingly, pheromone exposure does not alter the levels of circulating *dILP2* in the hemolymph, suggesting pheromones modulate *dFoxo* through an insulin-independent mechanism (Figure 2.6 A). Indeed, mutation of three important insulin-like peptides (*dILP2*, *dILP3*, and *dILP5*) did not prevent the effects of pheromone exposure on longevity (Figure 2.6 B) or starvation resistance (Figure 2.6 C)[38]. These results suggest that pheromone perception modulates *dFoxo* activity (and aging) through an insulin-independent mechanism.

Discussion

In this chapter, we have demonstrated that, at least in male *Drosophila*, the "costs of reproduction" are not actually due to reproducing, but instead are the result of pheromone perception through the *ppk23* receptor. Mating in the absence of female pheromones (or the ability to perceive them) has no negative effect, and in the presence of female pheromones, mating is beneficial, opposing the negative consequences of pheromone exposure. Pheromones induce a change in the neurometabolome, and this change is opposed by mating. Specific neural circuits mediate these pheromonal effects, with *npf*-expressing neurons associated with the negative effects of pheromones and *crz*-expressing neurons required for the beneficial effects of mating. Downstream, pheromones alter physiology though decreased *dFoxo* signaling, which is mediated through a dILP2/3/5 independent mechanism. Finally, these effects on longevity can be separated from its effects on fat stores, as demonstrated by mutation of *AkhR*.

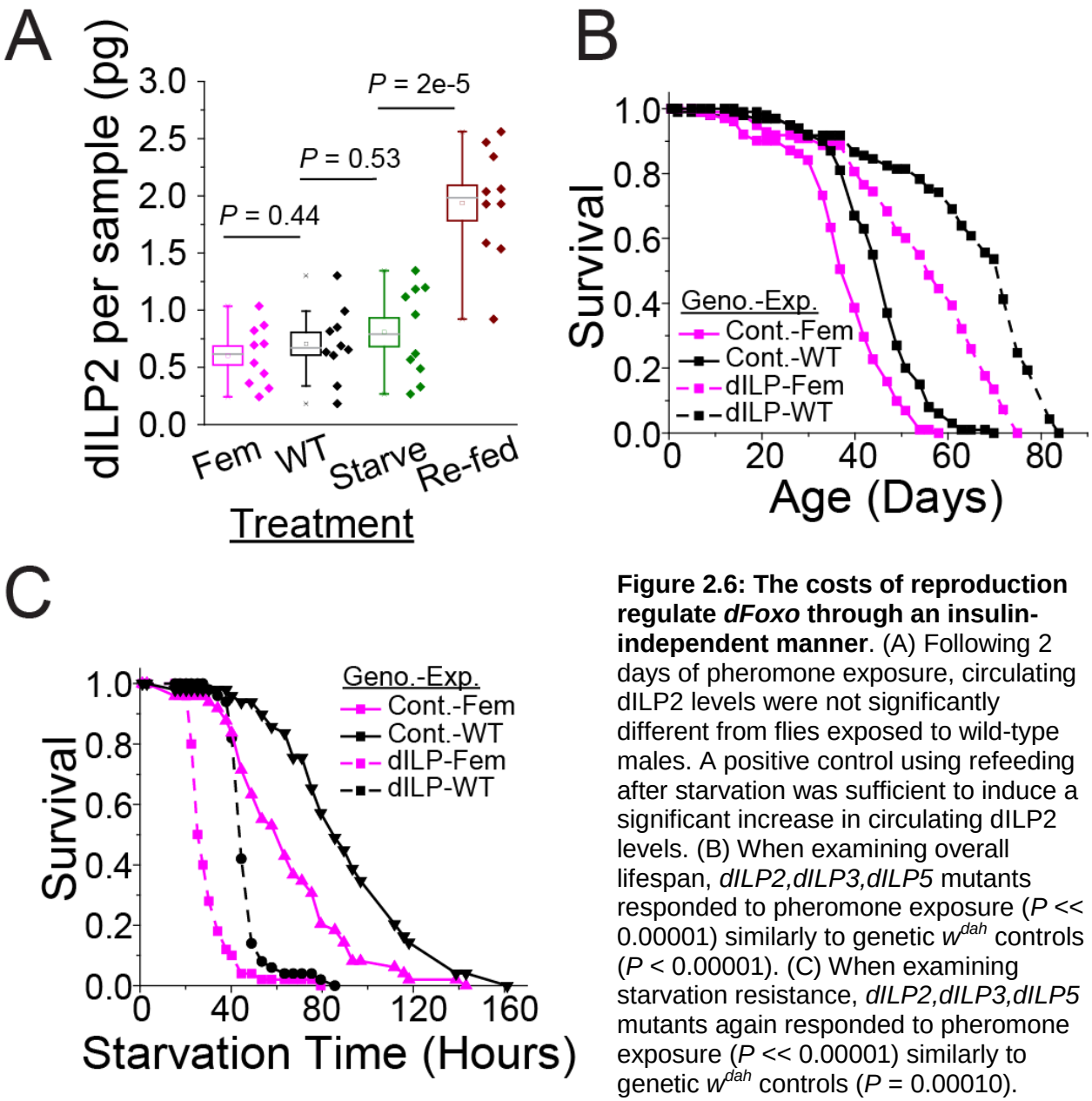


Figure 2.6: The costs of reproduction regulate *dFoxo* through an insulin-independent manner. (A) Following 2 days of pheromone exposure, circulating dILP2 levels were not significantly different from flies exposed to wild-type males. A positive control using refeeding after starvation was sufficient to induce a significant increase in circulating dILP2 levels. (B) When examining overall lifespan, *dILP2,dILP3,dILP5* mutants responded to pheromone exposure ($P << 0.00001$) similarly to genetic w^{dah} controls ($P < 0.00001$). (C) When examining starvation resistance, *dILP2,dILP3,dILP5* mutants again responded to pheromone exposure ($P << 0.00001$) similarly to genetic w^{dah} controls ($P = 0.00010$).

These studies identify multiple signaling pathways that mediate the costs of reproduction, but the connections underlying how these signals interact is still unclear. Expression patterns suggest that *ppk23*-expressing neurons do not interact directly with *npf*-expressing neurons[20,41], and to our knowledge this is the first study to suggest a link between NPF signaling and *dFoxo* activity. How *crz*-expressing neurons act to oppose these effects is also unclear, though CRZ signaling is known to regulate insulin-

producing cells[30], and thus the beneficial effects of *crz*-expressing neurons may be insulin dependent even though the negative effects of pheromones are not. By dissecting these signaling pathways, future studies may identify complete neural circuits that mediate how reproduction, social interaction, and the brain regulate the aging process.

Reproductive costs are often considered from an evolutionary viewpoint, with the idea that the consequences of mating must be balanced with survival[6]. In this chapter, we found that these trade-offs are not a requirement of reproduction, but instead are regulated by specific neural circuits in the resulting from perception of the environment. In fact, our results suggest that successful mating actually partially reverses the costs of reproduction through a process mediated in part by *crz*-expressing neurons. By improving the survival of males that successfully mate over those that fail in courtship, more attractive males gain even more of an advantage. This in turn could accelerate the selection of advantageous alleles, and could be an important driver of evolution.

The pheromonal effects could also serve as a window into the evolution of primitive emotional states. The effects of pheromones are scalable, persistent, general phenotypes that induce a change in the neural state and appear to be valently opposed by mating. Thus, the neural state induced by pheromones is consistent with that of primitive emotions[26], perhaps best described as a drive to reproduce as evidenced by the increased courtship of males previously exposed to female pheromones. These neural states are likely to be evolutionarily selected upon in certain scenarios, and thus provides a potential evolutionary link between the development of emotion, reproduction, and aging.

In addition to the more general link between "emotions" and aging, it is of interest that we found a positive relationship between sex and physical health in an invertebrate model. The prevailing wisdom generally suggests that mating is dangerous and costly. This is a common observation across taxa but especially in invertebrates, and has typically been supportive of the concept of costs of reproduction[6,8,10]. However, by separating mating and sexual perception we demonstrated that copulation itself appears to be beneficial. This has been observed in humans as well[42,43], but moreover we have found a mechanism through which the benefits of sex may be mediated, which happens to be an evolutionarily conserved pathway. While studies in humans are difficult for numerous reasons, the ability to use invertebrate models to study the benefits of sexual and emotional health could lead to rapid progress in treating depression, anxiety, and sexual dysfunction. The fact that these diseases are common (and commonly untreated) diseases of aging emphasizes the link between emotional health and healthy aging. Discovery of the underlying physiologic processes causing these emotional issues would only serve to improve both mental and physical health amongst the elderly.

Methods

General fly husbandry: All flies used in this paper (including donor flies) were collected using the same method. Eggs were collected from yeasted grape juice agar plates, and 32 µl of eggs were placed onto bottles containing CT food. All flies except the experimental flies in temperature sensitive experiments (i.e. neuronal activation/inhibition) were raised in a controlled humidity incubator at 25C in 12 hour light/dark conditions. Flies were collected into bottles containing 10% yeast/sucrose food within 24 hours of

emergence, and (unless virgin) were allowed to mate for 2-3 days, after which the flies were sexed into groups of 25 in vials containing 10% yeast/sucrose food.

Production of donor flies: Male donor flies were produced by crossing either *w-;UAS-TRA;+* virgin females or *y-w-;UAS-TRA;+* virgin females to either *w-;OK72-Gal4;+* males (to create feminized male donor flies) or to *w-;+;+* genetic controls (to create wild-type male donor flies). Female donor flies were produced using the same methods, except using *w-;+;UAS-TRA^{DSRNA}* virgin females, which produce masculinized donor females when crossed to *w-;OK72-Gal4;+* males and wild-type donor females when crossed to *w-;+;+* genetic controls.

Exposure to donor flies: Experimental flies were exposed to donors in a ratio of 5 experimental flies to 25 donor flies, unless otherwise stated. For short-term assays, exposure began on day 8-10 after eclosion, except when performing the screen, at which point flies were placed on 30% sucrose/5% yeast food for 4 days starting at day 8-10. Short-term exposure lasted 48 hours, after which donor flies were removed and the experiment was performed. For lifespan experiments, exposure began on day 2 after eclosion, following sexing the flies. Exposure lasted the lifetime of the flies, with donor flies being replaced when ~25% of feminized donors had died (approximately day 28 in 25C experiments, day 21 at higher temps).

Lifespan assays: After beginning exposure (see "Exposure to donor flies"), lifespans were tracked using the *Dlife* computer software[44]. For each lifespan, 100 experimental flies (20 replicate vials of 5 experimental flies each) were established for all treatment/genotype groups. Flies were transferred to fresh vials/food every 2-3 days, at

which time deaths were recorded. Donor flies were replenished when approximately 75% of feminized male donor flies remain (~28 days at 25C, ~21 days at 29C). Experiments continued until no experimental flies remained alive, at which time donor flies were discarded.

Starvation resistance assays: Following 48 hours of exposure (see "Exposure to donor flies"), experimental flies were placed in fresh vials without donor flies containing 1% agar. For each assay, 50 experimental flies (in vials of 10 flies/vial) were established for all treatment/genotype groups. The number of dead flies in each vial was recorded every 2-4 hours until no experimental flies remained alive.

Fat store (TAG) assays: Following 48 hours of exposure (see "Exposure to donor flies"), experimental flies were quickly frozen in a dry ice bath, then homogenized in 100 µl PBS/0.01% Triton-X in groups of 5 flies per sample, with 5-10 samples per treatment/genotype. Afterward, 5 µl of homogenate was added to 150 µl of Infinity Triglyceride Reagent (Thermo Electron Corp.) and incubated at 37°C for 10 minutes with constant agitation. Concentrations of Triacylglycerides (TAG) were determined by comparing the absorbance at 520 nm of experimental samples to known triglyceride standards.

Neurometabolomic analysis: Following 48 hours of exposure (see "Exposure to donor flies"), experimental flies were quickly frozen in a dry ice bath, and stored at -80C overnight. Heads were removed via vortex and separated from body parts by filtering through meshes. 40-50 heads were then homogenized for 20 seconds in 200 µl PBS using the Fast Prep 24. Following the addition of 800 µl of methanol, samples were

incubated for 30 minutes on dry ice, then homogenized again. The mixture was then spun at 13000 RPM for 5 minutes at 4C, then the soluble extract was collected into vials. This extract was then dried using a speedvac at 30C, over approximately 3 hours. Samples were then sent for mass-spec and data analysis at University of Washington with the Promislow and Rafferty laboratories.

Temperature-dependent manipulations (i.e., corazonin and npf activation/inhibition experiments): For temperature-dependent experiments, experimental fly eggs were collected and raised in either 18C, 12h:12h light:dark incubators (*crz* experiments) or 23C 12h:12h light:dark incubators (*npf* experiments). 2 days post-eclosion, flies were sexed and sorted as described above. Following experimental set-up, flies were shifted to a 29C incubator for the remainder of their lifespan.

Quantitative PCR: Following 48 hours of exposure (see "Exposure to donor flies"), experimental flies were quickly frozen in a dry ice bath. RNA was extracted using Trizol (Invitrogen) from 5 samples per genotype/treatment, with each sample containing 25 experimental flies. cDNA was then synthesized using the Superscript III first strand synthesis kit (Invitrogen). Quantitative real-time PCR was performed with SYBR green from SA Biosciences. Expression was normalized to expression of the housekeeping gene *rp49*. The following primers were used:

npf Forward: TGAACCAGAACTATGTGCCAAA

npf Reverse: TTGTCCATCTCGTGATTCCTC

4EBP Forward: CGAACAGCCAACGGTGAACA

4EBP Reverse: TTCCGCTGGACGTGTAAGCA

RP49 Forward: ACTCAATGGATACTGCCAG

RP49 Reverse: CAAGGTGTCCCACTAATGCAT

RNA-sequencing: After the 48 hour exposure period (see "Exposure to donor flies" above), RNA was extracted from three independent sets of five flies per treatment group (w^{1118} and *ppk23* mutant flies each exposed to wild-type and feminized males). Thus, for each of four treatment groups we had three biological replicates. RNA was first isolated using Trizol (Invitrogen) and then further purified using the RNeasy kit (Qiagen). Aliquots of 1.1ug of RNA (as determined by NanoDrop) from each sample were delivered to the University of Michigan Sequencing Core, which performed cDNA library creation using Poly-A selection followed by 50bp paired-end high-throughput sequencing using an Illumina HiSeq 2000 sequencer. Sequencing was completed in three separate lanes, each multiplexed with samples prepared from each of the four treatment groups. Sequencing data were processed using the Bowtie-TopHat-Cufflinks pipeline[45]. Sequences were aligned to NCBI build 5.2 of the *Drosophila melanogaster* genome, and the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) per annotated gene was used as the expression measure. Cluster/Go analysis was carried out using cummerbund software to identify genes differentially expressed upon pheromone exposure, and functional classification analysis was completed using DAVID and a significant category enrichment Bonferonni $P < 0.001$.

Circulating dILP2 ELISA: Following exposure for the listed time period, hemolymph was extracted by skewering flies with a large needle, then immediately spinning at 5000 x g for 5 minutes at 4C in a .6 ml tube perforated at the bottom with a 16-gage needle,

placed in a 2 ml tube. The hemolymph will flow to the 2ml tube. The ELISA assay was performed as described previously, using flies with FLAG-tagged dILP2[46].

Statistics: Unless otherwise indicated, pairwise comparisons between both lifespan and starvation survivorship curves was performed using the DLife computer software[44]. P-values were obtained using cox-regression tests. Additionally, interaction terms between genotype and treatment were determined using the survival statistical package within the software program R (also using the cox-regression test).

Hydrocarbon extraction: At the time of extraction, 5 flies were placed in a single vial with 100 µl hexane containing 10 µg/ml of synthetic hexacosane (Sigma-Aldrich) as a spike-in standard. Extracts were allowed to incubate at room temperature for 30 minutes, after which the extract was removed and placed in a clean glass vial, where the solvent was allowed to evaporate. Extracts were then stored at -80C and re-dissolved in 70 ul of hexane prior to GC-MS analysis, which was performed by Dr. Joanne Yew. GS-MS analysis was performed with a QP2010 system (Shimadzu) equipped with a DP-5 column (5%-Phenyl-methylpolysiloxane column; 30 m length, 0.25 mm ID, 0.25 um film thickness; Agilent). Ionization was achieved by electron ionization (EI) at 70 eV. One microliter of sample was injected using a splitless injector. Chromatograms and mass spectra were analyzed using GCMSsolution software (Shimadzu).

Courtship assay: In order to count the number of courtships, we exposed flies to either feminized or wild-type males for 48 hours, then isolated flies using CO2 anesthesia and allowed 1 hour recovery. One male exposed to wild-type males and one male exposed to feminized males were then added to each vial containing one virgin wild-type female,

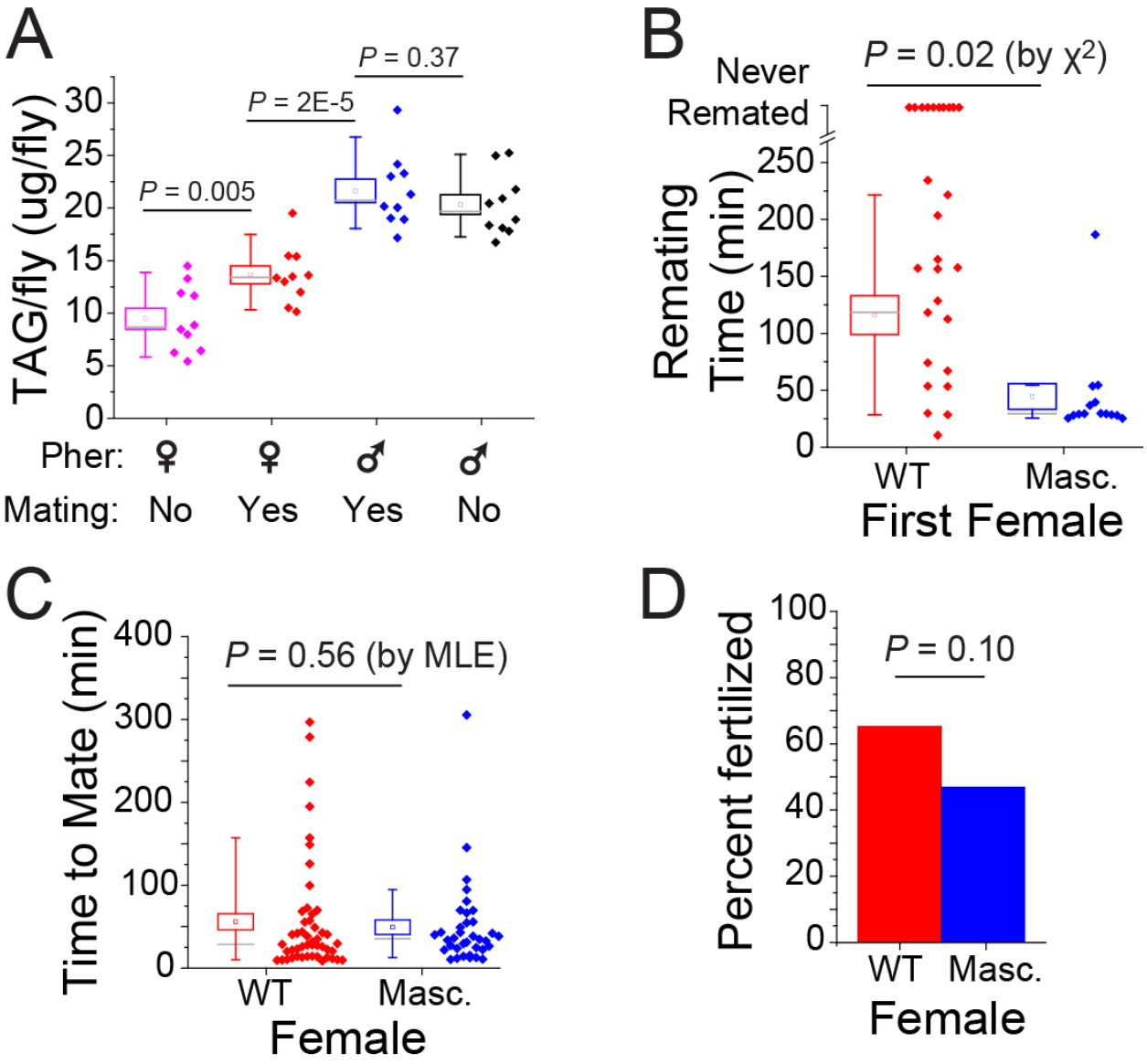
and the number of courtship attempts were counted by visual inspection under red light conditions. Flies were differentiated by green or red colored florescent powder (with the color randomized for each vial). Experiment was performed in the evening to avoid mating, and no mating was observed over the course of the experiment.

Mating latency assays: In order to measure mating latency, we flipped individual Canton-S male flies into vials containing either a single wild-type female, a single masculinized female, or one wild-type female and one masculinized female (i.e. competitive vials). Interaction times were increased by pushing the flug to within 1 inch of the food. Flies were then under constant observation (each vial checked at least once every 10-15 minutes), and time of copulation and female genotype (if the vial was competitive) were recorded when the male was seen to successfully mount the female. Flies were observed for 6 hours, after which the experiment was concluded.

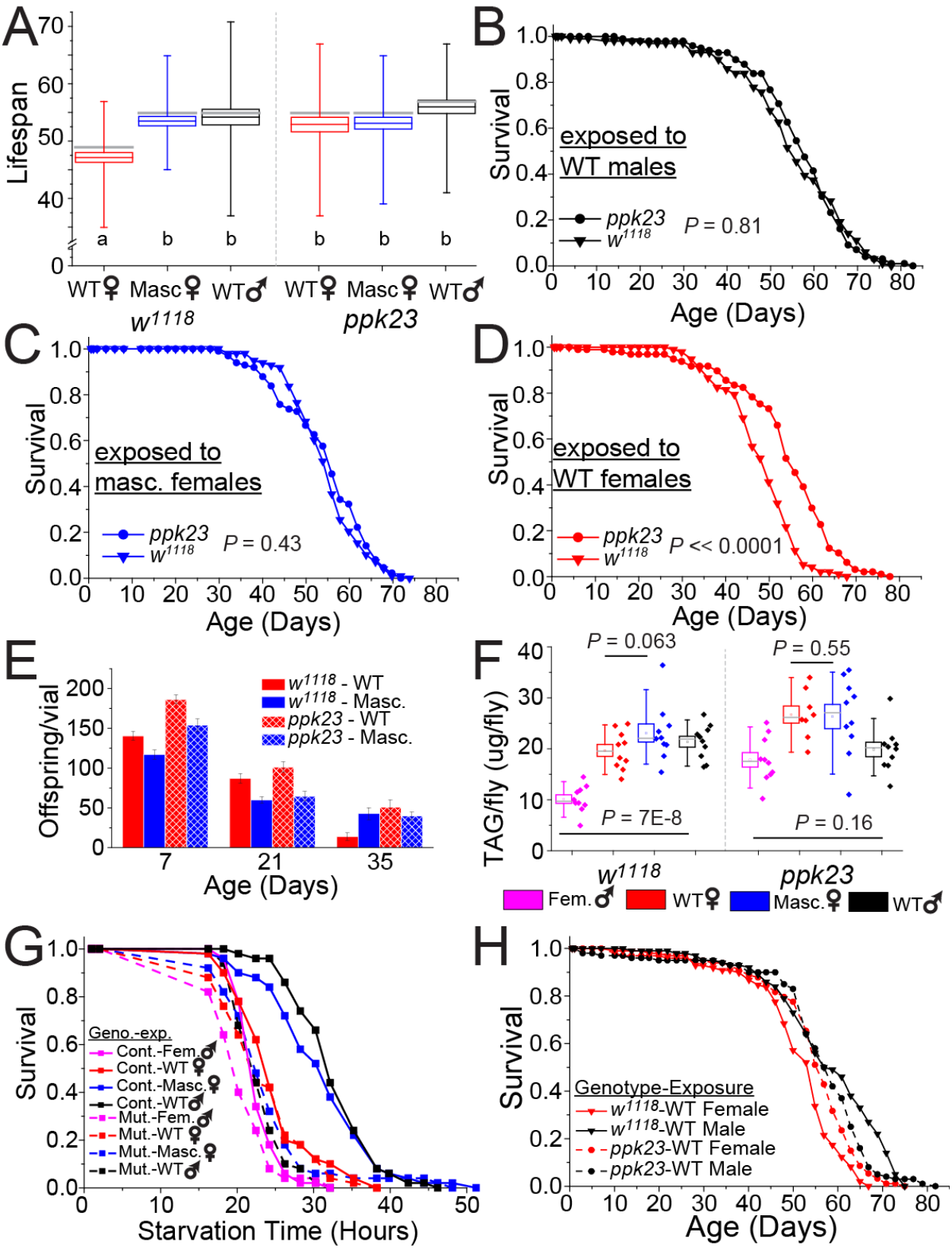
Fertilization assays: Fertilization assays to identify *successful* mating attempts were performed by adding 1 male ($y^{-}w^{-}$) and 1 female (either wild-type or masculinized) to a vial. Flies were then allowed to interact overnight, after which the male was removed. If evidence of larval development was not observed after 1 week, it was assumed no successful fertilization occurred.

Offspring assays: In order to measure the number of offspring produced during a lifespan experiment, all vials were saved on pre-determined dates and kept at 25C following transfer. Offspring were allowed to emerge over the course of 2 weeks. After 2 weeks, flies were frozen and then counted later at our convenience.

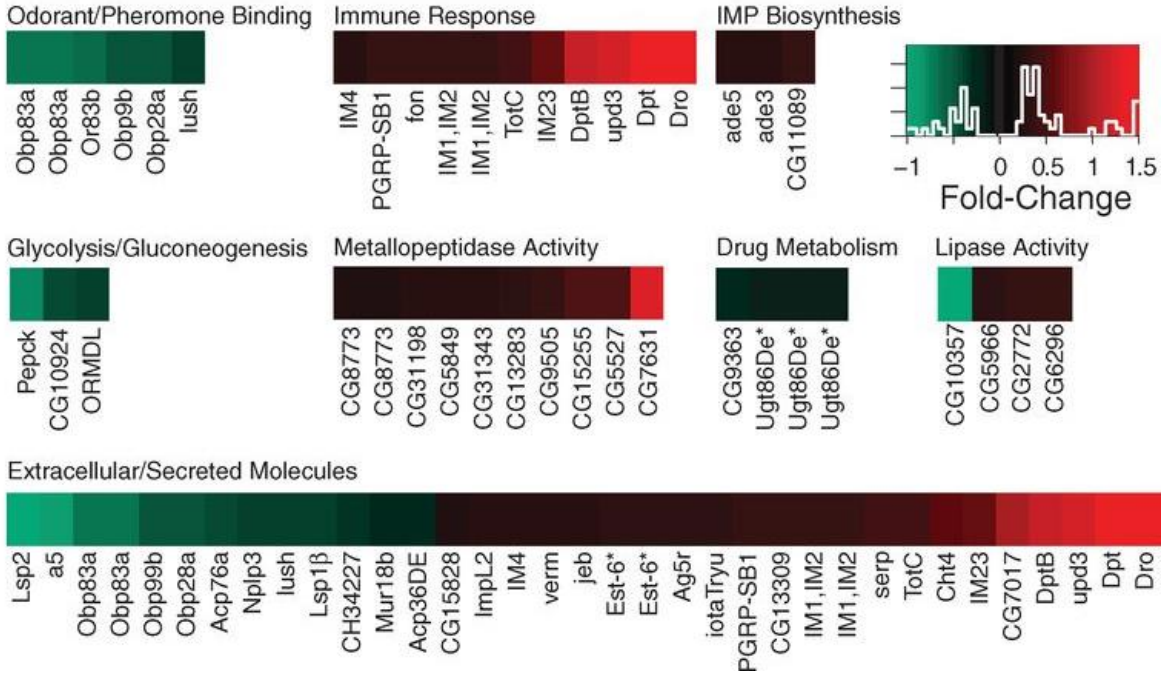
Supplementary Figures and Tables



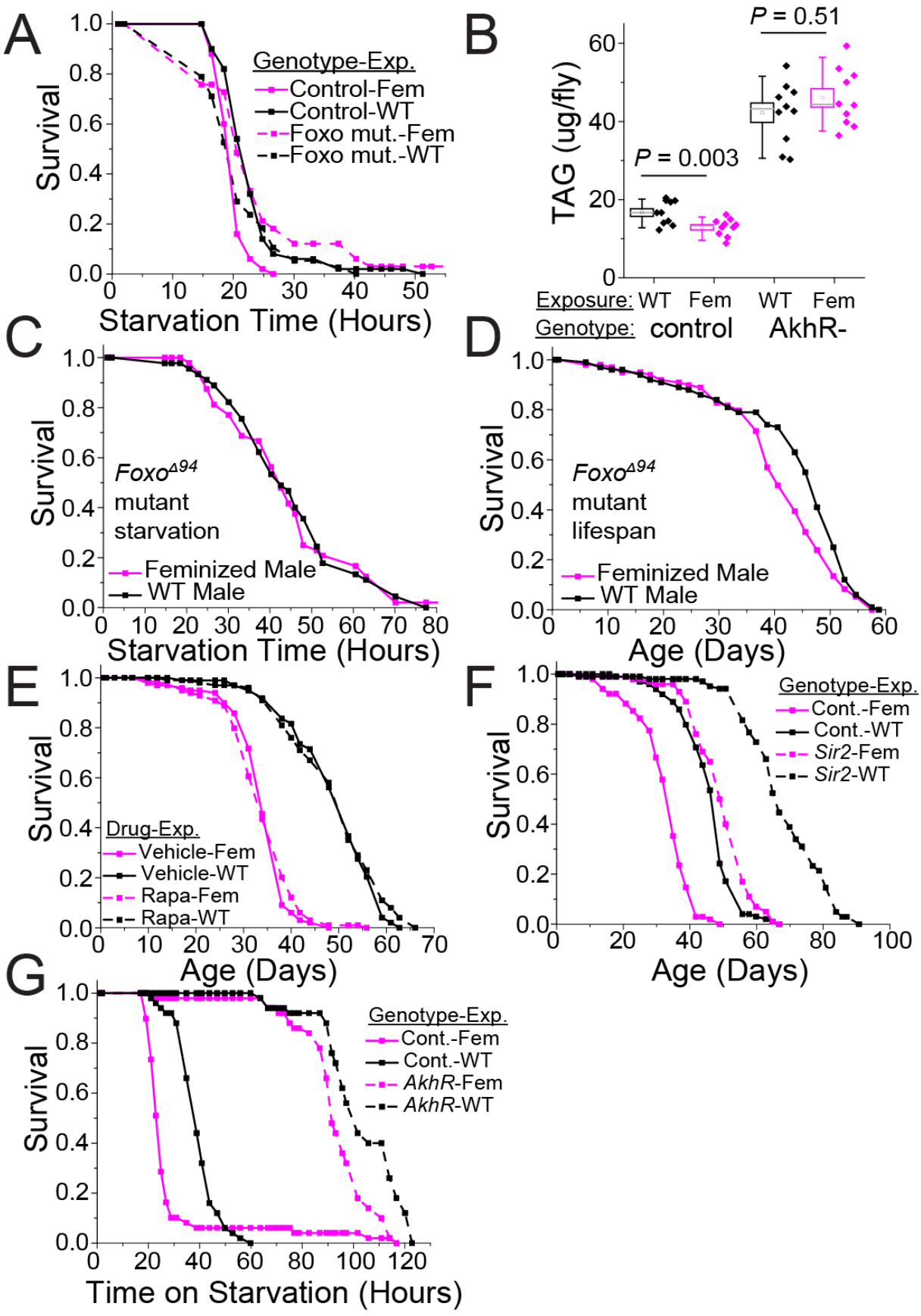
Supplementary Figure 2.1: Males mate with masculinized females at a similar rate as with wild-type females. (A) A second genotype (w1118) responds similarly in terms of fat stores to mating in the presence and absence of pheromones. (B) In competitive environments, males prefer mating with wild-type females over masculinized females (N = 46). The majority of flies (70%) mated with both females, though males that mate with a single female always select wild-type females (9 to 0). Additionally, males that first mated with masculinized females were significantly faster to re-mate with wild-type females. (C) In non-competitive environments, time-to-mating is similar for males exposed to wild-type or masculinized females that mated (by maximum likelihood estimation). In both sets of flies, over 70% mated during the course of the 6 hour experiment. (D) If left in a large vial with a single female, males have similar success at fertilizing that female whether it is wild-type or masculinized. For all box plots, box represents SEM, whiskers represent 10%/90%, and the grey line represents the median.


A
Lifespan
70
60
50
40
0
a
b
b
b
b
b
WT♀
Masc♀
WT♂
WT♀
Masc♀
WT♂
w1118
ppk23
B
Survival
1.0
0.8
0.6
0.4
0.2
0.0
exposed to
WT males
ppk23
w1118
P = 0.81
0
10
20
30
40
50
60
70
80
Age (Days)
C
Survival
exposed to
masc. females
ppk23
w1118
P = 0.43
Age (Days)
D
Survival
exposed to
WT females
ppk23
w1118
P << 0.0001
Age (Days)
E
Offspring/vial
200
150
100
50
0
w1118 - WT
w1118 - Masc.
ppk23 - WT
ppk23 - Masc.
7
21
35
Age (Days)
F
TAG/fly (ug/fly)
40
30
20
10
0
P = 0.063
P = 0.55
P = 7E-8
P = 0.16
w1118
ppk23
Fem.♂
WT♀
Masc.♀
WT♂
G
Survival
Geno.-exp.
Cont.-Fem.♂
Cont.-WT♀
Cont.-Masc.♀
Cont.-WT♂
Mut.-Fem.♂
Mut.-WT♀
Mut.-Masc.♀
Mut.-WT♂
0
10
20
30
40
50
Starvation Time (Hours)
H
Survival
Genotype-Exposure
w1118-WT Female
w1118-WT Male
ppk23-WT Female
ppk23-WT Male
Age (Days)

Supplementary Figure 2.2: The costs of reproduction act through the pheromone receptor ppk23. (A) Average lifespans of w^{1118} males exposed to wild-type females are significantly different than all other groups by Tukey's Post-Hoc ($P < 0.005$ comparing WT females to all other groups, whereas comparisons between other groups have $P > 0.3$ and thus are insignificant). (B) *ppk23* and w^{1118} males live similar lifespans upon exposure to wild-type males. (C) Similarly, *ppk23* and w^{1118} males live similar lifespans upon exposure to masculinized females. (D) In the presence of wild-type females, the *ppk23* mutation extends the lifespan of males. (E) Reproductive output is not decreased in *ppk23* mutants. *Ppk23* mutants produce more offspring than w^{1118} controls on day 7 in both groups, and on day 35 specifically in the wild-type female group. This could be explained by a decrease in health in the w^{1118} males exposed to wild-type females, though there are still over 90% of males alive at this point, so it is not due to male death. Wild-type females generally produced more offspring than females in both genotypes, suggesting the differences are either due to the females themselves or other pheromone signals not transmitted by *ppk23*. (F) Pheromone exposure also fails to affect *ppk23* mutant fat levels. (G) *ppk23* mutants are resistant to the negative effects pheromone exposure on starvation resistance. $P = 0.014$ comparing *ppk23* exposed to wild-type and feminized males, $P = 0.91$ comparing *ppk23* exposed to wild-type and masculinized females. For w^{1118} controls, $P << 0.00001$ comparing wild-type to feminized males, and $P < 0.00001$ comparing wild-type and masculinized females. (H) In a repeat lifespan performed on non-virgin females, *ppk23* mutation again prevents the negative effects of wild-type female exposure ($P = 0.071$ comparing exposures for *ppk23* mutants, while $P << 0.00001$ for w^{1118} controls). Again, *ppk23* mutation extended lifespan specifically when exposed to females ($P = 0.0026$ comparing *ppk23* and w^{1118} males exposed to females, whereas $P = 0.090$ comparing the male exposures). For all box plots, box represents SEM, whiskers represent 10%/90%, and the grey line represents the median.



Supplementary Figure 2.3: Pheromone exposure alters several groups of transcripts. This figure shows significantly enriched Gene Ontology pathways and functions whose genes are differentially regulated after pheromone exposure.


A
Survival
Starvation Time (Hours)
Genotype-Exp.
Control-Fem
Control-WT
Foxo mut.-Fem
Foxo mut.-WT
B
TAG (ug/fly)
P = 0.003
P = 0.51
Exposure: WT Fem WT Fem
Genotype: control AkhR-
C
Survival
Foxo^Δ94 mutant starvation
Feminized Male
WT Male
Starvation Time (Hours)
D
Survival
Foxo^Δ94 mutant lifespan
Feminized Male
WT Male
Age (Days)
E
Survival
Drug-Exp.
Vehicle-Fem
Vehicle-WT
Rapa-Fem
Rapa-WT
Age (Days)
F
Survival
Genotype-Exp.
Cont.-Fem
Cont.-WT
Sir2-Fem
Sir2-WT
Age (Days)
G
Survival
Genotype-Exp.
Cont.-Fem
Cont.-WT
AkhR-Fem
AkhR-WT
Time on Starvation (Hours)

Supplementary Figure 2.4: The costs of reproduction are mediated through *dFoxo* and AKH signaling. (A) Repeat of initial screening experiment demonstrating that $dFoxo^{w24}$ mutants are resistant to the effects of pheromones on starvation when compared to genetic controls (P = 0.12 for *dFoxo* mutants, P = 0.00006 for controls). (B) Repeat of initial screening experiment demonstrating that *AkhR* mutants are resistant to the effects of pheromones on fat stores when compared to revertant controls. (C) A second, more severe mutation, $dFoxo^{\Delta 94}$, is also unaffected by pheromones in terms of starvation resistance (P = 0.90). (D) $dFoxo^{\Delta 94}$ mutants are also largely resistant to the effects of female pheromones on overall lifespan (P = 0.018). (E) Rapamycin treatment does not prevent the effects of pheromone exposure on lifespan (P << 0.00001 for both drug and vehicle control). (F) *Sir2* mutant males are affected similarly by pheromone exposure as wild-type males (P = 0.73 for interaction between genotype and exposure, P << 0.0001 comparing exposures for both genotypes). (G) *AkhR* mutation partially prevents the effects of pheromone perception on starvation resistance (P = 0.015 for interaction, P < 0.00001 for control and P = 0.00005 for *AkhR*). For all box plots, box represents SEM, whiskers represent 10%/90%, and the grey line represents the median.

Supplementary Table 2.1: List of RNA-sequencing hits from pheromone exposure. List of all genes that were significantly different in w^{1118} wild-type male vs feminized male exposures, but were not significantly different in *ppk23* exposures.

Gene	Ppk23 WT	Ppk23 fem	W1118 WT	W1118 fem	ln(FC)
Lsp2	18.4851	20.5231	9.91674	3.61318	-1.00964
CG32564	1.33219	1.61952	0.514243	0.187932	-1.00662
CG10357	2.81864	1.81536	1.95172	0.740976	-0.9685
a5	7.27351	5.35667	7.58137	3.04695	-0.91155
Cpr11B	1.13449	1.36544	2.00407	0.861776	-0.84394
Pepck	889.889	799.119	534.296	238.412	-0.80695
NA	0.0399	0.06809	2.37005	1.08876	-0.77787
CG13905	292.996	264.716	42.0605	20.1132	-0.73773
CG11391	5.08345	4.21514	6.41624	3.0822	-0.73319
CG14245	317.345	322.784	126.103	61.7769	-0.71357
Os-E, Pbprp3	86.675	63.341	119.492	58.7361	-0.71019
CG34342	1.5168	1.15922	0.847074	0.432746	-0.67164
CG17752	95.6106	84.6091	50.2622	26.39	-0.64427
NA	12.7697	16.421	16.0471	8.45187	-0.64114
Ir64a	0.266624	0.234914	0.374947	0.198946	-0.63375
CG4465	0.615302	0.601186	1.35968	0.736021	-0.61375
Or83b	4.77395	3.60404	4.41344	2.40256	-0.60812
CG11892	222.507	227.08	180.501	99.2947	-0.59764
CG10513	271.725	295.523	148.282	82.6862	-0.58406
CG10514	151.022	164.055	154.418	86.2566	-0.58234
NA	3.57682	4.08485	5.2567	3.01608	-0.55555
Obp99b	1547.12	1707.12	433.803	253.075	-0.5389
NA	3.88055	3.36011	3.23676	1.89744	-0.53407

Gene	Ppk23 WT	Ppk23 fem	W1118 WT	W1118 fem	ln(FC)
CG9259	158.349	187.281	121.917	71.5284	-0.53325
CG17999, CG9993	12.4172	14.827	33.1901	19.7614	-0.51852
CG13177	14.3477	12.9058	8.61139	5.18754	-0.50683
Pbprp5	21.7269	19.4164	25.042	15.2667	-0.49488
Acp76A	18.4283	26.3131	39.5143	24.7143	-0.46928
CG10924	31.6671	25.8429	3.20739	2.01544	-0.46462
NA	0.197477	0.412427	8.80586	5.57008	-0.45801
CG9497	24.8662	21.8348	21.7878	14.2012	-0.42802
ect	3.89644	3.66442	3.39285	2.2126	-0.4275
NA	8.17206	8.27784	14.0387	9.18878	-0.42383
CG10516	30.2544	38.5115	36.9116	24.2739	-0.41912
CG42351	158.117	127.637	118.899	78.2447	-0.41843
NA	1.92614	1.93158	1.84916	1.23338	-0.40497
Nplp3	64.4671	69.6306	124.028	83.2533	-0.39862
CG4459	3.1279	2.80857	3.12937	2.10306	-0.39744
CG32444, ORMDL	178.079	188.437	415.218	282.527	-0.38503
CG15358	17.0557	17.8597	7.32134	4.98541	-0.38428
lush	11.0355	8.90242	17.9281	12.2352	-0.38205
antdh	21.8272	17.3741	20.6504	14.1057	-0.38116
Lsp1beta	80.2162	85.8068	32.0493	22.0054	-0.37599
lectin-28C	79.9227	86.9089	97.2558	67.2193	-0.36938
CG11864	11.0749	11.4934	13.0336	9.15667	-0.35305
CG15408, CG3285	42.4552	43.1641	62.3424	44.2661	-0.34242
CG7402	7.68028	7.8601	10.3564	7.42856	-0.33227
CG8654	67.4885	56.817	46.0369	33.2188	-0.32633
CG34227	72.029	76.3033	101.04	73.3836	-0.31982
CG42852	947.323	1268.07	1619.61	1177.33	-0.31894
CG15263	89.883	99.3789	89.8117	65.3531	-0.31791
GRHR	47.5709	41.2825	34.5689	25.4099	-0.30782
p24-2, unc-115	5.79825	4.9882	8.18506	6.02409	-0.30654
yellow-e	7.76176	8.25281	11.4539	8.4325	-0.30624
CG10477	422.745	434.763	140.543	104.151	-0.29967
sug	70.0168	81.7427	63.743	47.5667	-0.29273
Mur18B	1624.5	1530.22	1254.33	937.841	-0.29078
NA	7.37661	10.83	21.3503	15.9941	-0.28885
Oat	17.4301	15.8923	30.6931	23.0159	-0.28785
Spn3	103.241	121.843	164.139	123.5	-0.28447
CG9363	27.1333	26.9159	35.869	26.9894	-0.28443
CG18302	8.49127	9.44619	13.933	10.5806	-0.27524
fln	98.8999	89.1933	25.8711	19.6834	-0.27335

Gene	Ppk23 WT	Ppk23 fem	W1118 WT	W1118 fem	ln(FC)
lectin-29Ca	57.7371	65.4393	77.9166	59.7197	-0.26598
CG42481, Sfp70A4	1334.48	1590.9	1847.67	1428.04	-0.25762
Acp36DE	175.703	207.017	269.091	208.12	-0.25693
CG14375, CG9799	124.248	119.538	101.224	78.3786	-0.25578
BG642378	36.8862	40.2426	68.9099	53.5807	-0.25161
Acp53C14a, Acp53C14b	1022.59	1192.84	1490.41	1159.35	-0.25119
CG5770, CG5773	146.606	129.624	81.4824	63.5499	-0.24856
CG17472	110.99	121.219	172.985	137.385	-0.23042
CG5002	28.5753	27.0755	31.4127	24.9595	-0.22996
Sodh-2	24.9628	25.1599	44.8699	35.72	-0.22806
Ugt35b, Ugt86De, Ugt86Dg	55.3646	61.7574	69.6867	55.7821	-0.22256
CG15829	5.56522	1.37155	1.59186	13.7829	2.158526
Dro	36.0014	26.4552	6.40961	43.9095	1.924332
Dpt	31.4648	19.8772	4.81251	25.7186	1.675996
CG3290, CG3292	147.569	95.4801	56.7608	241.8	1.449265
CG8147	21.8328	28.4524	13.3984	57.0295	1.448433
CG7631	3.24689	4.34092	1.69522	6.08294	1.277676
Acp65Aa	0.490463	0.59203	0.457505	1.62533	1.267678
CG10182	1.97201	0.502403	0.857792	3.03126	1.262372
upd3	1.12146	0.583076	0.236674	0.820685	1.243456
GstD2	17.7265	11.8532	8.28383	26.8345	1.175383
CG8665	19.1742	18.5022	9.95288	32.1247	1.171763
DptB	65.6043	33.363	5.84361	18.7764	1.167252
CG14219	0.39321	0.324288	0.40587	1.25754	1.13088
Cpr49Ac	0.627826	0.903991	0.503941	1.56113	1.130706
NA	0.434359	0.534973	2.33101	7.0582	1.107888
Jon25Bi	10.8423	10.5187	8.83207	25.981	1.078976
CG9466, CG9468	42.4464	46.5581	39.3338	113.062	1.055852
CG7017	1.45702	1.94314	4.13908	11.2687	1.001555
Jon25Bii	12.5047	14.7441	13.1707	35.1409	0.981371
NA	0.631388	0.535263	0.323292	0.841323	0.95642
Cyp12d1-p	7.40516	5.10263	0.305585	0.676246	0.794329
CG31463	3.2421	3.82536	3.4185	7.05432	0.724438
GstD5	10.7773	9.52975	4.65304	9.53133	0.717063
CG13215	26.8232	25.647	5.644	11.3788	0.701159
Jon65Ai	16.7306	16.2315	7.36664	14.7129	0.691763
CG5150	59.3993	66.7245	28.6197	56.1716	0.674316
CG10592	85.6122	96.0429	36.7439	71.0474	0.659375

Gene	Ppk23 WT	Ppk23 fem	W1118 WT	W1118 fem	ln(FC)
CG14205	3.34347	3.93634	2.06458	3.9493	0.648612
CG33510	0.782923	0.666298	0.799559	1.47075	0.609467
IM23	14.4021	12.2735	17.85	32.7725	0.607586
CG42397	45.6283	37.8227	31.7405	57.5414	0.594911
CG8389	19.8882	16.6723	20.4059	36.9634	0.594104
CG6640	17.2809	4.85551	17.3625	31.2234	0.586855
CG14120	9.73733	9.51839	6.84707	12.0896	0.568525
NPC1b	1.60676	1.31493	1.47144	2.59564	0.567592
CG9498	125.921	106.811	81.3928	142.669	0.56124
Cht4	9.7863	7.89094	16.6648	28.8607	0.549182
CG5527	7.53773	7.68766	5.09611	8.64175	0.528128
CG32107	1.35591	0.975674	1.14633	1.93344	0.522735
CG31041	14.9375	10.3254	6.96687	11.6465	0.51384
CG31974, CG31975	60.7403	56.3518	56.0585	93.606	0.512699
CG13324	35.8898	31.8793	41.0332	68.4552	0.511798
CG17560, CG17562	234.374	232.402	117.668	195.929	0.509885
CG12057	116.038	135.899	120.535	200.265	0.507701
CG15255	33.6634	28.8375	21.3877	35.4586	0.50555
Act88F	756.653	537.571	255.983	409.045	0.468714
CG31086, CG31323	46.7134	45.4141	27.7772	44.1127	0.462532
Socs36E	11.4851	9.14186	8.49202	13.4063	0.456598
CG30008	1144.86	1029.45	603.361	947.614	0.451432
CG33301	43.7295	47.272	34.6781	54.2975	0.44837
TotC	14.9298	14.8899	77.0418	120.411	0.446563
Aph-4	209.878	192.267	142.916	222.02	0.44051
serp	4.95289	5.57295	4.85094	7.43433	0.426936
Cyp6a19, Cyp6a23	58.5442	52.3729	80.0596	122.672	0.426743
CG15043	180.69	129.179	141.518	216.293	0.424207
CG6296	0.031168	0.040072	1.58528	2.41246	0.419886
lcs	3338.54	3593.14	3531.12	5332.7	0.412243
IM1,IM2	634.522	618.915	855.596	1286.55	0.407921
fon	136.722	143.287	94.7293	142.195	0.406176
CG11089	30.3616	30.2027	38.9426	58.2852	0.403259
CG2772	7.36533	8.35616	6.67501	9.97596	0.401807
CG14949	70.4992	60.7985	88.0956	129.933	0.388596
CG10912	109.723	83.8481	140.096	206.625	0.388578
CG5535	15.4314	16.2618	24.5488	35.7457	0.375767
l(2)08717	64.2931	64.8207	56.009	81.4992	0.375081

Gene	Ppk23 WT	Ppk23 fem	W1118 WT	W1118 fem	ln(FC)
CG10910, CG5084	66.1835	66.5391	30.9857	45.0856	0.375037
CG13309	209.468	175.545	188.968	274.683	0.37404
CG7882	119.407	105.757	74.0974	107.255	0.369829
CG9505	5.37983	4.94381	5.69422	8.2264	0.367897
CG5767	19.5444	22.0015	48.029	69.3398	0.367214
PGRP-SB1	52.1773	44.5469	34.7939	50.0293	0.363167
Cpr	755.47	666.419	412.516	591.613	0.360578
iotaTry	25.1443	25.6784	15.4822	21.9219	0.347795
CG14691, CG31272	108.643	109.941	63.515	89.2065	0.339678
CG8317	193.313	148.003	177.623	248.831	0.337111
Ag5r	210.688	214.334	332.074	464.386	0.335358
CG16743	34.5477	32.4792	43.5692	60.6331	0.33049
CG16904, CG42857, CG9458, CG9459, CG9461	922.313	795.025	587.528	816.985	0.329697
CG7900, CG7910, CG7918	385.378	341.088	244.352	338.018	0.324489
CG18594	56.0877	64.9663	59.1987	81.6576	0.321635
CG13283	8.45729	5.43759	11.4055	15.6364	0.315506
vanin-like	6.32601	7.3704	8.20086	11.1889	0.310683
Irc	65.3992	61.9223	47.5638	64.7543	0.308528
CG6910, Est-6, Est-P	753.95	755.725	1123.98	1528.57	0.307457
CG5966	49.0426	27.3426	18.7017	25.4149	0.306721
CG6188, CG6225	303.072	322.173	229.187	309.883	0.301657
Zip3	7.92795	7.54763	8.3444	11.2375	0.297666
jeb	2.69775	2.73962	2.03985	2.74667	0.297513
Smvt	129.136	85.6756	115.806	155.039	0.29176
CG17108	695.708	710.91	555.482	743.484	0.291511
CG31343	74.9501	75.8037	63.0368	84.2539	0.290116
Proct	34.5219	33.5418	27.0815	36.1921	0.28999
verm	8.97998	8.9869	10.0496	13.3536	0.284253
ade3	16.9562	16.1841	20.5859	27.3407	0.28377
IM4	930.785	859.044	888.542	1177.11	0.281236
CG2765, CG30424	663.143	625.647	338.763	446.713	0.276616
Cyp6a21, Cyp6a8	61.9889	52.9422	175.498	231.006	0.274816
CG31004	22.4662	20.8612	16.6877	21.9167	0.272577
CG5849	6.40462	5.99985	5.56034	7.25905	0.26659
ImpL2	123.491	116.374	93.1039	121.131	0.263157
CG14687	123.241	123.399	101.373	131.524	0.260383

Gene	Ppk23 WT	Ppk23 fem	W1118 WT	W1118 fem	ln(FC)
CG15531, CG9743	228.734	233.187	179.616	231.128	0.25215
CG10911	554.811	498.412	491.075	631.185	0.251002
CG13323	274.944	265.998	244.006	313.493	0.250584
Ela	34.664	36.4629	45.9426	58.9224	0.248829
ade5	224.742	203.042	205.09	262.758	0.247785
CG31198	140.826	144.789	119.409	152.056	0.241694
CG1678	2428.21	2627.74	6425.02	8176.1	0.241016
v	30.7637	30.0786	30.7576	39.0241	0.238042
CG10650	68.9945	65.4904	46.1618	58.4878	0.236666
CG6432, Dis3	60.0479	52.3198	27.9928	35.4654	0.23661
CG15828	15.0901	14.6035	9.74673	12.3459	0.236392
cwo	21.2498	21.69	20.9266	26.4686	0.234938
CG8773, CG8774	39.5927	44.2956	32.1503	40.419	0.228878

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Chapter III: A Computational Approach to Studying Aging at the Individual Level[3]

While genetic experiments are a powerful method for dissecting out mechanisms underlying longevity, it would be a mistake to ignore the usefulness of computational methods for understanding how interventions affect aging. In fact, the computational work that follows was essential for two findings in the previous chapter. First, our computational results demonstrated that the effects of pheromones on an individual's chance of death persist for a period of time before reversal. Notably, we found that mortality rates reverse much slower than short term phenotypes like fat stores and starvation resistance, leading to the second finding: the effects of pheromones on lifespan and short-term physiology are separable using genetic methods. If not for the different dynamics, we might not have followed up on the relationship between AKH signaling and short-term pheromone effects. Beyond these mechanistic findings, the following results exemplify the dangers of conflating individual and population statistics, while demonstrating a method to identify when such issues might arise.

[3] This chapter originally appeared in the following publication: **Z. M. Harvanek***, M. A. Mourão*, S. Schnell and S. D. Pletcher (2016). Disentangling individual and population mortality dynamics in aging Drosophila. Proceedings of the Royal Society B 283, 20152346. DOI: 10.1098/rspb.2015.2346. (asterisk indicates equal work). Work was done as follows: Conceptualization: Z.M.H., S.S., and S.D.P.; Methodology: Z.M.H., M.A.M., S.S., and S.D.P.; Software: M.A.M. and S.S.; Formal Analysis: Z.M.H., M.A.M., and S.D.P.; Investigation: Z.M.H. (in vivo) and M.A.M. (in silico); Writing – Original Draft: Z.M.H. and M.A.M.; Writing – Review & Editing: Z.M.H., M.A.M., S.S., and S.D.P.; Visualization: Z.M.H.; Supervision: S.S. and S.D.P. Final Approval: Z.M.H, M.A.M., S.S., and S.D.P.

Abstract

The aging process is actively regulated throughout an organism's life, but studying the rate of aging in individuals is difficult with conventional methods. Consequently, aging studies typically make biological inference based on population mortality rates, which often do not accurately reflect the probabilities of death at the individual level. To study the relationship between individual and population mortality rates, we integrated *in vivo* switch experiments with *in silico* stochastic simulations to elucidate how carefully designed experiments allow key aspects of individual aging to be deduced from group mortality measurements. As our case study, we used the recent report demonstrating that pheromones of the opposite sex decrease lifespan in *Drosophila melanogaster* by reversibly increasing population mortality rates. We showed that the population mortality reversal following pheromone removal was almost surely occurring in individuals, albeit more slowly than suggested by population measures. Furthermore, heterogeneity among individuals due to the inherent stochasticity of behavioral interactions skewed population mortality rates in middle-age away from the individual-level trajectories of which they are comprised. This article exemplifies how computational models function as important predictive tools for designing wet-lab experiments to use population mortality rates to understand how genetic and environmental manipulations affect aging in the individual.

Introduction

Research over the last decade suggests that aging is exceptionally malleable. Changes in mitochondrial function during the larval stages of the nematode worm *Caenorhabditis elegans* can regulate the health and longevity of the adult animal [1]. In the fruit fly

Drosophila melanogaster, specific genetic and environmental factors, such as dietary restriction [2-4], insulin signaling [5], and mating status [6] rapidly and reversibly affect population mortality rates, often within hours or days. A thorough understanding of the dynamics of the normal aging process, and how those dynamics respond to experimental manipulation, would provide insight into its underlying molecular mechanisms and help guide the development of anti-aging interventions.

In addition to developing interventions, scientists and physicians interested in broadly treating age-related decline must also consider variability in the targets of their interventions. Aging, unlike many diseases, will eventually affect all individuals, so the simplest answer is to provide everyone with such an intervention once it is developed. However, this is unlikely for many reasons, including the possibility that not everyone would benefit equally, if at all. In traditional aging experiments, a drug that improved lifespan 15% in half the population and simultaneously reduced lifespan by 10% in the other half of the population would be difficult to distinguish from a drug that increases lifespan across the population by 5%. Thus, it is imperative for aging researchers to determine whether their population measurements reflect heterogeneous or homogeneous responses to their interventions.

Unfortunately, individual patterns of aging are difficult to measure directly. By far the most common descriptor of aging, particularly in simple model systems, is age-specific mortality, which has been used to document rapid changes in aging following interventions such as dietary restriction and temperature manipulation [2,4,7]. Because individual animals are either alive or dead, typical aging experiments measure population mortality rates, which are estimates of the instantaneous risk of death

calculated as a function of the fraction of individuals in a cohort that survive a given census interval. Individual aging rates are often assumed to mirror the population mortality trajectory, which is only justified if all individuals in the population are identical not only genetically but in every facet, which is untenable even in the most tightly controlled aging experiments. In most cases, however, individuals vary in their mortality characteristics. This heterogeneity, together with demographic selection (i.e., a change in cohort composition due to deaths of select individuals), make it difficult to infer individual dynamics from population measures [8].

Herein we present a computational and experimental approach to infer how manipulations affect aging at the individual level using population measurements. Thus, we needed to carefully design experiments that will determine the extent to which a population is aging in a heterogeneous fashion and that would identify the relationship between individual-level characteristics and population-level observations. We used as our case-study the recent report demonstrating that exposure of male fruit flies to female pheromones affects lifespan by increasing mortality rates throughout the exposure period [9]. Interestingly, two weeks after pheromone removal population mortality rates in the treated flies reverted to levels observed in cohorts of unexposed animals. We asked whether these data are indicative of changes at the level, which would suggest a reversal of the aging process. To determine whether pheromone manipulation affects how fast or slow individual flies age, we tested a series of predictions from a stochastic, 2-dimensional lattice-based simulation that models the known effects of pheromones on mortality. We found that the reported effects of pheromones on aging likely derive from both population heterogeneity and individually-

changing mortality rates. To our knowledge, these results are the first to effectively infer individual aging patterns primarily from population mortality rates, and they provide a template for future investigations of the dynamics of aging.

Experimental Procedures

In a broad sense, in this paper we ask whether the patterns of demographic aging that we observed in our population-level measures (as represented by age-specific mortality rates) following pheromone exposure accurately reflect the biological process of aging at the individual level, and if not, how they differ. Unfortunately, to answer this question with certainty we must understand in detail how the effects of age influence an individual's probability of dying at each instant, which is a near-impossible task *in vivo*. It is, however, straightforward to monitor *in silico* when the researcher defines aging parameters. Thus, we designed a computational model that simulates the death rates of individual flies based on age and pheromone exposure. Using this simulation we studied how stochastic interactions with conspecifics and variability in the magnitude and duration of pheromone effects influences heterogeneity among individuals and population mortality rates. In so doing, we seek to specify the most appropriate *in vivo* experiments that provide insight into individual-level patterns of aging and that distinguish them from population-level phenomena. Thus, in the narrow sense, by combining *in silico* and *in vivo* experiments, we set out to determine whether transient increases in population mortality following pheromone exposure, as previously observed by Gendron et al. (2014), are more likely reflective of a reversible process of aging on the individual level or an emergent property of aging of a highly heterogeneous population.

Computational Model Design

With this goal in mind, we designed a computational model to investigate the dynamics through which pheromones affect the aging rates of individuals. Gendron et al. (2014) reported that exposure of male *Drosophila* to female pheromones affected population mortality rates, and several of the results of that work are relevant to our formulation. First, male population mortality rates increased exponentially with age, and mortality rates were consistently higher in cohorts of male flies when they were exposed to female pheromones. Second, removal of female pheromones was followed by a gradual reduction in population mortality such that, in roughly two to three weeks, mortality in previously exposed and unexposed cohorts was similar. Third, fly pheromones are detected by contact, through taste receptors. Fourth, genetically modified flies that lacked the required taste modality were unaffected by pheromones. Accordingly, our model incorporates individual behaviors such as movement through the environment and social contact with other flies, as well as dynamics of individual mortality and how they are affected by pheromone exposure.

Simulation environment: We model an experiment using a 200x200 2D triangular lattice (each vertex has six neighboring vertices) with cyclic boundary conditions, which maintains the fly density observed in vials used for *in vivo* experiments while allowing for a similar sample size. Fly movement is modeled as a random walk, under the assumption there is no movement bias. The triangular nature of the lattice, when compared to square lattices (each vertex has only four neighboring vertices), provides a better approximation to continuous diffusion, allowing for improved dispersal of the

flies [10]. This simulation was developed using C++, and can be downloaded freely at the link provided in the data accessibility section.

In our simulations, three distinct types of flies inhabit the lattice and age over time. Feminized males (FM) serve as pheromone donors. These animals are genetically male but produce female pheromones. They were used by Gendron et al. to expose male flies to female pheromones without the confounding effects of mating. Wild-type males (WM) produce normal male pheromones and have normal perceptive capabilities. Sensory mutant males (SM) produce normal male pheromones but lack the ability to detect pheromones in their environment. In our simulations, we use SM flies as a control, as they are completely unaffected by pheromone exposure. We are interested in investigating how individual and population levels of age-specific mortality are affected in WM and SM given differential exposure to FM.

Time: During each time step, our experimental flies (i.e., WM and SM) age and then have a chance to die, interact with FM, and move around the lattice. Donor flies (i.e., FM) also move around the lattice but do not age or die, which reflects the supplementation of feminized males that Gendron et al. used to maintain a consistent level of exposure in the environment.

1. *Survival*: At the beginning of each time step, we increase the age of each experimental fly by one, and update its probability of death (P_{death}, which is defined below). A random number (r) between zero and one is drawn for each individual, and if $r < P_{death}$ the fly dies and is removed from the lattice.

2. Interactions with FM and movement: After experimental individuals have aged, surviving flies are chosen at random to evaluate social interactions and to move. If an experimental male is chosen, the number of FM in the six adjacent positions is determined, and that number is added to that individual's level of pheromone exposure (*I*), which depending on genotype may influence probably of death (P_{death}). For each selected fly a destination site is randomly chosen among the six adjacent locations. If the destination is vacant, the fly will move to that site. Otherwise, it will remain in its current site. The number of randomly chosen flies at each time step is equal to the number of experimental and donor flies alive. Therefore, while an individual fly may be selected multiple times, on average each fly will move/interact once each time step. This selection process guarantees that each fly is moved on average once each time step.

Death rates: The Gompertz equation has been used for decades to study how intrinsic and extrinsic factors influence patterns of aging. Examples include descriptions of historical trends in human population mortality rates [11] and investigations into how specific genetic manipulations impact aging rates in nematode worms, fruit flies, and mice [12]. The equation itself describes mortality rates (μ_t) as exponentially increasing with age (t):

Equation 3.1: Gompertz equation

$$\mu_t = \alpha e^{\beta t}$$

where α represents an age-independent, or intrinsic, mortality rate and β represents the rate at which mortality increases with age. With this formulation and our discrete simulations, the probability of death for a fly between age *t* and *t*+1 is (7, 17)

Equation 3.2: Probability of death

$$P_{death,t} = 1 - e^{-\frac{\alpha_t}{\beta}[e^{(t+1)\beta} - e^{t\beta}]}$$

In this equation, *t* is the number of time-steps that have occurred beginning from 2 days post-eclosion (when pheromone exposure begins in our *in vivo* experiments). Five time steps occur each simulated 'day' in the fly's life, which allows for social interactions to occur on a finer timescale. The effects of pheromone exposure on mortality rates observed in Gendron et al. more closely resemble a shift in the intercept of log-mortality (determined by α) than a change in slope (determined by β). Thus, we model the effects of pheromone exposure in terms of the age-independent component, such that

Equation 3.3: Defining alpha in terms of Mating Expectation (ME)

$$\alpha_t = e^{-7}\,(1 + ME_t)$$

Equation 3.4: Defining Mating Expectation (ME) in terms of the duration, quantity and strength of pheromone exposure

$$ME_t = \sum_{n=t-D}^{t} (I_n * Z_n)$$

where *I* represents the number of encounters an experimental fly has with FM at a given time step *n*, *D* represents the duration of the effects of pheromone exposure, and Z_n represents the magnitude of effect each pheromone encounter will have on mortality. *ME* is therefore the Mating Expectation, i.e., the cumulative effect of pheromone perception on an individual fly. The parameter values β = 0.04 and α = e^{-7} were estimated as those best fit from the empirically observed mortality rates in cohorts of male flies that were not exposed to female pheromones. By varying the size of the duration, *D*, pheromone effects can be made to be long-lasting or quickly lost, with subsequent effects on individual patterns of aging that range from permanent (*D* = 70

days, the full simulation length) to rapidly reversible (e.g., $D \leq 7$ days). Differences in Z_n reflect the impact of pheromone exposure; SM flies, for example, do not sense pheromones and therefore have $Z_n = 0$. For experiments where we simulate mortality rates following pheromone removal, we set $Z_n = 0$ for all ages thereafter.

Initial conditions and population measures: Unless otherwise noted, we seeded simulations with 80 WM or SM flies and 400 FM flies, occupying 0.2% and 1% of the lattice points, respectively. These conditions are similar to those in pheromone exposure experiments, where the number of FM outnumbered experimental flies 5 to 1, and they maintain a similar density if a lattice location simulates 0.04 cm^2, or approximately 1 body length (0.2 cm) in all directions. We designed the simulations to investigate the effects of pheromone exposure over a time frame reflective of a normal fly lifespan of roughly 70 days. Five time steps per day allowed flies to explore and interact over a finer timescale, with each simulation step therefore corresponding to approximately 4.8 hours. All population measures and test statistics were based on 100 replicates of each condition to obtain averages at each time step.

For each time step of the simulation, we determine the state (living or dead) for each fly, as well as the number of mating expectations accumulated. We calculate population mortality rates as the number of flies that died during a given time step divided by the number of flies alive at the beginning of the time step.

During any single simulation, individual flies may accumulate differences in *ME* based on their chance interactions with FM and the effect of each interaction, *Z*. These differences in *ME* will translate into heterogeneity in death rates among individuals in

the population, and they will influence the extent to which population mortality rates reflect individual aging. Because we have access to *ME* and P_{death} for all simulated flies, we can directly calculate heterogeneity as the variance in *ME* across the live flies. When required, we greatly reduce heterogeneity by adding the same number of exposures (the average population value) to I_n for each fly when they are selected to move.

In vivo experimental procedures

We used the following procedures for the *in vivo* experiments to test hypotheses generated by the computational work and to differentiate population- and individual-level changes in mortality rates upon manipulation of pheromone exposure.

Fly Strains: We derived all experimental flies from the *yw* strain from Bloomington Stock Center (#1495). Donor flies used for pheromone exposure expressed a feminizing transgene (*UAS-TRA*) in pheromone-producing cells called oenocytes, marked by *OK72-GAL4*. They were obtained as progeny from mating female *w⁻;UAS-TRA;+* with male *w⁻;OK72-GAL4;+* flies. Wild-type male donor flies (for the "never exposed" controls, as well as replacement of feminized donor flies at pheromone removal times) were obtained from the progeny of mating female *w⁻;UAS-TRA;+* with male *w⁻;+;+* flies.

General Pheromone Exposure Protocol: To expose experimental males to female pheromones in the absence of mating, we housed five experimental flies with 25 donor flies expressing either male or female cuticular hydrocarbons. We reared experimental and donor flies separately under controlled larval conditions, and transferred adults from each group to 10% Sugar/Yeast (SY) food for two days where they were allowed to mate freely. At two days post-eclosion, we separated the sexes using light CO_2

anesthesia. Experimental flies were immediately housed with donor flies, and remained with donor flies throughout their lifespan. All donor flies were replenished when approximately 20% of donor flies had died. For cohorts where the donor type was switched, flies were again lightly anesthetized and feminized male donors were replaced with wild-type male donors. At day 28, all donor flies were replaced (regardless of cohort) in order to maintain a constant number of donor flies.

Lifespan Assays: We performed lifespan analyses using empirically optimized protocols established by our lab [13]. We established 20 replicate vials (100 experimental flies) for each treatment. Lifespans were measured from the second day post-eclosion, when exposure to donor flies began. Flies were transferred to fresh media every 2-3 days at which time dead flies were removed and recorded.

Results

Models of pheromone exposure demonstrate realistic increases in mortality rates. We first used our computational model to examine population mortality dynamics from cohorts of simulated male flies that were not affected by female pheromones (i.e., sensory mutant males, $Z_n = 0$). Not surprisingly given the assumptions in equations 3.1 and 3.2, mortality rates were Gompertzian, and statistical estimation of model parameters from the population-level data are generally close to the values used to determine P_{death} for individuals (see Supplementary Materials). We therefore asked how exposure to female pheromones influenced population-level mortality rates by allowing flies to be affected by pheromone exposure ($Z_n > 0$). We found that when male flies were exposed to female pheromones throughout life and the effects of exposure were long-lasting ($D = 35$ days), population mortality rates were significantly increased compared

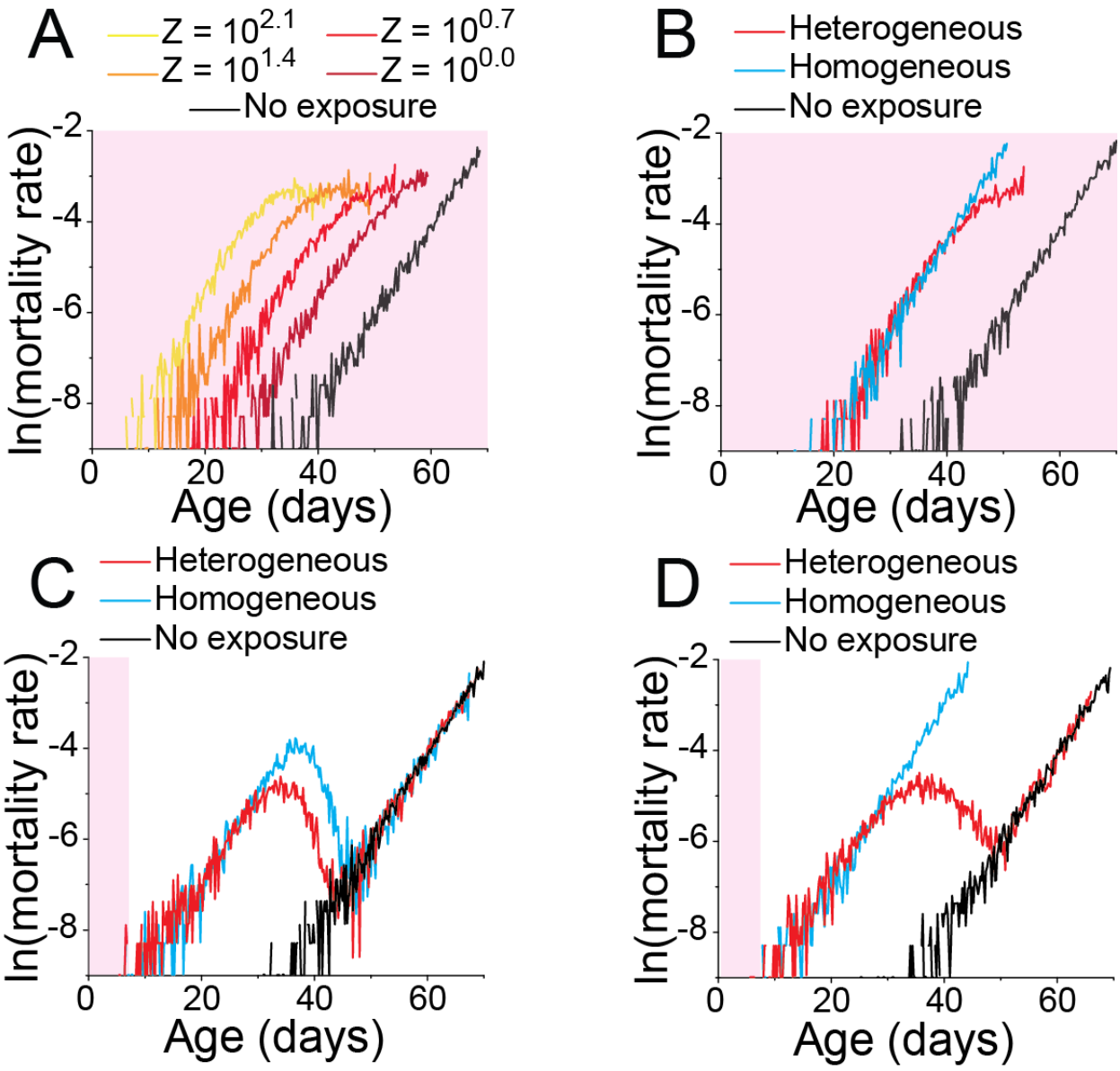


Figure 3.1: Mortality dynamics can be explained by individual reversibility and/or population heterogeneity. (A) Increases in mortality rate from continuous pheromone exposure are proportional to the magnitude of pheromone effect, Z_n. Exposure is throughout life, with long-lasting pheromone effects (D = 35 days, eqn. 4). (B) Late-life mortality deceleration is driven by heterogeneity in mating expectation. Removal of heterogeneity (light blue line) restores Gompertzian dynamics reflective of aging at the individual level. Exposure is permanent with D = 35 days and $Z_n = 10^{0.7}$. (C) Removal of pheromone exposure at day 7 leads to reversion of mortality rates to never exposed control rates in both heterogeneous and homogenous populations when the duration of pheromone effects is long (D = 35 days), using $Z_n = 10^2$. (D) Removal of pheromone exposure at day 7 when pheromone effects are permanent (D = 70 days) leads to reversal of mortality rates in heterogeneous, but not homogeneous, populations, using $Z_n = 10^2$. Plotted values for all panels represent averages of 100 replicates each running for a maximum of 350 time steps (70 days), with WM and SM = 0.2% and FM = 1% on a 200x200 triangular lattice. Shading in panels represent when pheromones were present.

to those unaffected by pheromones, with increasing values of Z_n resulting in progressively greater increases in population mortality (Figure 3.1 A). When $Z_n > 0$, population mortality rates deviate significantly from Gompertz dynamics, with mortality deceleration becoming apparent at advanced ages. This pattern of deceleration is a population-level phenomenon because the expected value of individual mortality rates increases exponentially throughout life. Population-level mortality estimates are expected to level off when individuals vary significantly in their mortality characteristics [8]. In our simulations the source of this heterogeneity is variability in Mating Expectation. Each time step, experimental flies on the lattice experience between 0 and 6 encounters with FM. Each interaction augments Mating Expectation, which leads to a distribution of mortality rates among same-age individuals in the population (Supplementary Figure 3.1). To determine whether variability in Mating Expectation is driving mortality deceleration, we repeated the simulations but removed this source of heterogeneity. Reducing heterogeneity in this manner resulted in a return to strict Gompertzian dynamics, with mortality rates continuing to increase at an exponential rate even at older ages (Figure 3.1 B).

Heterogeneity in individual mortality characteristics can produce population-level reversals. We next investigated the conditions for which, in our simulations, removal of the pheromone stimulus at a given age resulted in a reversal of mortality increases, as was observed in the published work [9]. We began by limiting pheromone exposure to the first 7 days of life ($Z_n = 0$ for $n > 7$) while maintaining their long-lasting effects ($D = 35$ days). We found that population mortality rates in exposed cohorts remained high until age 30, after which they rapidly declined such that at age 42 they were indistinguishable

from cohorts comprised of flies that had never been exposed to pheromones (Figure 3.1 C). This is not surprising considering that the effects of exposure at the individual level were reversible. They could not, for example, persist after 42 days of age (as the latest possible age of exposure is day 7 and mating expectations effects are defined to last for 35 days).

The observation that heterogeneity in Mating Expectation generated late-life deceleration in population mortality that is not present at the individual level led us to examine to what extent heterogeneity might be influencing the dynamics of mortality reversal after removal of the pheromone stimulus. We therefore repeated the simulations (removing pheromone perception at day 7 and constraining D = 35 days) with heterogeneity removed. We found that heterogeneity contributed to an earlier and more abrupt mortality reversal (Figure 3.1 C). These results prompted us to ask whether heterogeneity alone is sufficient to cause population mortality rates to exhibit a mortality reversal after removal of the pheromone stimulus. For these simulations we eliminated individual reversibility by making pheromone effects permanent (D = 70 days, which is the length of the simulation) and stopped pheromone exposure at day 7. We found that overall population mortality reverted to levels observed for unexposed cohorts (Figure 3.1 D) despite individual death probabilities that do not decrease (Supplementary Figure 3.2 A). Without heterogeneity the reversal did not occur (Figure 3.1 D & Supplementary Figure 3.2 B).

Multiple switch experiments can distinguish population from individual mortality dynamics. Our results highlight the potential discordance between patterns of aging that are occurring in the individual and those observed at the population level. We

showed that the observed reversal of pheromone effects on mortality rates can result either when effects are permanent but the population is highly heterogeneous, when effects are temporary and individual death probabilities are reversible, or some combination of both. To identify experimental designs that would allow us to distinguish these competing effects, we used the simulation structure to explore predicted mortality dynamics when the age of pheromone removal was manipulated.

Our simulations revealed that patterns of individual aging may be inferred from population mortality when a set of wet-lab experiments are executed, each of which involves removing pheromone exposure at a different age. When pheromones were removed at day 7, for example, we found that individual reversibility alone (i.e., no heterogeneity) could be clearly differentiated from a pure heterogeneity model (i.e., D = 70 days) if the duration of pheromone effects was sufficiently short. If pheromone effects lasted 5 days (D = 5 days), then pheromone exposure early in life had no effect on population mortality rates. If, however, pheromone effects lasted longer (e.g., 35 days), then population mortality trajectories were similar to those observed from the pure heterogeneity model (i.e., effects are permanent); mortality rates were significantly increased for much of early life before reverting to unexposed control levels (Figure 3.2 A). On the other hand, when pheromones were removed later in life (at day 30), then the slower individual reversibility condition could be differentiated from a pure heterogeneity model (i.e., no reversal, D = 70 days). Whether pheromone effects were quickly reversed without heterogeneity (D = 5 days) or permanent with heterogeneity (D = 70 days), population mortality dropped soon after pheromone removal. However, if D = 35 days there was no significant reversibility in population mortality rates in the

absence of heterogeneity (Figure 3.2 B). Together, these results indicate that an experiment in which pheromones are removed early in life (i.e., before flies begin to die) should differentiate rapid reversibility at the individual level from heterogeneity as the primary cause of population mortality reversal. Removal in middle age (i.e., in the midst of the exponential rise in mortality rates) should distinguish slower individual reversibility from a pure heterogeneity model.

In vivo experiments rule out rapid individual reversal of pheromone effects. We next applied the predictions from our model to a set of *in vivo* experiments, with removal of pheromones occurring at days 7, 28, and 35. Cohorts that were exposed to pheromones throughout life and those never exposed served as positive and negative controls, respectively. We observed that male flies exposed to female pheromones only during the

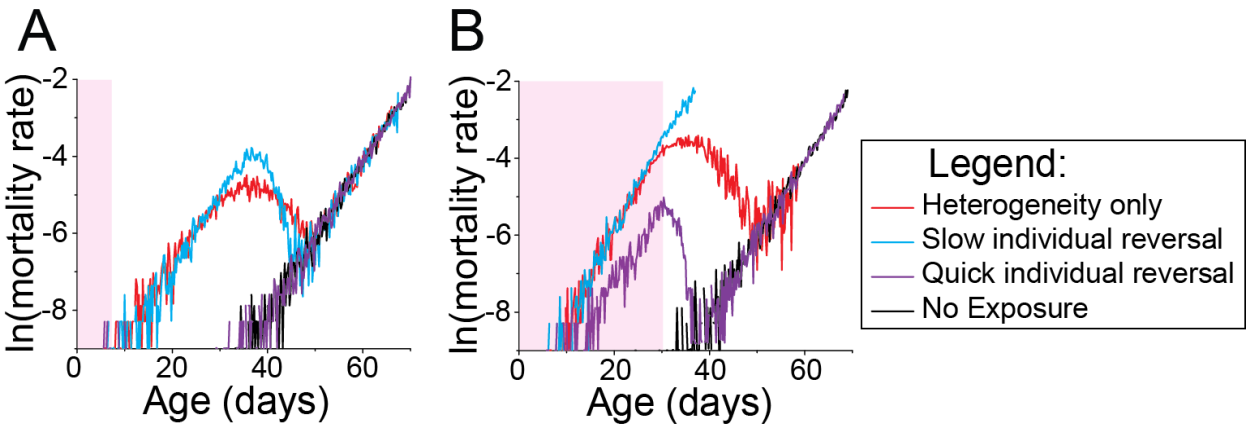


Figure 3.2: Age of pheromone removal differentiates aging patterns. (A) Removal of pheromone exposure early in life (day 7) differentiates quickly reversible individual mortality rates from slow or non-reversible individual mortality rates. (B) When pheromones are removed in middle age (day 30), populations with heterogeneous mortality rates can be differentiated from slowly reversible homogeneous populations. In all panels, $Z_n = 10^2$. Plotted values represent averages of 100 replicates each running for a maximum of 350 time steps (70 days), with WM and SM = 0.2% and FM = 1% on a 200x200 triangular lattice. The "slow" homogeneous group is defined as $D = 35$, whereas the "quick" homogeneous group is defined as $D = 5$. For the heterogeneous group effects are permanent. Shading in panels represent when pheromones were present.

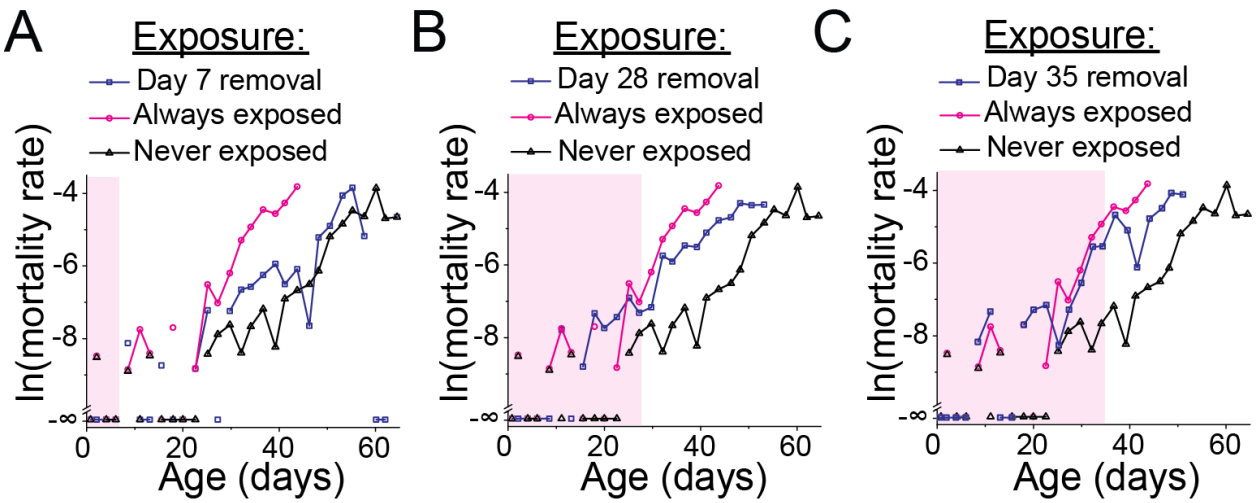


Figure 3.3: *In vivo* experiments demonstrate varying reversibility depending on age of pheromone removal. (A) When pheromones are removed at day 7, mortality rates are increased early in life, but eventually revert to those of never-exposed controls (P = .0002 by log-rank comparing day 7 and non-fem groups). When pheromones are removed at day 28 (B) or day 35 (C), mortality rates never completely revert to those of never-exposed controls (P << .0001 by log rank comparing day 28 or day 35 and non-fem groups). However, both the day 28 and day 35 groups are significantly longer lived than the life-long feminized exposure group (P < .0001 and P = .012 respectively, by log-rank). N = 94-100 for all groups. Log-rank statistical tests compare entirety of lifespans. Shading in panels represent when pheromones were present for switch group.

first week of life exhibited significantly higher post-exposure mortality rates than flies that were never exposed to female pheromones (Figure 3.3 A). These results are not consistent with the predictions generated when individual effects are rapidly reversed.

When pheromone exposure was terminated during middle-age (28 or 35 days post-eclosion) mortality rates began to separate from the life-long exposed cohort roughly one week after pheromone removal; they trended downward toward (though did not join) the mortality curve associated with non-feminized exposure for the remainder of the animals' lifespan (Figure 3.3 B, C). These observations are consistent with both slow reversibility at the individual level (on the order of weeks but not days) and a pure heterogeneity model.

One necessary condition of our simulations is that pheromone exposure will lead to increased heterogeneity in the probability of death of individuals. As this prediction cannot be directly tested *in vivo*, we instead examined a measure of health correlated to aging: the variance of climbing ability (see Supplemental Information). We found that 28 days of pheromone exposure led to a large decrease in average climbing ability when compared to both never exposed and seven-day-exposed cohorts (Supplementary Figure 3.3 A, B). 28 days of exposure was also sufficient to increase the variance proportional to the mean when compared to both never-exposed and seven-day exposed cohorts ($P <$ .0001 and $P = .008$ respectively, comparing % deviation by F test). Exposure for 7 days (followed by 21 days of recovery) led to a slight, but not statistically significant, increase in variance compared to the never exposed control ($P = 0.16$, comparing % deviation by F test). Thus, the condition demanded by the simulation appears to be fulfilled *in vivo*, and these experiments allowed for an additional, somewhat unexpected observation. It is notable that both exposed populations, (but not the never exposed population) display significant bimodal tendencies as determined by Hartigan's dip test ($P = 0.05$ for 28 day exposure, $P = 0.01$ for 7 day exposure, $P = 0.71$ for never-exposed). This suggests the development of distinct populations of flies that are affected versus unaffected by pheromone exposure. This is consistent with the distributions observed in our heterogeneous simulations (see Supplementary Figures 3.1 & 3.2).

Pheromone effects on mortality reverse over the course of weeks. To better examine the extent to which heterogeneity and individual reversibility contribute to the observed changes in population mortality, we undertook a more detailed analysis of our new experimental data using simulations designed to reflect the observed dynamics. We first

examined the values of duration (D) and magnitude (Z_n) of pheromone effects that best fit the *in vivo* mortality rates from males exposed to female pheromones throughout life. Simulations were executed using parameter values chosen from a grid comprised of 5 values of D (ranging from 5 days to permanent) and 25 values of Z_n ($Z_n=10^x$ where x={0, 0.1, 0.2, …, 2.4}). For each set of parameter values, we executed 100 simulations (with pheromone exposure throughout life) and obtained the average mortality rate at each age. We then calculated the McFadden Pseudo R^2, also known as ρ^2 (Figure 3.4 A) and the mean average deviation (Supplementary Figure 3.4) between the simulated and the observed *in vivo* mortality rates for each set of parameters. We found that the best fits were obtained along a ridge (red/orange colored in the plot) where a larger magnitude of pheromone effects was offset by a shorter duration (Figure 3.4 A).

We next chose the combination of parameter values along this ridge of best fit and executed simulations where the effects of pheromones were removed at day 7, 28, or 35. Results of these simulations were then compared to the observed data from the pheromone removal experiment, again using mean average deviation (Supplementary Table 3.1) and ρ^2 (Supplementary Table 3.2). We found that simulations in which D = 35 days and $Z_n \approx 5$ most accurately predicted the effects of pheromone removal at all three time points (Figure 3.4 D-F) as determined by both mean average deviation and ρ^2. These results suggest that pheromone effects on individual mortality patterns dissipate, albeit slowly.

To determine the extent to which heterogeneity is also influencing population mortality rates in these experiments, we repeated these simulations without heterogeneity. In the absence of heterogeneity, removal of pheromone exposure early in life led to population

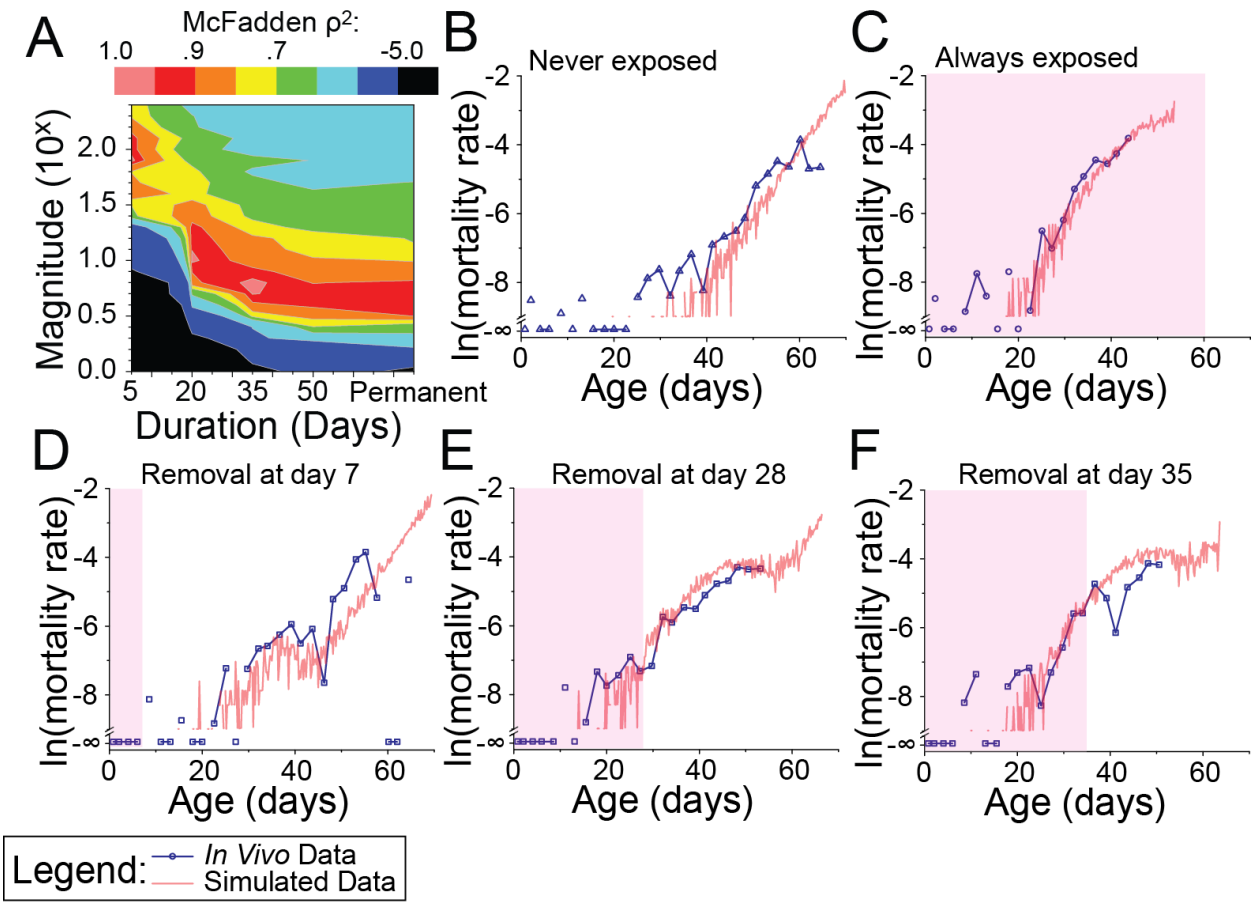


Figure 3.4: *In silico* analysis of *in vivo* data reveals individual mortality rates reverse slowly. (A) McFadden's Pseudo R^2 statistic, also known as ρ^2, [18] colormap as a function of the duration of pheromone effects D (x axis, in days) and magnitude of pheromone effects Z_n (y axis). Outcomes represent the difference between experimental fly mortality rates data with permanent pheromone exposure and simulation outcomes for combinations of D and Z_n. Further simulations (see Supplementary Tables 1 and 2) examining best fit to the *in vivo* effects of pheromone removal determined that the optimal D and Z_n correspond to 35 days and $10^{0.7}$, respectively (Shown in panels B-F). *In vivo* mortality data closely match simulated data with a pheromone effect duration of 35 days for no exposure (B), constant pheromone exposure, (C), 7 day removal (D), 28 day removal (E), and 35 day removal (F). See Supplementary Tables 1 and 2 for fitting statistics. Simulated values represent averages of 100 replicates each running for a maximum of 350 time steps (70 days), with WM = 0.2% and FM = 1% on a 200x200 triangular lattice. Shading in panels represent when pheromones were present.

mortality rates that accurately reflect reversibility seen on an individual level (Figure 3.5 A). However, when pheromone exposure is terminated later in life, the absence of heterogeneity leads to divergence from the experimentally observed mortality dynamics (Figure 3.5 B, C). Indeed, removing heterogeneity significantly worsens the fit of the middle- age removal curves, but not the early removal curves (Supplementary Table 3.3). Thus, we have demonstrated through a combination of *in vivo* and *in silico*

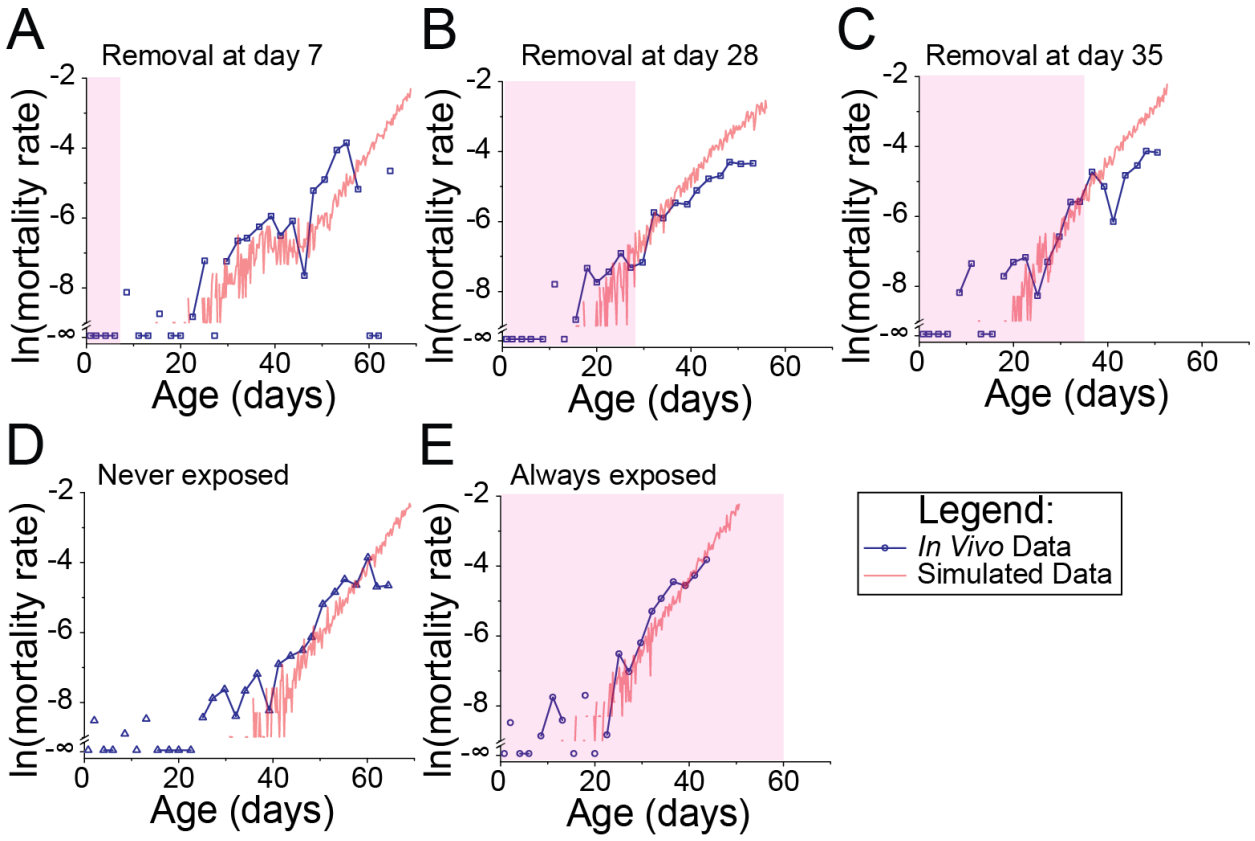


Figure 3.5: Heterogeneity drives late reversibility but not early reversibility. (A) When heterogeneity is removed from the simulation, the in vivo effects of early (day 7) pheromone removal are still replicated. However, when pheromone removal occurs on day 28 (B) or day 35 (C), removal of heterogeneity leads to deviation from the observed in vivo mortality rates (see Supplementary Table 3). Removal of heterogeneity does not prevent replication of never-exposed (D) or constantly exposed (E) mortality rates. Simulated values represent averages of 100 replicates each running for a maximum of 350 time steps (70 days), with WM = 0.2% and FM = 1% on a 200x200 triangular lattice. Shading in panels represent when pheromones were present.

experiments that pheromone exposure leads to highly heterogeneous, reversible changes in individual mortality rates, with individual reversibility driving changes in population-level mortality early in life while heterogeneity dominates population-level dynamics once flies reach middle-age.

Discussion

Understanding how genes and the environment influence the dynamics of aging in individuals is essential to many areas of aging biology. Reliable biological indices of the rate of aging, often called biomarkers of aging in the scientific literature, have proven elusive, and population measures of age-specific mortality have served as the primary method to infer patterns of aging at the individual level. Indeed, roughly 17 years ago it was observed that mortality rates decelerated in the oldest-old of several species, suggesting that the pace of aging slows in individuals of advanced age [14]. However, it was recognized at the time that population heterogeneity could produce the observed deceleration even when individual mortality rates continued to rapidly increase. Subsequent efforts to disentangle these influences have been largely unsuccessful, predominantly because demographic models of population heterogeneity are descriptive and focus on retrospective analyses.

Empirical loss of pheromone effects on mortality rates in male Drosophila result from individual reversibility and heterogeneity with demographic selection. We reexamined the conclusions from Gendron et al. [9], who used demographic analysis to show that exposure of male fruit flies to female pheromones caused a reversible increase in population mortality rates. They used this information to assert that pheromone perception had short-term effects on individual aging. However, when we simulated life-

histories of individual flies for which social interactions and the effects of pheromone exposure were systematically manipulated we found that transient changes in population level mortality rates can be caused by a combination of heterogeneity (i.e., variability in mortality parameters among individuals) and demographic selection (i.e., those individuals with the highest mortality tend to die first) even when effects on individual aging were permanent. In other words, for simple experiments, individual- and population-level mortality dynamics are confounding.

We then explored, *in silico*, experimental designs that would distinguish these two phenomena. We found that carefully timed pheromone exposure in flies of multiple, specific ages would do so, and the appropriate experiments were subsequently carried out. Our results support the original conclusion that exposure to female pheromones transiently affects individual mortality rates in male *Drosophila,* but we find that these effects are longer-lasting than previously suggested (likely on the order of five weeks rather than two weeks). This distinction is important. Pheromone exposure alters other physiologic phenotypes, such as fat stores and stress resistance, on a time scale of approximately two days. Thus, the longer-lasting effects of pheromones on mortality suggest that distinct mechanisms drive these different phenotypic outcomes.

Computational methods enhance the power of acute experimental manipulations to discern mechanisms of aging. Our results reinforce the benefits of "switch experiments," in which genetic and/or environmental manipulations known to affect lifespan are applied only at specific ages and for defined durations. While switch experiments have long been recognized as a key component to understanding aging dynamics [1,4,15], such experiments are time consuming and can be difficult to design and

interpret. However, by using computational modeling to closely examine the parameter space of an experimental design, parameters can be selected to maximize the inference obtained from these experiments. By using this method to identify the appropriate timing of switch experiments, researchers could, for example, explore whether manipulations early in life that have long-lasting effects on population mortality rates have "escapers" that are unaffected or the entire population is affected similarly.

Stochastic model simulations can help address ever more complex questions of aging. Using simulations to select experimental parameters and enhance experimental design has uses beyond examining the timing of switch experiments. For example, if a researcher were interested in exploring how the effects of diet composition on longevity varied over time, computational methods such as the one outlined here could help select both the timing of switches as well as the specific diets to be used. If used properly, such computational methods allow researchers to "test" a wide range of experimental conditions *in silico*, then use the timings and diets which are most informative in the simulations when the experiments are performed *in vivo*. For example, intuitively it might make sense to perform such an experiment with only two, extreme diets. Simulations would either support this intuition, or provide evidence that a third, moderate diet would provide additional insight.

With the growing recognition that behavior and the environment are important modulators of aging, the complexity of experiments investigating the interaction between aging, behavior, and the environment will continue to expand. Our work, along with that of Gendron et al., leave open the question of whether the beneficial effects of mating are as fast-acting and dynamic as the negative effects of pheromones, which

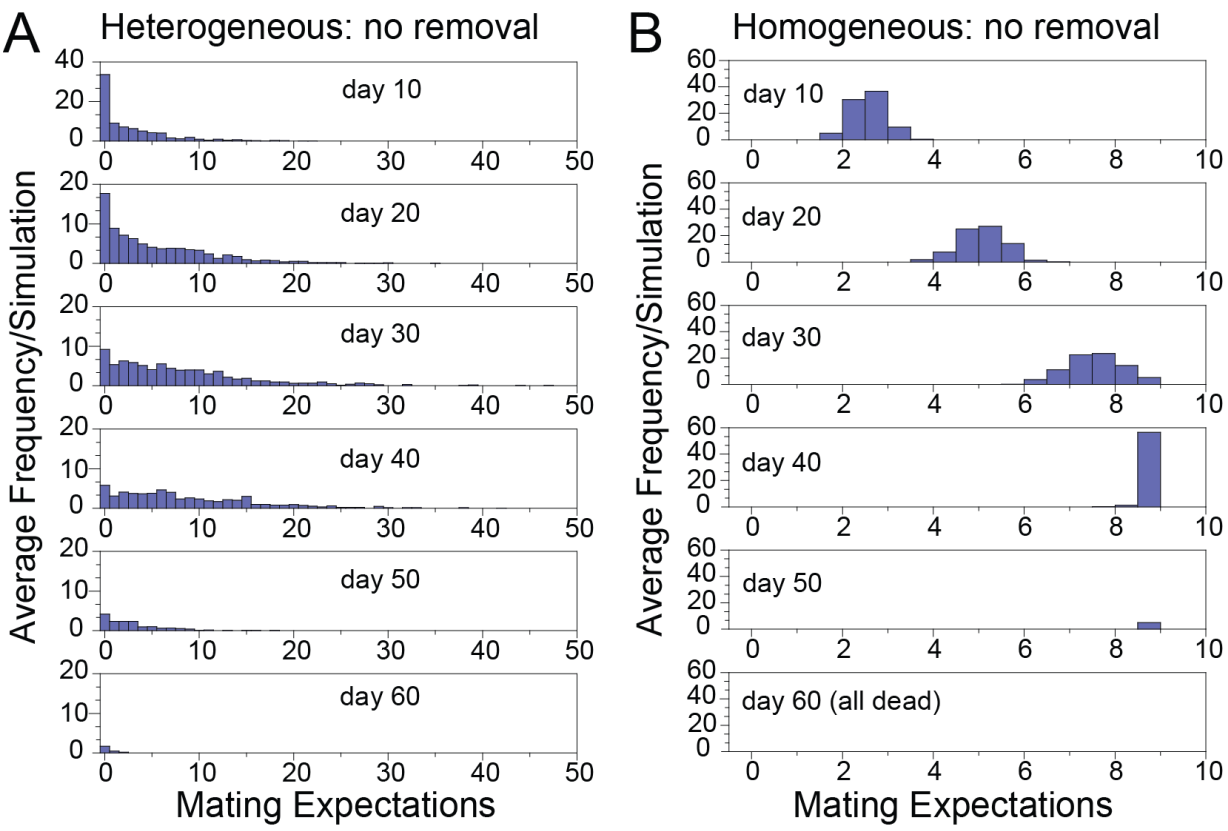
would increase complexity by adding an “outcome” (mating versus no mating) to each interaction and potentially changing how that encounter affected mortality. Along a similar vein, exploration of how complex social hierarchies and dominance amongst individuals affect lifespan would significantly increase the potential number of confounding factors, which would make it difficult for experimentalists to generate specific hypotheses and corresponding predictions. Here we demonstrate a computational tool capable of generating testable predictions while incorporating the intrinsic heterogeneity resulting from varying behaviors and life-history experiences.

Beyond the laboratory setting, heterogeneity must be considered when analyzing human interventions or treatments in middle-aged populations, as such interventions are by their very nature a type of switch experiment. With the recent increased focus on both personalized medicine and manipulations that can alter the aging process during middle age, it is becoming more important than ever to be able to ensure subtleties between individual and population effects are distinguishable. Identification of individuals that are (or conversely, are not) affected by various interventions will not only improve our understanding of mechanisms of action, but also enhance the efficacy of treatment guidelines. For example, studies have found differential effects of cardiovascular medications between various ethnicities [16]. It is reasonable to expect such heterogeneous effects may also occur between groups that are not so obviously differentiated, such as variation between patients’ gut microbiomes, which may alter the effectiveness of dietary regimens in treating type-2 diabetes [17]. It is our hope that this article can become a roadmap for improving the design, methodology, and analysis of experiments examining the effects of various treatments on an individual level.

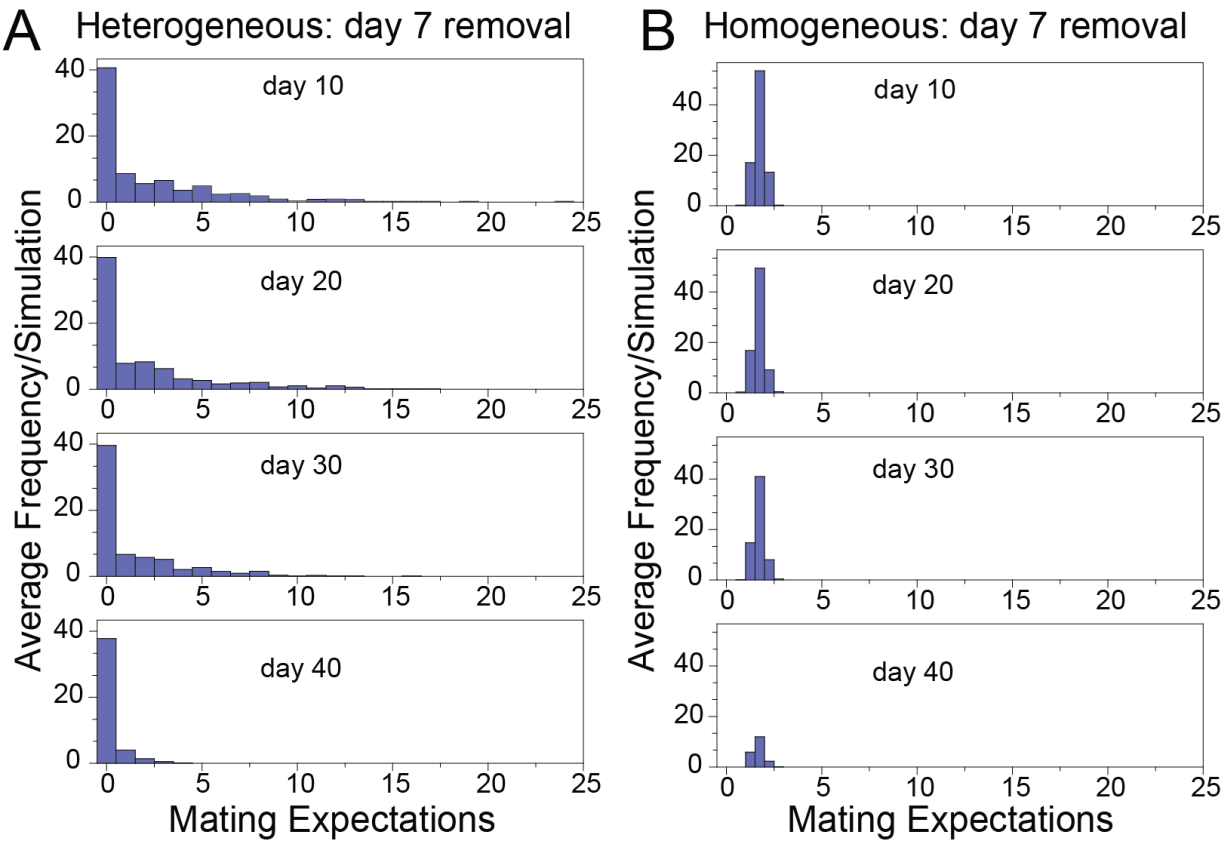
Supplementary Methods, Figures, and Tables:

Negative Geotaxis Assay: We measured vertical distance traveled by recording video footage of flies for 10 seconds after they were forcefully banged to the bottom of their lane, and then analyzing the number of vertical pixels traveled. We measured flies in cohorts of 14, with a single fly in a lane so distance could be tracked on a per-fly basis. We performed 5 replicates for each set of flies, giving 10 seconds of recovery time between each trial.

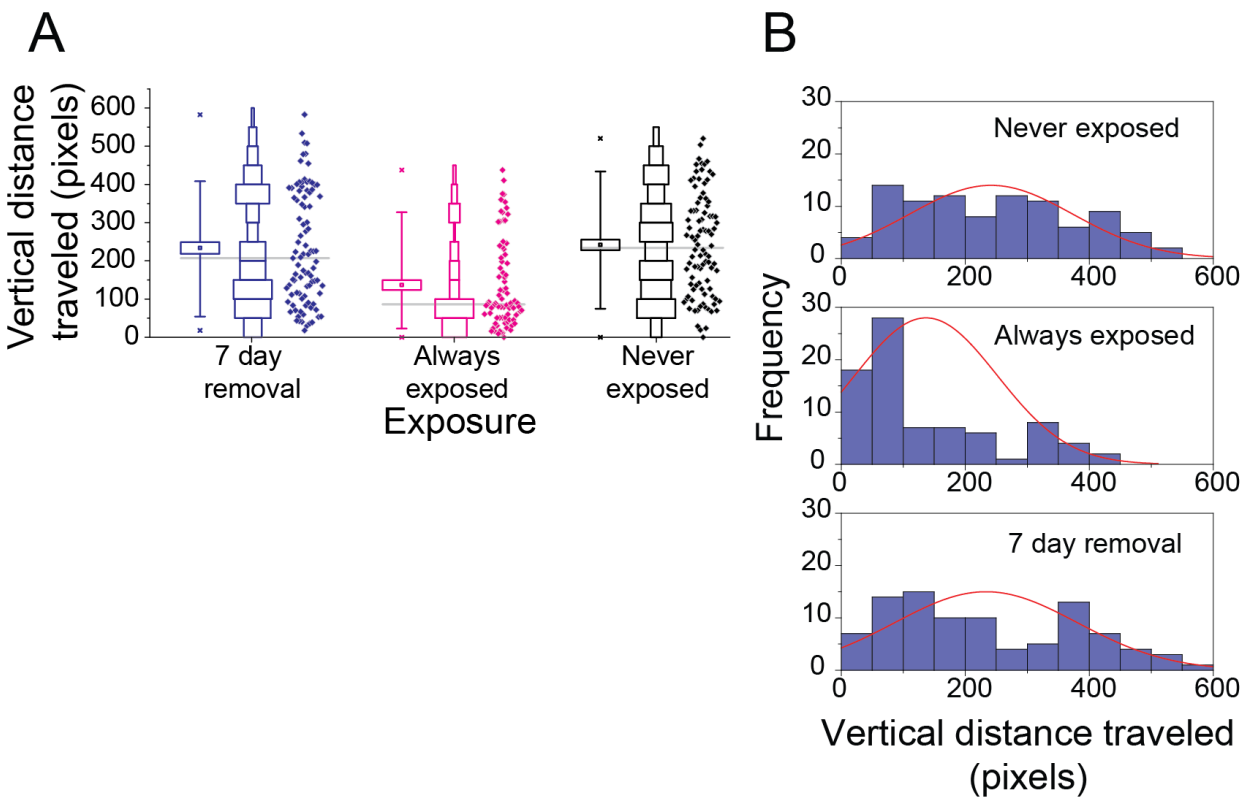
Statistical Estimation of Model Parameters from Population Results: We fit the "No Exposure" data in Figure 3.1 A using maximum likelihood, which led to an estimate for $\alpha = 5.72e\text{-}8$ (95% CI: 3.08e-8, 1.06e-7) and $\beta = 0.0424$ (95% CI: 0.04042, 0.0447). These values are similar to the values used to calculate P_{death}. The P_{death} values are $\alpha = 1e\text{-}7$, which is within the estimated CI, and $\beta = 0.04$, which is only 6% away from the estimated value of β, though not within the estimated CI. This implies that our simulated fly population may be aging slightly faster than would be expected of individual flies.



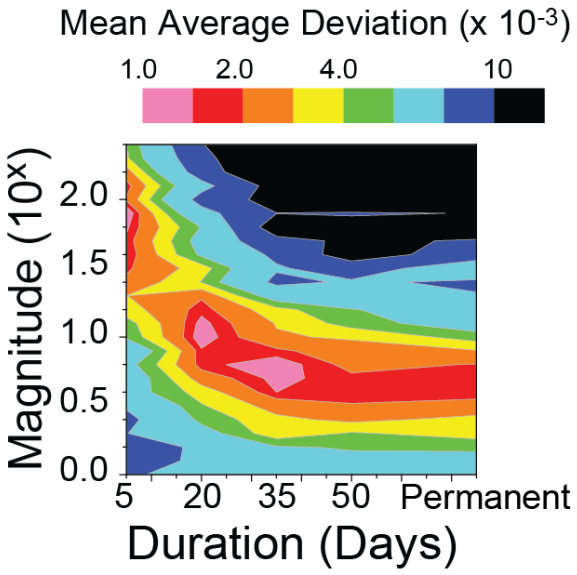
Supplementary Figure 3.1: Simulated populations are largely heterogeneous, and this heterogeneity is controllable. (A) Simulations allowing for heterogeneity lead to a population of flies with largely disparate mating expectations. (B) Our method of decreasing heterogeneity leads to a tightly controlled and normally distributed set of mating expectations. The tight normal distribution observed in the "homogeneous" set of simulations is a consequence of the stochastic nature of the simulation.



Supplementary Figure 3.2: Heterogeneity exists even after a 7-day exposure, and is slowly reversed. (A) Simulations allowing for heterogeneity lead to a wide range of mating expectations after only 7 days, and over time the flies with higher mating expectations selectively die off, leading to the reversal of the population mortality rates seen in Figure 3.1 D. (B) This same reversal is not observed when we reduce heterogeneity.



Supplementary Figure 3.3: Pheromone exposure increases heterogeneity in Drosophila populations. Flies exposed to pheromones for a full 28 days (P = 0.05) or for 7 days and then not exposed for 21 days (P = 0.01) have significantly bi-modal populations, whereas flies never exposed to pheromones do not (P = 0.71). (A) Each dot represents the average distance climbed by a single fly over 5 trials. Box is SEM, whiskers represent 10% and 90%, gray line is the median. N = 81-94, depending on the treatment. (B) Histograms with normal curves demonstrate how well the data fit a normal distribution. Significance was determined by Hartigan's dip test using MATLAB, bootstrap = 100.



Supplementary Figure 3.4: Mean average deviation identifies similar ideal values as ρ^2. Mean average deviation colormap as a function of the duration of pheromone effects D (x axis, in days) and magnitude of pheromone effects Z_n (y axis). Outcomes represent the difference between experimental fly mortality rates data with permanent exposure and simulation outcomes for combinations of D and Z_n. The optimal values of D and Z_n found here are similar to the optimal values found using other methods, including ρ^2 (Figure 3.4 A) and residual sum of squares (not shown).

Supplementary Table 3.1: Mean absolute deviation of hazard rates. Mean absolute deviation measured on hazard rates (not log-mortality) over entire duration of lifespan. Highlighted values are best (dark green) and second best (light green) fits for each switch date. Very similar results are obtained if analysis is limited to later in life when mortality rates are higher.

Pheromone removal time	Duration in model (*D*)				
	5 days	20 days	35 days	50 days	permanent
Remove @ day 7	0.0057	0.0065	0.0050	0.0060	0.0058
Remove @ day 28	0.0036	0.0024	0.0017	0.0043	0.0045
Remove @ day 35	0.0036	0.0022	0.0034	0.0058	0.0057

Supplementary Table 3.2: McFadden Pseudo R^2 (also known as ρ^2) values of hazard rates. ρ^2 calculated from hazard rates (not log-mortality) over entire duration of lifespan. McFadden's test is effective for comparison of multiple models, with the higher ρ^2 value representing the better fit for the data. However, unlike traditional R^2 tests, McFadden's test considers values over 0.4 to be of "good" fit (similar to an R^2 value of 0.8) [18]. Highlighted values are best (dark green) and second best (light green) fits for each switch date. Similar results are obtained if analysis is limited to later in life.

Pheromone removal time	Duration in model (*D*)				
	5 days	20 days	35 days	50 days	permanent
Remove @ day 7	0.2861	0.2922	0.3196	0.3925	0.4832
Remove @ day 28	-7.2344	-0.3168	0.9293	0.7687	0.7461
Remove @ day 35	-2.3244	0.7538	0.8106	0.6930	0.6850

Supplementary Table 3.3: Differences in goodness of fit measures between heterogeneous and homogeneous populations. Using ρ^2 and mean average deviation, we calculated the percent change between simulations using heterogeneous populations and those with homogeneous populations. Large differences were found in the goodness of fit in the late switches, but not the early switch, indicating that heterogeneity plays a more important role in the population mortality dynamics later in life.

Pheromone removal time	% Change in Measure of fitness	
	Mean Average Deviation	McFadden's Pseudo R^2 (ρ^2)
Switch @ day 7	21.9%	-0.35%
Switch @ day 28	76.4%	-74.3%
Switch @ day 35	63.8%	-71.6%

Data Accessibility

We have made the code for our simulations freely available as a supplemental zip file uploaded to Dryad, at the link below. The unzipped folder includes a Readme file with a brief explanation of how to compile and run the simulations with the corresponding expected inputs and outputs. The link is as follows:

http://datadryad.org/handle/10255/dryad.105631

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Chapter IV: FLIC: High-throughput, continuous analysis of feeding behaviors in Drosophila[4]

As the previous chapter demonstrates, it is common for the science in a field to push beyond the standard assays and analyses, and often furthering science requires developing new technologies. Such is the case with the studying of feeding behavior in fruit flies, where the minuscule quantities consumed lead to difficulties measuring feeding, especially in individual flies. As aging and diet appear to be tightly intertwined, the ability to accurately measure what, when, and how much an animal eats can provide important insights into the mechanisms of food perception, dietary restriction, and aging. In an effort to solve these issues, we designed a new method that allows for continuous analysis of individual fly feeding behavior with millisecond level resolution.

[4] Originally published in PLoS ONE (2014 9(6): e101107, doi: 10.1371/jmynal.pone.0101107) with authors listed as Ro, J.*, **Harvanek, Z.M.*,** Pletcher, S.D. (asterisk indicates equal contribution). Conceived and designed the experiments: JR SDP. Performed the experiments: JR ZMH. Analyzed the data: JR ZMH SDP. Contributed reagents/materials/analysis tools: JR ZMH SDP. Contributed to the writing of the manuscript: JR ZMH SDP.

Abstract

We present a complete hardware and software system for collecting and quantifying continuous measures of feeding behaviors in the fruit fly, *Drosophila melanogaster*. The FLIC (Fly Liquid-Food Interaction Counter) detects analog electronic signals as brief as 50μs that occur when a fly makes physical contact with liquid food. Signal characteristics effectively distinguish between different types of behaviors, such as feeding and tasting events. The FLIC system performs as well or better than popular methods for simple assays, and it provides an unprecedented opportunity to study novel components of feeding behavior, such as time-dependent changes in food preference and individual levels of motivation and hunger. Furthermore, FLIC experiments can persist indefinitely without disturbance, and we highlight this ability by establishing a detailed picture of circadian feeding behaviors in the fly. We believe that the FLIC system will work hand-in-hand with modern molecular techniques to facilitate mechanistic studies of feeding behaviors in *Drosophila* using modern, high-throughput technologies.

Introduction

The ascent of the fruit fly, *Drosophila melanogaster*, as one of the most powerful model systems in which to dissect neural mechanisms of complex behavior has uncovered a need for innovation at the roots of the science. Technical advances in neurobiology have outpaced those that facilitate basic observation. Consequently, challenges identified as recently as five years ago as primary obstacles to discovery, such as the ability to temporally manipulate the expression of genes in specific brain regions or to alter the excitatory properties of individual neurons, have become standard practice[1]. In

contrast, many experimental procedures that have been used for decades to characterize behaviors such as courtship, locomotor activity, and circadian rhythm have proven less than ideal for modern analysis. This is either because they fail to capture subtleties in the behavior that were not previously recognized or because they are not easily "scaled-up" and automated for genetic or pharmacological screens.

Measurement of fly feeding behavior is one area that is overdue for improvement. There is arguably no reliable and agreed upon method for measuring total food intake of flies in undisturbed, steady state conditions[2-5] and preference assays lack qualities appropriate for high-throughput analysis[6]. The most common methods use tracers, such as non-digestible dye, to quantify food intake and, by analysis of abdominal color, to distinguish the extent of food choice[7,8]. Tracer methods are most effective when exposure periods are short, otherwise they report gut size rather than feeding rate[2]. Strong preference behavior is easily identified by two-dye choice assays, but intermediate preference is difficult to quantify because one must often assess different shades of the mixed color. The Capillary Feeder (CAFE) method, which requires flies to feed from calibrated capillary tubes suspended from the top of the chamber, has been proposed as a viable alternative[9]. However, it is physically challenging for the flies to access the food, which can bias data in favor of healthy flies and make long-term studies difficult. Visual assessment of feeding behavior, based on proboscis extension, has also been suggested[10,11], but this approach is labor intensive and may confound feeding and tasting events.

Here we propose the FLIC (Fly Liquid-Food Interaction Counter), a general purpose system for accurately and continuously measuring feeding-related behaviors in

Drosophila. The FLIC device uses a simple electronic circuit that can be monitored continuously to signal when a single fly or a group of flies interacts with a liquid food. Single flies are placed in feeding areas in which one or more sources of liquid food are provided, and they are subsequently monitored indefinitely and without disturbance. Data from each food source are collected independently, allowing for simultaneous, automated analysis of thousands of flies. We thus obtain continuous trajectories for individual flies that reflect what they eat, when they eat it, and how much they consume. For simple choices, the FLIC faithfully reproduces results obtained using traditional methods. Moreover, the system provides the power and flexibility to quantify many new aspects of feeding behavior, including temporal dynamics of food assessment and circadian feeding patterns. We envision that the FLIC system, and the principles behind it, will promote discovery in fields as diverse as aging, metabolism, and neurobiology, which require detailed analysis of food intake. It will also enable researchers to study mechanisms of feeding preference and behavior using modern, high-throughput genetic and pharmacological means.

Methods and Materials

Drosophila stocks. For technical reasons, short-term experiments that required starvation were done using female flies (we found that their choice patterns were more clear-cut), and longer-term experiments (e.g., 24hr and circadian analyses) were conducted using male flies to avoid potential problems with egg-laying and to facilitate comparison with most published activity data. Unless otherwise noted, choice experiments used Canton-S female flies. Female flies carrying a loss of function mutation in the trehalose receptor *Gr5a*, *ΔGr5a*, were a gift from A. Dahanukar[12]. This

mutation was backcrossed to the w^{1118} control strain for 8 generations prior to analysis. Circadian rhythm experiments used male *Canton-S*, *yw* , and Per^{01} flies, which carry a loss of function mutation in *Per*. Per^{01} flies were obtained from P. Hardin[13].

FLIC system details. The FLIC system is comprised of four components (Figure 4.1 A). The first component, the *Drosophila* Feeding Monitor (DFM), is the physical unit that is responsible for detecting feeding behavioral events. The primary characteristics of the DFM are a set of 12 feeding wells, a dedicated signal detection circuit for each well, and a microcontroller board that controls the signal detection circuitry and that integrates data from all wells. The second component, the Master Control Unit (MCU), is responsible for coordinating up to 128 DFM, providing simple data processing, and forwarding data to the third component of the system, the PC monitoring software. The FLIC computer software allows the experimenter to control all the parameters of the system and records the data to the computer hard drive. The final component is a package for the statistical software, R, that simplifies visualization of the data and statistical analysis of feeding behavior.

The behavior arenas in the DFM are formed from an aluminum common plate, a solid plastic food reservoir base, a printed circuit board, and a plastic cover (Figure 4.1 A; Supplementary Figure 4.1). The food reservoir base is formed from 12.7mm thick high-density polyethylene (HDPE). The twelve feeding wells, 4mm in diameter, are placed in two parallel rows of six. Each row of wells is connected by a channel on the underside of the base, which connects them as a common food source. A large (8mm) hole extending into each channel is provided at the end of the device to allow liquid food to be loaded and, for longer experiments, to provide an attachment point for an external food

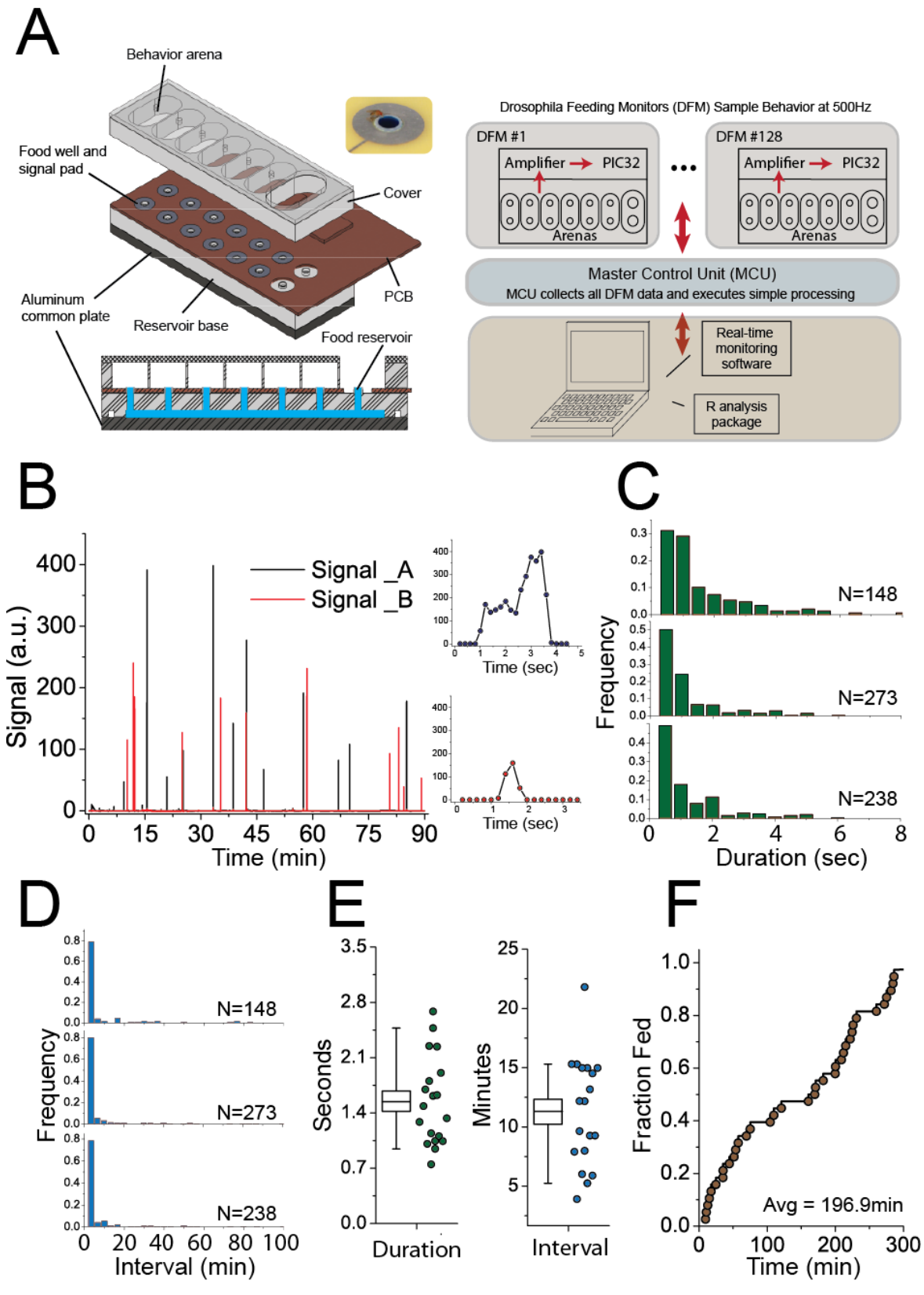

A
Behavior arena
Food well and signal pad
Cover
PCB
Aluminum common plate
Reservoir base
Food reservoir
Drosophila Feeding Monitors (DFM) Sample Behavior at 500Hz
DFM #1
Amplifier → PIC32
Arenas
DFM #128
Amplifier → PIC32
Arenas
Master Control Unit (MCU)
MCU collects all DFM data and executes simple processing
Real-time monitoring software
R analysis package
B
Signal _A
Signal _B
Signal (a.u.)
Time (min)
Time (sec)
Time (sec)
C
Frequency
N=148
N=273
N=238
Duration (sec)
D
Frequency
N=148
N=273
N=238
Interval (min)
E
Seconds
Duration
Minutes
Interval
F
Fraction Fed
Avg = 196.9min
Time (min)

Figure 4.1: Illustration of the FLIC system. (A) Cartoon of the *Drosophila* Feeding Monitor (DFM) from the top- and side-view along with a flowchart of data collection and processing. Analog signals from all DFMs are collected by the Master Control Unit (MCU), which relays the information to the PC where the signals are visualized and recorded by the real-time monitoring software. (B) Representative signals from each of two feeding wells within a single feeding arena taken from a 90min subset of a 24-hour feeding measurement. Close-up signal patterns representative of two distinct classes of feeding behavior events are presented as insets. (C) Histograms representing the distribution of durations for individual feeding behavior events (an event is a set of contiguous signals above baseline) over a 24hr measurement period. Each plot represents values from a single fly, and distributions for three flies are presented. N represents the number of behavior events observed. (D) Histograms representing the size of the intervals between successive feeding behaviors over a 24hr measurement period. Each plot represents values from a single fly, and distributions for the same three flies as in panel C are presented. (E) Among-fly variability in the average feeding duration and average time between feeding events. Each point represents the average value over a 24hr period (N = 21). (F) Event-time distribution that represents the fraction of the population that has experienced at least one feeding at a given elapsed time from a randomized point between 12pm-2pm (N = 21). It took roughly 197min for 50% of the population to feed at least once during this time of the day.

reservoir. A 6.35mm thick aluminum plate is secured to the bottom of the reservoir base and provides a low-resistance connection between each liquid food source and, therefore, among all of the feeding wells. Elastomer O-rings around each of the two channels prevent leakage and cross-contamination of food.

A printed circuit board (PCB) is fixed to the surface of the reservoir base and serves both as mechanical support for the electronic circuitry and as a floor for the feeding arenas. Holes in the PCB achieve a press-fit around each of the individual feeding wells, which extend 0.2mm above the floor. This design allows the liquid food in each well to achieve a modest meniscus that is easily accessible to the flies and that is isolated from the PCB floor. Surrounding each of the feeding wells on the surface of the PCB is a 13mm diameter conductive metal pad, which is connected by standard traces to the detection circuitry.

A machined plastic cover is placed on top of the PCB to separate the floor into a number of distinct arenas depending on the design of the cover. We have constructed covers that form 6 two-choice areas (Figure 4.1 A) as well as covers that form 12 single-choice areas. The cover is composed of 12.7mm thick HDPE that forms the walls of the arenas and 3.2mm thick acrylic that is used for the ceiling. The clear acrylic allows personal observation or video monitoring of the flies during the experiments. A small hole in the acrylic ceiling above the center of each choice arena is used to insert the flies. A large opening in the ceiling at the end of the DFM allows access to the pair of food-loading holes.

To taste or consume the food in any particular well, the fly must stand on the conductive metal pad and extend a leg or proboscis into the liquid. When this occurs, the fly itself completes a simple voltage divider circuit and the resulting voltage is detected and recorded. Each individual arena houses two liquid food wells, and both foods are given a common 5V charge through the aluminum common plate. Each metal pad is grounded through a 10 MΩ resistor, which ensures a low input voltage when the fly is not touching the food. When the fly physically interacts with the food the voltage across the 10 MΩ resistor serves as the input to a simple, non-inverting operational amplifier (op-amp) with a gain of approximately 1.2. The op-amp output voltage is detected by an 11-bit, analog-to-digital converter of a PIC32 microcontroller (PIC32MX320F128; Uno32 board from Digilent, Inc). One PIC 32 is capable of monitoring all 12 feeding wells at a rate of 500KHz (i.e., every 2µsec). With our circuit design, electrical current through the fly is negligible (< 0.001mA), and feeding behavior is unaffected.

The MCU coordinates data collection from each of the DFM and forwards processed signals to a personal computer for final visualization and recording. The core of the MCU is a PIC32 microcontroller, and it communicates with up to 128 DFM by Inter-Integrated Circuit Protocol (I^2C). Each of the individual DFM employs a simple low-pass filter by maintaining a running average over a fixed number of measured signal intensities. The MCU queries each DFM several times per second to obtain and coordinates the collected data from each, and it then forwards them to a personal computer (PC) via wireless serial communication or TCP/IP. The FLIC computer software provides a real-time view of the data from each feeding well and stores signal data from each well to an appropriate text file. An open source package for the statistical software, R, provides visualization and quantitative analysis of raw and processed data. The number of active DFM in an experiment, the rate at which the DFM collects feeding signals, and the frequency at which the MCU queries each DFM are configurable.

Solid model and circuit design: Detailed SolidWorks part files of all machined components (metal base, plastic base, and plastic/acrylic cover) and PCB designs are available at www.wikiflic.com.

FLIC Signal Data Processing

Baseline calculation: Analog signals were recorded as 11-bit integers ranging from 0-1023. These values are called signal intensities, and they represent voltages ranging from 0-3.3V. In practice, nearly all signal intensities fell into a range of 0-700. To adjust for background fluctuations in the readings, corrected intensities from each signal pad were calculated by subtracting the signal baseline. Signal baseline was determined using a median smoother of fixed window size (normally 5 minutes). Because of the high

temporal resolution of the data (for the examples presented here signals were obtained every 200ms) signal intensities that indicated feeding behaviors were rare in any given window, and the median intensities within any 5min interval accurately represented background.

Behavior identification: Feeding behaviors were identified by signal intensities that surpassed a defined threshold value above baseline. For all of the experiments except those involving circadian rhythm, we used a fixed threshold. By (*i*) manually recording an exact behavior at the instance of a fly-food interaction, (*ii*) matching that to raw FLIC signals, then (*iii*) dissecting the flies gut afterward to verify a presence of blue dye to categorize characteristics of tasting and feeding signals, we established that the longer, high intensity signals corresponded to feeding events, while the ephemeral spikes were most often associated with tasting events. Thus, feeding events were defined as periods in which a particular analog signal intensity exceeded a value of 200, while signal intensities from 20-100 were considered tasting events. While we found these values to be effective for our studies, differences in the conductivity of the experimental food, for example, could necessitate an adjustment. More sophisticated algorithms are certainly possible for detecting and categorizing feeding behaviors, and we developed one for circadian behaviors, which we designed to account for modest changes in the average signal intensities that occur over the course of a multiple-day experiment. This adaptive threshold algorithm identified a significant signal as one that exceeded three times the 90% percentile of signal values over a five minute window. A minimal threshold was specified to avoid spurious events when there were zero interactions with the food in a five minute window. While the adaptive threshold performed better over the course

these experiments, it does not escape our notice that there are likely more effective ways to be developed that better extract feeding information from millions of data points. Nevertheless, it is reassuring that the general characteristics of the observed phenotypes and the resulting biological inference are apparently robust to changes in the details of the analysis.

Behavioral Assays

CAFE choice assays: Our CAFE assay was modified from Devineni and Heberlein[14]. The choice chamber consisted of a plastic vial with a fine metal mesh floor for air exchange and a size 0 rubber stopper capped ceiling with 2 drilled holes, which were fitted with truncated 200µl pipet tips that allowed a snug fit for two 5µl graduated capillary tubes (Analtech Inc., Neward, DE). The vials were placed above water to increase local humidity and reduce evaporation from the capillary tubes. Each chamber was loaded with three flies. Flies were given access to water-filled capillaries for 24hrs prior to food choice experiments to induce modest starvation and enhance intake. Water-filled capillary tubes were then replaced with tubes filled with either 10% or 1% sucrose solution. A small amount of mineral oil was placed on top of each capillary tube to minimize evaporation. The 3hr choice assay was performed in 25°C, 60% relative humidity, and uniform lighting. After 3hr, the capillaries were removed and the displacement of liquid was measured to estimate the food consumption. The food displacement from each capillary tube was divided by number of flies in each vial to obtain the estimated volume consumed per fly, and preference index (PI) was calculated as "[(Volume of 10% sucrose consumed/fly)-(Volume of 1% sucrose consumed/fly)]/[Total volume consumed/fly]". Two vials without flies were used to

measure evaporation of each food solution and to adjust estimates of consumption accordingly.

Two-dye choice assays: We labeled 10% sucrose and 100μM denatonium with either 0.05% FD&C #1 brilliant blue or 0.1% sulforhodamine. Each DFM was loaded with either blue 10% sucrose and red 100μM denatonium or (the converse) red 10% sucrose and blue 100μM denatonium. The choice assay was performed for 3hr in the FLIC, after which flies were anesthetized by CO_2 and inspected under a microscope to determine their abdomen color. We assigned a PI of 1 to flies with intense abdominal color matched to 10% sucrose, 0.5 to less intense color with a shade of purple, and a PI of 0 to flies with purple abdomen. Scores of -0.5 and -1 were given to flies with abdomen the color of the 100μM denatonium food label.

FLIC assays: When monitoring simple feeding behaviors (i.e., for experiments that did not involve food choice), we either filled both channels of the DFMs with the same liquid food or we blocked one set of food wells with a plastic plug. In all the other cases, each of the two channels was filled with a particular food of interest. After loading the foods, an individual fly was introduced in an arena through a hole in an acrylic celling using an aspirator. We began signal collection software before flies were loaded to ensure that no signals were lost. In general, loading 8 DFMs with 48 flies took less than 5 minutes. Feeding PI values from the FLIC system were calculated as "[(Total feeding time from food A)-(Total feeding time from food B)]/Total feeding time". See below for selection of signals generated by feeding versus tasting.

Behavior statistics: The duration of a feeding event was defined as the width (in seconds) of a series of sequential signal intensities above threshold. To determine wait-time distributions (e.g., the fraction fed as a function of time), a Kaplan-Meyer survival estimate was used with flies that failed to feed within a particular experiment considered right-censored. The average wait time distribution to the next feeding event was calculated, for each fly, as the time elapsed from a randomized point between 12pm-2pm until the next feeding behavior. For testing whether PI is significantly different than 0, we used paired randomization test. Measures of total consumption for each fly were computed as the sum of the durations of all significant feeding during the assay period. Linear regression analysis was used to test whether different hours of starvation can predict total feeding estimation generated by either CAFE or FLIC assay. When comparing total consumption of foods from three different starvation groups, we performed One-way ANOVA followed by Bonferroni post-hoc test.

Circadian Analyses

Binning data: We used ClockLab to execute circadian analysis on the FLIC feeding behavior data. Because the resolution of our raw data is too high for ClockLab analysis, significant feeding behavior events for each fly were binned into 30 minute intervals by summing the total feeding time within that interval. Bins were defined in such a way that lights-on and lights-off occur at the junction of two bins. Binned data were output to a .txt file compatible with the ClockLab toolbox for Matlab. Transient periods of mis-communication among the DFMs, MCU, and the computer, were considered missing data, which ClockLab interprets as zero activity.

Normalization: Behavioral data were normalized within each fly to ensure an equal influence on the ZT plot and to avoid active individuals masking information from less active ones. Data for behavioral counts within each 30min interval were divided by the average 30min count for that individual over the entire experiment. A normalized behavioral count of 1 for a single 30min interval implies an average number of interactions over that interval, 2 indicates twice the average, etc.

External Food Reservoir: During circadian experiments, we attached an external food reservoir to the DFMs to ensure that the liquid food was maintained at a constant level (Supplementary Figure 4.2 A, B). We used a 15 mL glass scintillation vial containing the appropriate food (10% sucrose) attached to each channel by a short piece of flexible tubing. During the circadian experiments, the reservoirs were monitored under dim red light conditions and filled as necessary to maintain appropriate food levels.

Circadian analysis: Rhythmicity, periodicity, and power were determined using the ClockLab software as described previously[15]. Briefly, we used the power and significance values obtained using ClockLab's batch analysis functions to determine rhythmicity of individual flies. The period of all flies determined to be rhythmic was averaged to find the overall period of that genotype or treatment. Actograms present data from individual flies that were representative of the majority of flies from that genotype. They were obtained using ClockLab's scaled actogram function. Flies that died or escaped during the experiments were excluded from all analyses.

Results

FLIC allows reliable detection and differentiation of feeding and tasting events. The FLIC system provides an unprecedented amount of detail about a single fly's interactions with food in normal, undisturbed conditions. All of the experiments in this report used a configuration where the DFMs assess feeding signals every 2ms. A running average over 100 signal intensities was employed to remove high-frequency noise, and the MCU forwarded processed data to the PC every 200ms, where it was stored for future analysis. While this configuration did not utilize the full capabilities of the system, it provided sufficient resolution to distinguish tasting from feeding events (see below) without producing a crippling amount of data. Even at this limited resolution, a three hour feeding experiment using 30 flies produced 1.6 million data points. A similar-sized experiment measuring circadian feeding behavior (see below) surpassed 70 million data points.

To provide a flavor for the data produced by the FLIC system, food interactions with a 10% sucrose solution were measured for fully-fed male flies over 24hr without disturbance or operator interference. Every significant contact between a fly and the liquid food produced a signal spike, which was visualized on the PC software and recorded (Figure 4.1 B). A simple threshold was used to distinguish fly-food interaction events from background noise, and interactions as brief as 50μs were captured. We observed distinct types of events based on the characteristics of the signal including persistent signals of high-intensity as well as lesser-intensity, ephemeral spikes (Figure 4.1 B, inset). Low-intensity interactions were common, while sustained, high-intensity signals were substantially less frequent, resulting in an exponential distribution of

duration times for individual behavioral events (Figure 4.1 C). For starved flies, we also observed an approximately exponential distribution of interaction times, although most events were, on average, 5 times longer than non-starved flies. We also found that flies tended to interact with the food in high-frequency bursts that were punctuated by long interludes (Figure 4.1 D). Among individuals, the average duration of an event in our experiment was 1.5sec, while the average time between events was 11.3min (Figure 4.1 E). Finally, the average wait time from a randomized point between 12pm-2pm until the next interaction with the food was quite long (197min; Figure 4.1 F), although it should be noted that this analysis spanned the time of day when feeding behavior is thought to be least frequent (see below).

FLIC is more sensitive than the standard methods of analysis. The continuous nature of the analog signals from the FLIC system allows a broad range of feeding behaviors to be characterized. To simplify comparison with existing methods, which focus almost exclusively on total food consumption over a predefined period of time, we developed algorithms (see Methods and Materials) that categorize signal patterns into specific behaviors ; longer, high intensity signals were considered to represent feeding events, while the ephemeral spikes were most often considered tasting events (Figure 4.2 A).

We found that inference extracted from the FLIC data using our simple algorithms was consistent with that obtained using the traditional two-dye and CAFE assays. With each of the two feeding wells in an arena filled with a different food (A vs. B), choice was quantified by a Preference Index that ranged from 1 (complete preference for food A) to -1 (preference for food B) with a value of 0 indicating no preference[14] .When identical foods were placed in both wells the average PI among male and female flies was near

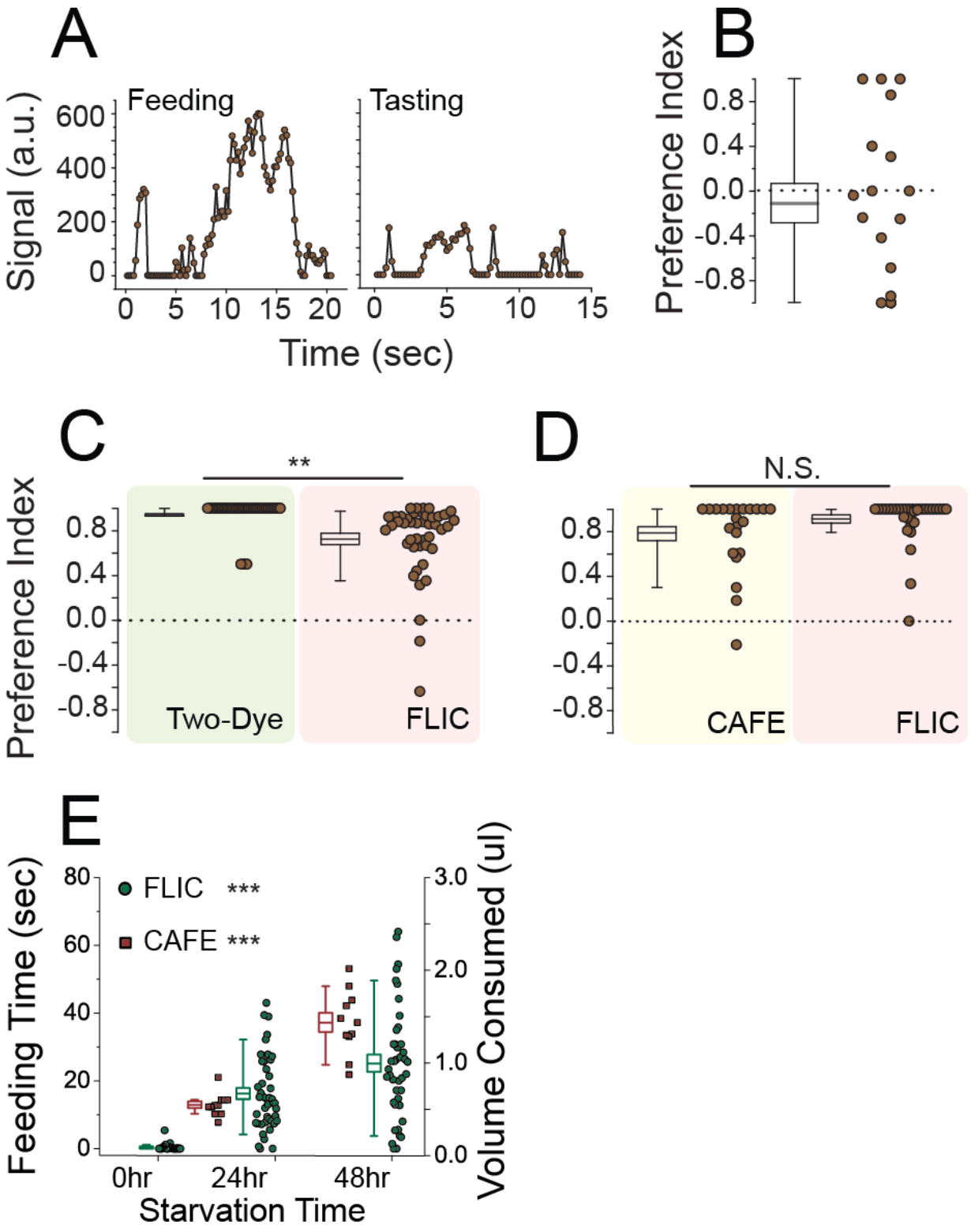

A
Feeding
Tasting
Signal (a.u.)
600
400
200
0
0 5 10 15 20
0 5 10 15
Time (sec)
B
Preference Index
0.8
0.4
0.0
-0.4
-0.8
C
**
Preference Index
0.8
0.4
0.0
-0.4
-0.8
Two-Dye
FLIC
D
N.S.
0.8
0.4
0.0
-0.4
-0.8
CAFE
FLIC
E
FLIC ***
CAFE ***
Feeding Time (sec)
80
60
40
20
0
3.0
2.0
1.0
0.0
Volume Consumed (ul)
0hr
24hr
48hr
Starvation Time

Figure 4.2. Comparison between traditional food choice assays and the FLIC system. (A) The analog signals from feeding (left) and tasting (right) behaviors have distinct characteristics. (B) When presented with identical food in both food wells, male and female flies do not exhibit a preference, which rules out systematic bias in the FLIC system (open symbol, male; closed symbol, female; pooled paired randomization test, $P = 0.97$). (C) Flies exhibited strong preference in favor of 10% sucrose over 100μM denatonium when measured using both two-dye and FLIC assays (Box charts represent mean, standard error of mean, and 10-90% quantile whiskers). (D) Flies demonstrated strong preference toward 10% sucrose over 1% sucrose when measured using both the CAFE and FLIC assays (Box charts represent mean, standard error of mean, and 10-90% quantile whiskers). (E) Estimates of food consumption using the CAFE and FLIC assays. Longer starvation resulted in increased food consumption as well as total feeding time (linear regression, $P < 1x10^{-5}$ for both assays). Changes in food volume in the capillary tubes was undetectable when fully fed flies were used, and only FLIC data are presented for that treatment. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

zero, indicating that there is no significant bias inherent in the FLIC design (Figure 4.2 B). To establish that the FLIC system reliably identifies non-zero preference behavior we exposed female flies following 24hr starvation to foods containing either 10% sucrose (sweet) or 100μM denatonium (bitter). Each food was simultaneously labeled with either 0.05% FD&C blue or 0.1% sulforhodamine red (food coloring was swapped for independent experiments) to allow direct comparison with dye color measures. After three hours the flies were removed, and a PI was determined for each individual fly based on the color of their abdomen (please see Methods and Materials for detail) as well as the feeding signals detected by the FLIC system.

While both methods produced an average PI that was substantially in favor of the sucrose food, the FLIC system was able to capture greater inter-individual variability in choice behavior. Indeed, the FLIC data suggested that some flies consumed modest amounts of the bitter food (Figure 4.2 C), which apparently could not be detected visually based on abdomen color. To confirm that a fraction of individual flies do indeed consume 100μM denatonium when presented as a choice against 10% sucrose, we

executed similar choice experiments using starved Canton-S female flies with one modification: we added 0.5% FD&C blue dye only to the denatonium food. Following one hour during which the flies were exposed to both foods, each animal's abdomen was examined for evidence of blue, which would indicate some consumption of the denatonium-laced food. We were able to visualize blue dye in 37.5% (6/16) of the flies.

The CAFE assay is often used when both foods are palatable because different shades of mixed colors that result from the two-dye approach are difficult to quantify. We therefore compared the FLIC system and CAFE assay in their ability to assess choice between a 1% and 10% sucrose solution, both of which are known to be appetitive for starved flies[7]. For the CAFE assay, we placed female flies following 24hrs starvation into a chamber with two calibrated capillary tubes, each filled with one of the two foods. After 3 hours, we measured the change in food volume in the tubes to calculate the amount of each food consumed per fly and the final PI. The PI estimates from CAFE and FLIC were similar in their distribution (Figure 4.2 D).

In addition to preference, measurement of the total amount of food a fly consumes is of interest. To illustrate how the FLIC system can be used to detect differences in overall consumption, we computed total feeding time from female flies that were starved for 24hrs or 48hrs as well as from flies that were fully fed. Assuming similar rates of food uptake per unit time, these estimates should be proportional to total consumption. We therefore compared the FLIC estimates with those obtained using the CAFE assay, the latter of which are based on measurements of the food volume lost in capillary tubes. To obtain a detectable change in liquid levels during a 3hr CAFE assay, three flies were housed in the same feeding chamber, and the volume of food consumed in each

chamber was divided by three to obtain per fly measures. Total feeding time (in seconds) from individual flies over the 3hr assay was obtained using the FLIC. Despite group housing, we were unable to detect measurable changes in liquid levels for the CAFE assay using fully fed flies, while the FLIC system detected a small number of feeding events (Figure 4.2 E; 0hr). Following 24hrs and 48hrs of starvation, a significant increase in feeding could be measured in both assays (Figure 4.2 E), and relative to the 48hr values, the differences were highly consistent (an average of 66% increase in feeding every 24hrs of starvation for the CAFE assay vs. a 50% increase for the FLIC). Notably, the distribution of data from the FLIC provides a direct estimate of the among-fly variability, and after taking into account the group measures in CAFE, the FLIC system resulted in a lower coefficient of variation (0.66 vs. 0.84, FLIC vs. CAFE, respectively).

FLIC allows for analysis of new dimensions of behavior. FLIC data represent feeding behaviors of variable nature and intensity as a rich set of analog signals with high temporal resolution. Having shown that simple summary statistics from these data recapitulate inference using traditional methods, we sought to propose new types of analyses that might be used to develop insight about more subtle aspects of feeding behavior. While it seems difficult to predict what kinds of hypotheses will eventually be tested using the FLIC, in this section we explore questions that interested us and that, in seeking their answers, provided a sense of the flexibility of the system and the principles involved.

How many times do flies taste each food before they discriminate between them? Can the very first feeding choice reliably predict food preference over a longer time period?

Although apparently uneventful, behaviors prior to food choice may provide insight into the biology associated with sensory evaluation of the food and linked with the animal's current nutritive state[16]. To explore these issues, we calculated the fraction of time flies spent in behaviors we characterized as tasting prior to consuming their first meal in the 1% versus 10% sucrose choice experiment described above. In most cases, flies devoted less than 10% of their time to tasting prior to making their first meal choice (Figure 4.3 A). Remarkably, nearly 90% of the time their first meal was taken from the same food that was preferred overall during the 3hr experiment. Flies also exhibited an increased number of estimated tasting events directed toward the food chosen for their first meal (Figure 4.3 B). These analyses suggest that initial ingestive behaviors result from measurable assessments and are strongly predictive of overall preference.

How does the preference behavior of a fly change during the course of an experiment? Given that feeding behavior may be driven by different mechanisms early and late in the assay[17], a method that provides a continuous estimate of preference is desirable. In such cases it is possible to calculate a time--dependent preference index (TDPI), which incorporates only events that occur within a specified time window. To illustrate this principle, we used the 1% vs. 10 % choice experiment to estimate a continuous preference index in which preference was calculated every 3 minutes using only the previous 30min of feeding behavior. While the cumulative PI measure was uniformly high throughout the experiment, (Figure 4.3 C, left panel), the TDPI measures revealed that preference for 10% sucrose changed during the experiment (Figure 4.3 C, right panel). Strong preference toward the higher concentration of sucrose solution was followed by a reduced preference after 90min, which may indicate that the preference

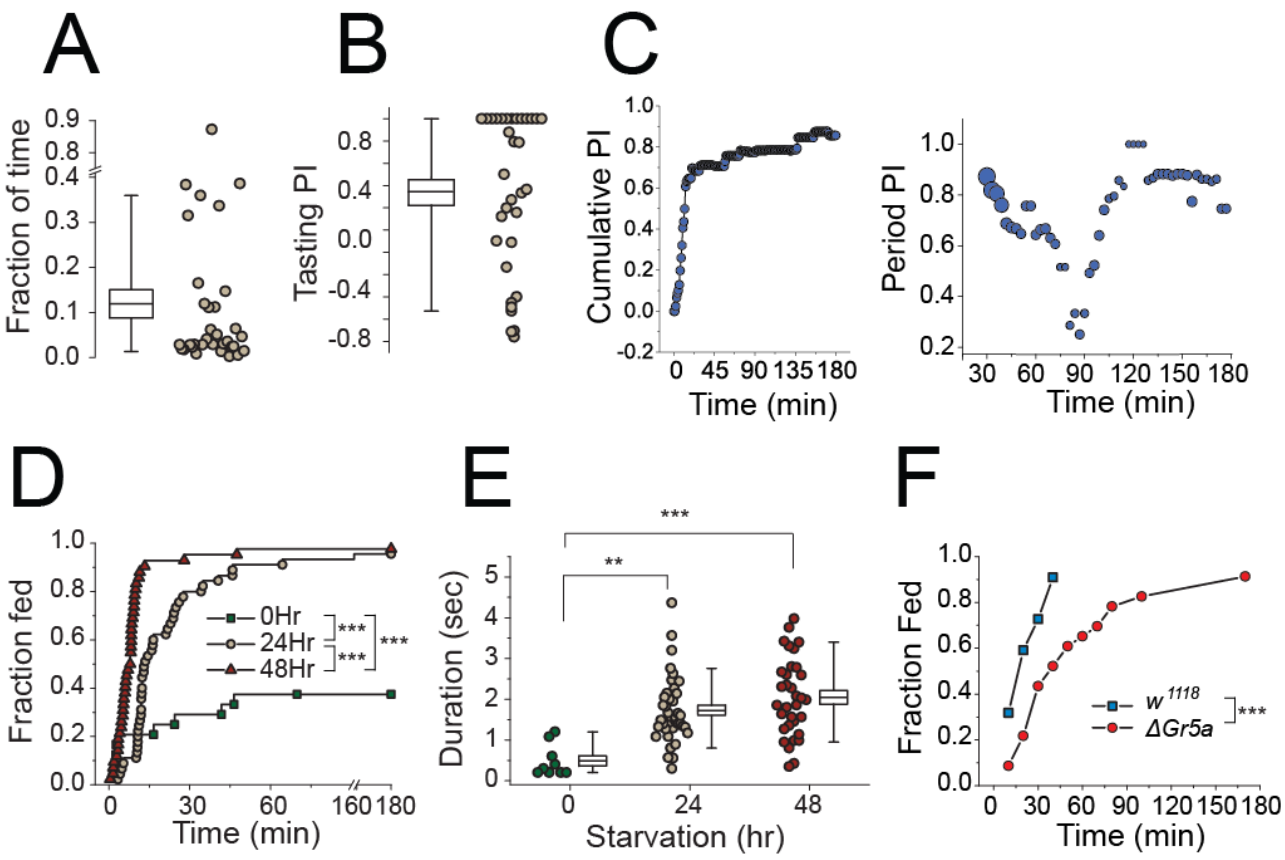


Figure 4.3: New types of behavioral inference from the FLIC system. (A) Flies spent 10% of their time in behaviors we categorized as tasting two foods prior to making their first meal choice. Fraction of time is calculated based on "total time spent tasting/ time until the first meal". (B) A greater fraction of tasting events were directed toward the food the flies choose to consume (mean Tasting PI = 0.35). A Tasting PI = 1 implies a fly tasted a single food before ultimately consuming that food. A Tasting PI = -1 implies that a fly tasted a single food before ultimately consuming the opposite food. (C) While a cumulative preference index (left panel) is effective at portraying overall feeding tendencies, time-dependent preference indices (right panel) reveal subtle differences in behavioral choices as the experiment progresses. Flies exhibited a strong preference toward 10% sucrose in the first 30min, which was attenuated in later time periods then returned to a strong preference (N = 34; the size of the symbol is proportional to the sample size contributed to calculate PI in a given period). (D) Flies with increased feeding motivation (through longer periods of starvation) experienced their first meal earlier than control flies. Flies starved for increasing periods (0hr, 24hr, or 48hr) exhibited reduced latencies until their first feeding event. Latency curves were found to be significantly different via log-rank test. (E) Flies with increased hunger (through longer periods of starvation) exhibited meals that were of significantly longer duration than control flies (One-way ANOVA followed by post-hoc test using a Bonferroni correction). (F) Taste input plays a role in motivation by decreasing latency to the first meal. Flies with loss of function in the trehalose receptor, *ΔGr5a,* were significantly delayed in their first meal of a liquid trehalose food compared with control animals (log-rank test). $^{**}P \leq 0.01$; $^{***}P \leq 0.001$

for 10% sucrose was enhanced by the importance placed on its nutritional value early on in the experiment (flies were starved prior to analysis). After satiation, the nutritional reinforcement may be lost and a lasting, but more modest, preference index is driven by taste. Notably all individuals were actively feeding when they were first introduced to the DFMs, perhaps due to hunger. After 40min, however, often less than a half of the population were feeding over any given 30min period, which indicates a reduction of feeding motivation after initial satiation (Figure 4.3 C, right panel; size of symbol).

Is it possible to quantify the motivation of a fly to feed? We reasoned that highly motivated flies would feed sooner and that the duration of early feeding events would be, on average, longer. To compare feeding event data from flies with putatively different levels of motivation, we measured female flies that were starved for 24hrs or 48hrs as well as flies that were fully fed. We found that flies starved for 48 hours fed significantly sooner than flies starved for 24 hours and that flies from both starved groups fed significantly sooner than fully fed animals (Figure 4.3 D). Indeed, over 60% of the fully fed flies failed to exhibit a significant feeding event during the 3hr experiment, while nearly all of the starved flies fed at least once during the first hour. Furthermore, the average meal duration increased significantly with increased starvation time (Figure 4.3 E).

Can sensory-dependent feeding behaviors be distinguished from those that are driven by hedonic or physiological reward? For example, prolonged starvation leads to preference for calorie-rich foods independent of taste inputs, while palatability determines choice under less stressful conditions[18]. Consistent with previous findings, we found that *ΔGr5a* mutant flies, who are unable to taste the sugar trehalose,

demonstrated a significantly longer latency to ingest their first trehalose meal compared to control flies, consistent with the idea that a lack of taste input reduced their motivation to feed (Figure 4.3 F) [18]. Mutant flies did not exhibit significant differences in interactions categorized as taste behaviors prior to feeding, and they eventually developed a strong preference for trehalose, suggesting a role for hedonic feedback later in the assay.

FLIC allows for unprecedented resolution in Drosophila circadian feeding analysis.

The FLIC system is particularly suitable for studying areas such as circadian biology, which require long-term, continuous measures of feeding activity without disturbance. To measure circadian feeding behavior in individual flies, we equipped each FLIC monitor with an external food reservoir, which served to maintain a constant volume of liquid food in the FLIC food channels throughout the duration of multiple-day experiments (Supplementary Figure 4.2 A, B). Male flies were loaded into DFMs containing a 10% sucrose solution, and the monitors were maintained under constant temperature and humidity as well as a controlled light cycle. Behavior was measured over two complete 12:12 light:dark cycles and 72 hours of complete darkness, and standard circadian analyses were applied to the data.

Circadian rhythms were clearly evident in actograms of individual *yw* flies (Figure 4.4 A). Indeed, 100% of the flies exhibited rhythmic feeding, with an average period of 23.3 hours and a combined power value of 64.6 (see Methods and Materials for statistical information). Much like general activity, feeding behaviors were concentrated near lights-on and lights-off (Figure 4.4 B). The periodicity remained through constant darkness, although feeding appeared to coalesce around the subjective evening at the

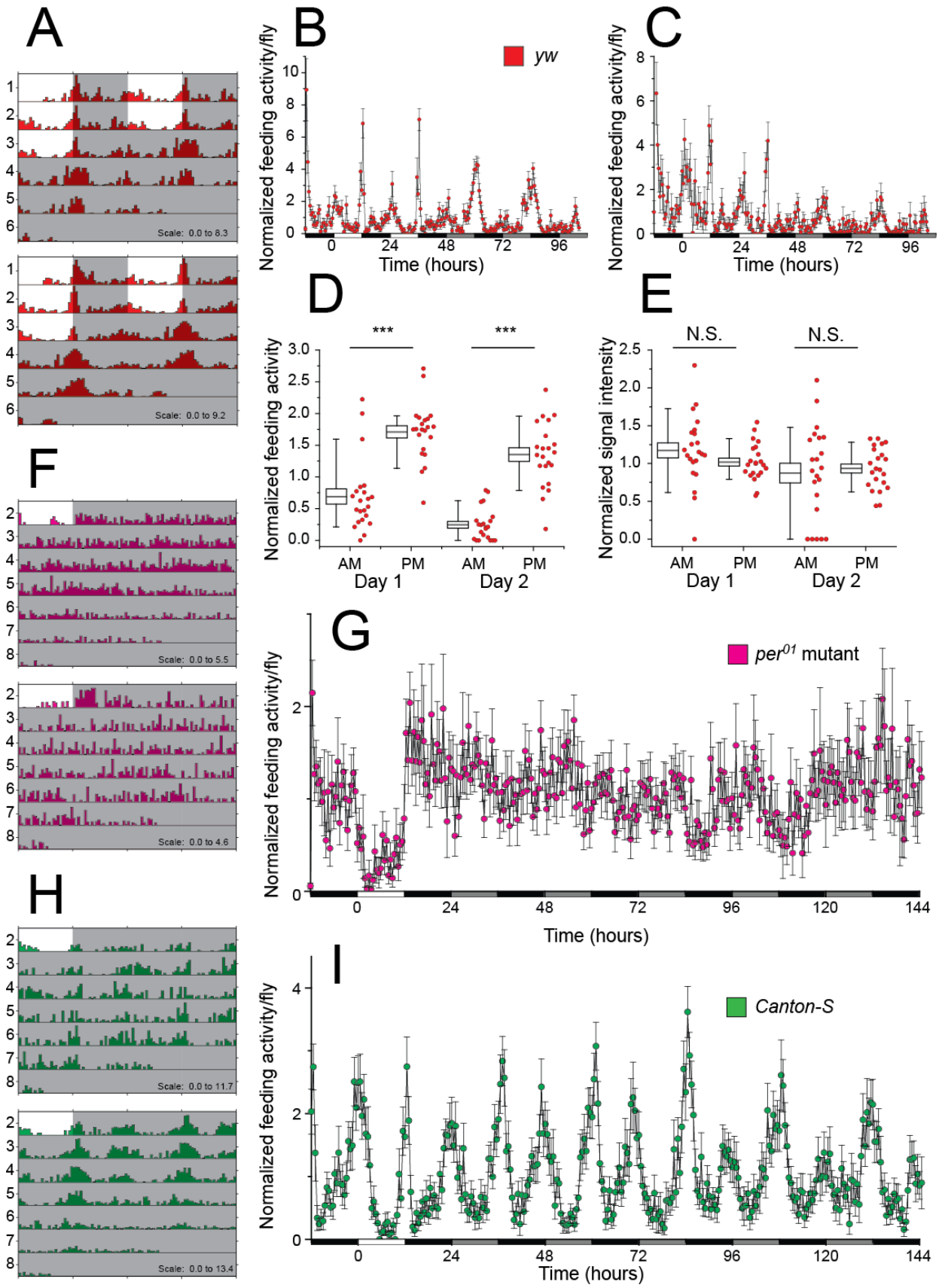

A
Scale: 0.0 to 8.3
Scale: 0.0 to 9.2
B
yw
Normalized feeding activity/fly
Time (hours)
C
Normalized feeding activity/fly
Time (hours)
D
***
***
Normalized feeding activity
AM
PM
AM
PM
Day 1
Day 2
E
N.S.
N.S.
Normalized signal intensity
AM
PM
AM
PM
Day 1
Day 2
F
Scale: 0.0 to 5.5
Scale: 0.0 to 4.6
G
per01 mutant
Normalized feeding activity/fly
Time (hours)
H
Scale: 0.0 to 11.7
Scale: 0.0 to 13.4
I
Canton-S
Normalized feeding activity/fly
Time (hours)

Figure 4.4. Feeding activity is circadian and dependent on the central pacemaker. (A) Representative actograms for two individual male *yw* flies depicting circadian feeding behavior during 12h light: 12h dark (LD) and constant darkness (DD) conditions. In all actograms, dark background indicates lights-off condition, and white background indicates lights-on conditions. Each horizontal line contains 48 hours of feeding activity data with the 2nd day of data on one line repeated as the first day of data on the following line to aid visualization of circadian period. (B) Averaged normalized feeding activity as a function of time reveals a strong circadian pattern of feeding behavior. Significant behavior events were determined using an adaptive threshold (N = 22). (C) Strong circadian patterns of feeding activity persist when a conservative criterion for behavior detection is used (N = 22). (D) Total feeding activity is higher in the subjective evening compared with subjective morning. Normalized feeding activity of each fly was obtained from the 2 hour window centered on the subjective lights-on and lights-off times during the first and second days of complete darkness (two-tailed paired-sample t-test; ***$P \leq 0.0001$). (E) The frequency distributions of feeding intensity signals are not different between morning and evening times. Normalized signal intensity of feeding events for each fly were from the 2 hour window centered on the subjective lights-on and lights-off times during the first and second days of complete darkness (two-tailed paired-sample t-test; $P > 0.05$). (F). Representative actograms of feeding patterns from two individual male Per^{01} mutant flies. (G) Averaged normalized feeding activity as a function of time for male Per^{01} mutant flies provides no evidence for feeding rhythms (N = 17). (H) Representative actograms of feeding patterns from two individual male *Canton-S* control flies. (I) Averaged normalized feeding activity as a function of time reveals consistent circadian rhythms for male *Canton-S* control flies (N = 22).

expense of morning. This conclusion is robust to particulars of the data analysis; circadian rhythms were evident when the criteria used for detecting a feeding behavior was made significantly more conservative (i.e., a higher defined signal threshold was used), suggesting that periods of increased feeding behavior are associated with increased consumption (Figure 4.4 C).

Our data revealed that under 12:12 light-dark conditions and constant darkness flies feed both in the morning and the evening. While the overall number of signals indicative of feeding activity was significantly higher in a two hour window surrounding subjective lights-off compared to lights-on, the distribution of their intensities was not significantly different between the two periods (Figure 4.4 D, E). These results indicate that the types of feeding behaviors that occur in the morning and evening are similar, but that the behaviors are more frequent in the evening. To verify that flies were actually consuming

significant amounts of food in the evening window, we loaded several DFMs with a 10% sucrose solution in the morning, and one hour prior to lights-off we added concentrated blue dye into the food-loading holes in the FLIC. The dye rapidly diffused throughout the food channel, thereby allowing us to introduce food tracer to each chamber without disturbing the flies. Two hours later, we found that 93.3% of the flies consumed a significant amount of dye, supporting our inference from the FLIC system.

To investigate whether the observed rhythms were circadian in nature, we measured feeding activity of Per^{01} mutant flies, which lack a functional core clock. We found that 47% of the Per^{01} mutant flies (N = 17) failed to exhibit any rhythmicity in feeding behavior (Figure 4.4 F), and the population as a whole was highly arrhythmic (Figure 4.4 G). When mutant flies did exhibit significant rhythms, they were weak (power = 24.6) and widely distributed (average period = 27.8 h, SEM = 1.86 h). It is notable that over half of the Per^{01} mutant flies that exhibited significant rhythms had a period between 31 and 33.5 hours. However, as the rhythms are weak and are based on only 5 days of data from constant darkness, these "rhythms" are most likely the result of random fluctuation. Feeding patterns of *Canton-S* males were similar to those previously observed for *yw* males (Figure 4.4 B); 100% exhibited rhythmic feeding with an average period of 23.4 hours (SEM = .042 h) and a power of 67.2 (Figure 4.4 H, I) [15]. Similar to *yw* males (Figure 4.4 D), Canton-S males also tended to feed more frequently in the evening, though the changes were more subtle and did not appear until the second day of complete darkness (Supplementary Figure 4.3). These data suggest that while qualitative circadian feeding behaviors are consistent across laboratory strains, genetic background must still be taken into account during these experiments.

Discussion

The FLIC system provides a precise and continuous quantification of the number and duration of interactions a fly has with food. It complements conventional methods of analysis, such as the CAFE assay and tracer dye approaches, by allowing comprehensive long-term studies of new and subtle aspects of feeding behavior. New measures of behavior, such as the time-dependent PI, revealed temporal aspects of food choice and suggest that preference toward a particular food can be determined within any defined period. The FLIC's temporal resolution allowed an examination of the duration of each feeding and tasting bout and an exploration of the flies' level of feeding motivation. By distinguishing and quantifying both feeding and tasting behaviors in this way, it may be possible to address questions relating food palatability with the impact of metabolic or hedonic feedback. Finally, the ability to carry out long-term experiments without operator interference led to evidence that, similar to circadian changes in sensory neuron sensitivity[19,20], feeding is prevalent both in the morning and evening and that circadian feeding is dependent on a functioning core clock. Surprisingly, we observed that a significantly greater amount of feeding in the evening, compared with the morning, which contradicts a previous report from Xu and colleagues who argued that flies concentrate nearly all of their feeding activity in the morning[21]. It seems likely that transferring flies onto the labeled food prior to data collection, as required by the protocol used by Xu et al., may have disrupted natural feeding behaviors and thereby confounded measures of food intake.

The FLIC measures represent individual behaviors and accurately capture individual variation. Although inter-individual variation in food choice is often observed by an

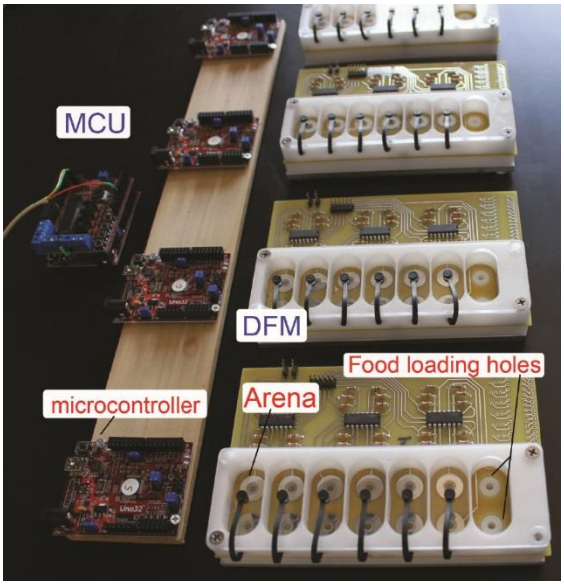
experimenter when performing a food choice experiment, conventional methods often focus on measures of preference based on groups of individuals, mostly due to an inherent lack of resolution in the methods. For example, a preference index of 0 for a group of flies can be obtained in two ways, with either each individual in that group consuming equal amounts of two foods or by half of the flies in a group exhibiting complete preference for one food and the remaining half showing complete preference for the other. Although the latter scenario may be an extreme case, it illustrates that group measures have the potential to be unrepresentative of individual behaviors and that dominant group behaviors can effectively eliminate measurable signal from rare individuals. The FLIC system may serve as a useful tool to circumvent these issues and to better address the causes for individual behavioral tendencies.

The principles embodied in our FLIC system might be adapted to expand its scope beyond feeding behavior. For example, the DFM could be modified to deliver an electric shock upon feeding, thus providing an individual-based aversive learning paradigm[22]. Food preference could be monitored continuously and simultaneously to measure the rate and extent of learning. Additionally, it is known that flies exhibit addiction-like behavior toward alcohol[1,14,23]. By coupling an aversive stimulus to a fly following alcohol consumption, one may be in a position to quantify motivation for alcohol consumption in the face of punishment. In this way researchers may be in a position to observe the origin of addictive behavior and measure its strength in response to genetic manipulation.

In summary, the FLIC system is a powerful tool for dissecting context-based feeding behaviors that encompass complex interactions among the characteristics of the food

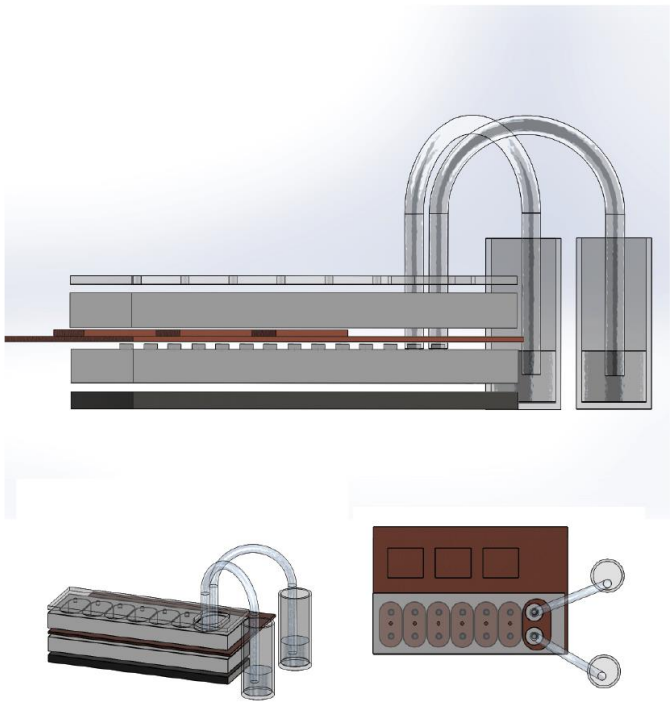
and the physiological drives of the animal. By combining the capabilities of the FLIC system with genetic tools available in *Drosophila* for manipulating gene function or neuronal activity, researchers can begin to address creative questions that will reveal important insights into neuronal and molecular mechanisms regulating feeding decisions.

Supplementary Figures

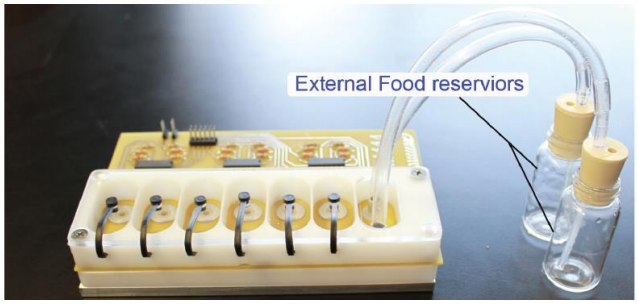


Supplementary Figure 4.1: An image of the FLIC system. A picture of the FLIC system showing a master controller unit (MCU), four microcontrollers, and four *Drosophila* feeding monitors (DFM) that consist of six behavioral arenas and a pair of food loading holes per DFM.

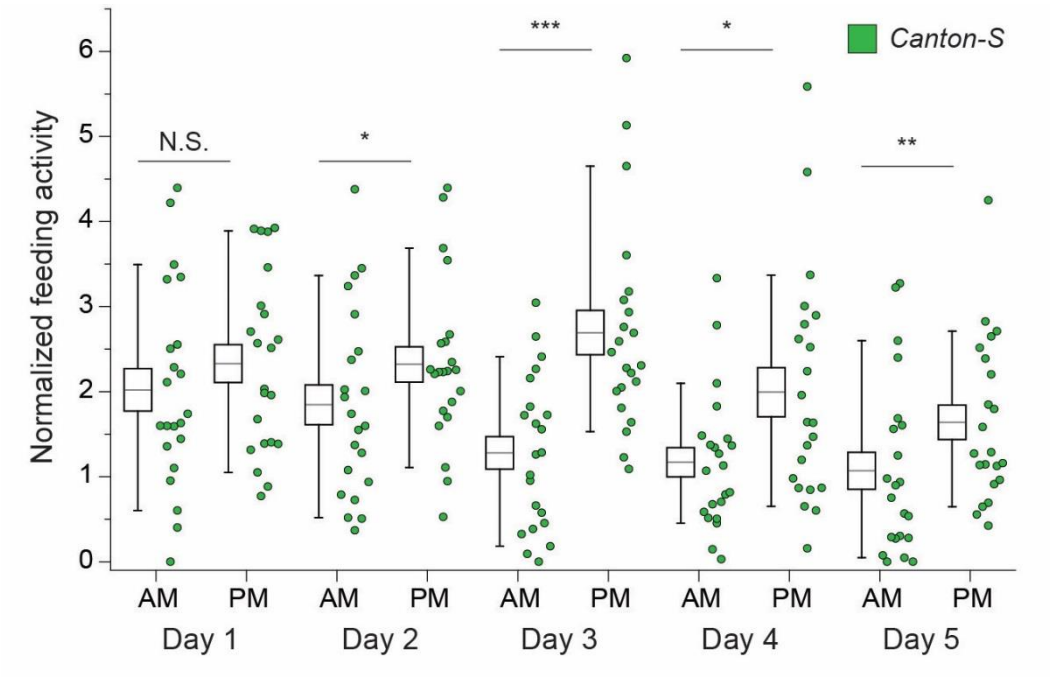
A



B



Supplementary Figure 4.2. Illustration of the FLIC system with external food reservoirs. (A) Cartoon of a DFM fitted with external food reservoirs shown from the side view (Top), the angled view (Bottom left), and the top view (Bottom right). (B) A picture of a DFM connected to two external food reservoirs.



Supplementary Figure 4.3. *Canton-S* males' feeding in morning and evening periods. Beginning on the second day of DD, total feeding activity in *Canton-S* males was significantly higher in the subjective morning than in the subjective evening (N = 22). Normalized feeding activity of each fly was obtained from the 2-hour window centered on the subjective lights-on and lights-off times for each day of complete darkness (one-tailed paired-sample t-test; **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001).

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Chapter V: Neuropeptidergic Signaling Regulates Aging and Lifespan in *Drosophila*[5]

While previous chapters demonstrated that the brain regulated the costs of reproduction in male flies, we cannot generalize from this single example that the brain is a key modulator of aging. *Drosophila* is an ideal system for studying aging because of both the rapid advancement of genetic tools and experimental methods as well as the speed and cost at which these experiments can be performed. By using these advantages of *Drosophila* research, we set out to determine how the brain as a whole might regulate the aging process. By analyzing the results of 288 different manipulations we identified specific structures and signaling molecules in the brain capable of regulating aging, demonstrating the importance of the central nervous system in the aging process.

[5] This chapter was written and edited by Zachary Harvanek (ZMH). Drs. Brian Chung (BYC) and Scott Pletcher (SDP) provided comments. This project has been a highly collaborative process between ZMH, BYC, and several undergraduate students, including Emily Feuka (EF), Lakshmi Guduguntla (LG), Sabine Hutter (SH), and Alex Standiford (AS). The screen and initial follow-up (e.g, Figure 5.1 and Figure 5.2 A and B) were performed by ZMH, BYC, EF, and AS. Lifespans involving mutants (e.g, Figure 2 C and Figure 4) were performed by ZMH, BYC, LG, and SH. Physiologic and behavioral experiments (e.g., Figure 5.2 D, E, Figure 5.3, and Supplementary Figure 2) were performed by ZMH and SH. Immunostaining and qPCR (e.g., Figure 5.5 and Supplementary Figure 5.1) were performed by BYC and LG. ZMH, BYC, and SDP conceived of this project and were primarily responsible for experimental design.

Abstract

Scientists generally accept that the brain is the origin of behavior and emotions, which result from changes in the neural state. Slowly, scientists are beginning to recognize that the central nervous system is also of prime importance in physiologic regulation, including regulation of the aging process. To determine how the neural state modulates aging, we examined the effects of manipulating 78 different subsets of neurons in *Drosophila melanogaster*. We found that defined regions of the brain are of prime importance for modulating aging, including the Pars Intercerebralis (PI), Subesophageal Ganglion (SOG), and Mushroom Bodies (MB). One specific subset of neurons was examined more closely, those neurons in the PI expressing the neuropeptide *Dh44*, which is the *Drosophila* homolog of the mammalian stress response hormone corticotropin-releasing hormone (CRH). Both inhibition of *Dh44*-expressing neurons and mutation of the *Dh44* gene itself extend lifespan in females. Mutation of *Dh44R1*, but not *Dh44R2*, fully recapitulated the longevity effects associated with loss of *Dh44*, suggesting that the aging effects of *Dh44* are transmitted through its receptor *Dh44R1*. Preliminary evidence suggests that *Dh44* modulates aging through insulin-like signaling, a common and well-conserved aging pathway. Thus, in this chapter we demonstrate that several neuronal subsets regulate longevity in general and specifically map out a neural circuit through which a conserved stress hormone regulates aging and physiology.

Introduction

Peptidergic signaling regulates a wide range of physiologic parameters and behaviors that can influence healthy aging. In humans, signaling peptides released from the brain

and other endocrine organs influence hunger[1], sleep[2], memory[3], reproduction[4], and a vast number of other functions important to health in old age[5,6]. Signaling peptides similar in form and function have been discovered in a number of other species, ranging from primates and mice to invertebrates. In the fruit fly, *Drosophila melanogaster*, many neuropeptides with human homologs, such as neuropeptide F (NPF) and the insulin-like peptides (dILPs), have been found to regulate sleep[7-9], nutrient stores[10,11], stress resistance[12,13], and lifespan[11,14]. As the homologous signaling peptides in humans often have similar functions[15-17], *Drosophila* physiology can be used to understand the underlying mechanisms through which these peptide signaling pathways modulate the aging process.

One new area of study regarding the effects of peptide signaling is how they can regulate mental states such as emotions and stress. While research is ongoing, there is evidence for relationships between specific peptide pathways and various mental states. For example, research suggests that neuropeptide Y (NPY, the homolog of *Drosophila npf*) modulates stress responses in humans[18,19], and it is currently being investigated as a potential mechanism for treating PTSD[20]. Additionally, studies suggest that another neuropeptide, oxytocin, may be useful for treating anti-social behavior[21-23], social fear[24,25], and even autism-spectrum disorders[26,27]. Beyond humans, there is interest in studying emotional states across the animal kingdom due to interest in both animal welfare[28] and understanding the origin of human emotions[29,30]. It has been proposed that central nervous system (CNS) states similar to emotions exist in simpler organisms such as fruit flies[29], and a likely mediator for these states is neuropeptidergic signaling. Thus, model organisms may provide an opportunity to dissect the neural circuits that

underlie emotional and mental states in humans, along with their effects on behavior and physiology.

To identify novel neuropeptides that regulate aging, we screened subsets of neuropeptidergic neurons for their ability to extend lifespan when activated or inhibited throughout adult life. Several neuronal assemblies were identified, and one was studied in detail: neurons that express the neuropeptide *diuretic hormone 44* (*Dh44*), which is homologous to human corticotropin-releasing hormone (CRH)[17]. Inhibition of neurons expressing *Dh44*, as well as a loss of function mutation of the *Dh44* gene, extends lifespan in female flies. Mutation of the *Dh44R1* receptor, but not the *Dh44R2* receptor, recapitulates the effects of *Dh44* loss on lifespan. These changes are not the result of changes in feeding behavior nor is *Dh44* in the same pathway as dietary restriction (DR). Instead, we hypothesize that *Dh44* regulates longevity by altering stress levels through changes in the internal state (or "emotional state") of the fly's brain.

Results

The "Fly Qi" screen identified specific brain regions that are important for regulating lifespan. We used the Janelia FlyLight GAL4 collection[31,32] to manipulate the activity of 78 individual subsets of neurons that putatively express known neuropeptides or neuropeptide receptors and measured the subsequent effects on lifespan. The selected GAL4 lines covered a wide range of neurons distributed throughout the brain in an unbiased manner. Neuronal subsets were either activated using the temperature-sensitive cation channel *TrpA1* [33] or inhibited using the potassium rectifying channel *Kir2.1* [34] in combination with a temperature sensitive *Gal80^{ts}* [35]to allow for adult-specific expression. Because GAL80^{ts} suppresses GAL4 activity at low temperatures, but

is inactivated at high temperatures a simple shift of the flies to a higher temperature effectively activates or silences the targeted neurons, respectively. Of 288 total manipulations (activation and inhibition for each sex in genotypes surviving development), 24 exhibited a significant increase in mean lifespan of 10% or greater, while 115 demonstrated a decrease in mean lifespan of 10% or greater (Figure 5.1 A, B, Supplementary Table 5.1).

To identify regions of the brain that are enriched in the manipulations that extended lifespan, we scored neurons a +1 for every manipulation in which they were targeted in a manipulation that increased lifespan following either activation or inhibition. We then created density maps of the brain that revealed concentrated areas of neuron that were commonly targeted following activation (Figure 5.1 C) and inhibition (Figure 5.1 D). These concentrations were not an artifact of an uneven distribution of GAL4 drivers used, as neurons that did not modulate lifespan were not enriched in these areas (Figure 5.1 E). Regions enriched in longevity-extending upon neuronal activation include the Subesophageal Ganglion (SOG) and the Mushroom Bodies (MB). The SOG receives inputs from many sensory neurons, including pheromone- and water-sensitive gustatory neurons, which are known to modulate lifespan[11,14,36-38]. The MB are important for higher-order processing of sensory information, and are known to receive inputs from the SOG[39,40]. In the inhibition image, there is a concentration of neurons in the Pars Intercerebralis (PI), which is the functional equivalent of the hypothalamus as it is responsible for mediating homeostasis within the fly[41]. The PI is also the location of the insulin producing cells (IPCs), and previous work has demonstrated that deletion of insulin-like peptides extend lifespan, serving as a positive control for our screen[41,42].

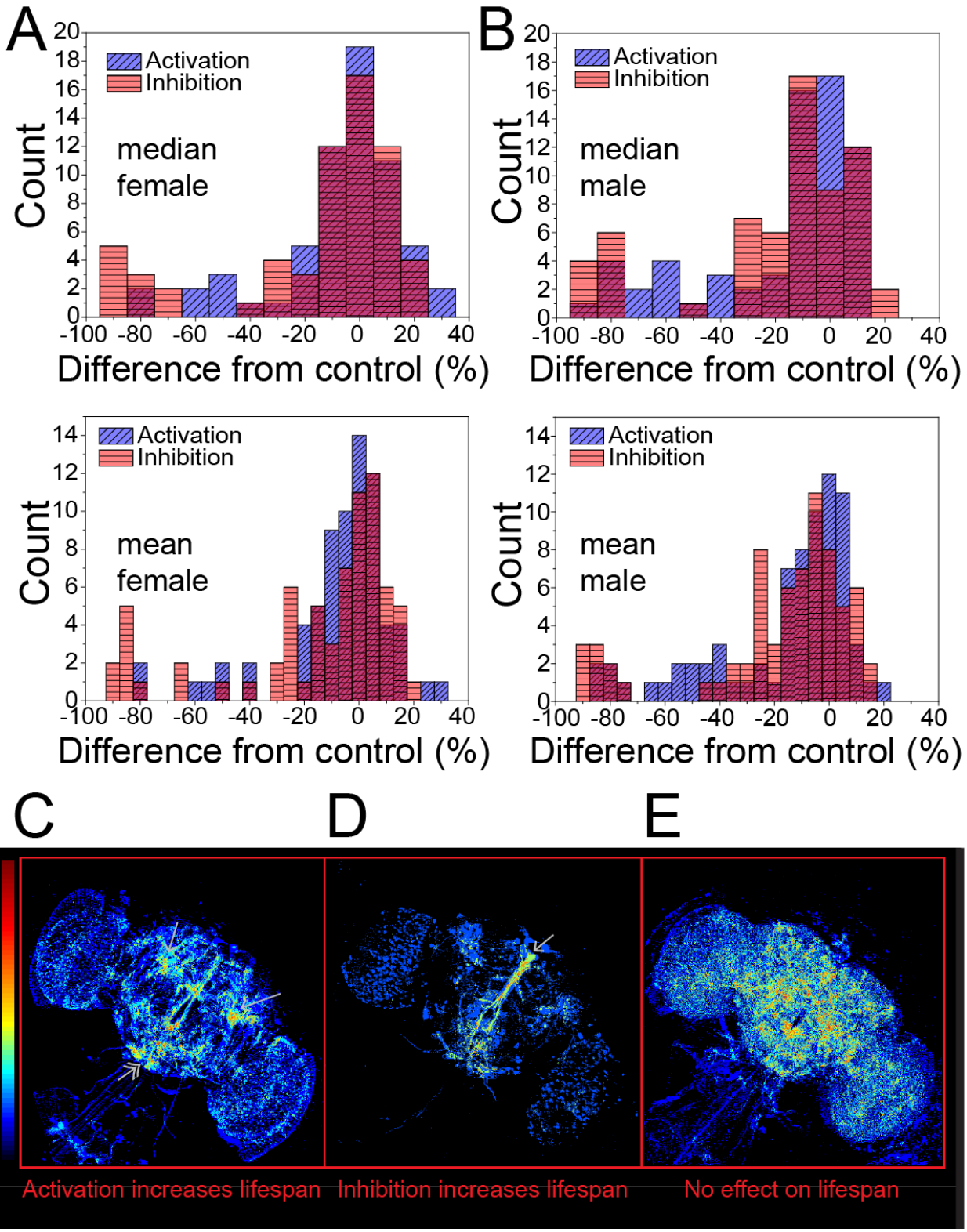

A
B
C
D
E
Activation
Inhibition
median
female
median
male
mean
female
mean
male
Count
Difference from control (%)
Activation increases lifespan
Inhibition increases lifespan
No effect on lifespan

Figure 5.1: Neuropeptidergic signaling in the PI, SOG, and MB regulates lifespan. (A) Overlaid histograms of median and mean changes in % lifespan from GAL4-only controls in females. Red indicates inhibition, blue activation, and purple is an overlap. (B) Overlaid histograms of median and mean changes in % lifespan from GAL4-only controls in females. (C) Heatmap of neurons that significantly increase lifespan upon activation. 11 brain images are overlaid in this image, with the maximum of 7 images overlapping at the reddest pixels. Single arrows point to mushroom bodies, double arrow points to subesophageal ganglion. (D) Heatmap of neurons that significantly increase lifespan upon inhibition. 6 brain images are overlaid in this image, with the maximum of 5 images overlapping at the reddest pixels. Arrow points to pars intercerebralis. (E) Heatmap of neurons that do not alter lifespan when either activated or inhibited. 10 brain images are overlaid in this image, with the maximum of 7 images overlapping at the reddest pixels. For all three heatmaps, red indicates the largest amount of overlap, and dark blue indicates the lowest. Specific lines included are labeled in Supplementary Table 5.1.

Both neurons expressing the neuropeptide Dh44 and Dh44 itself regulate lifespan. In addition to being the home of the IPCs, the PI also contains cell bodies expressing the neuropeptide *Dh44*. *Dh44* has previously been discovered to modulate feeding, excretion, reproduction, and activity levels[43-45]. In our screen, we found that expression of *Kir2.1* in neurons putatively expressing *Dh44* extended mean lifespan by 13.8% specifically in females (Table 5.1, green highlight, Figure 5.2 A). These neurons do in fact produce DH44 peptide, as identified by immunostaining (Supplementary Figure 5.1 A-C). In males, these manipulations significantly decreased lifespan (Table 1). These differences could be caused by known sexual dimorphism in neuronal targets of DH44, specifically *Dh44-R1*-expressing neurons[44]. The lifespan extension in females persisted after backcrossing the *Dh44-Gal4* line for eight generations into a controlled laboratory background (w^{1118}, Figure 5.2 B). Furthermore, female flies carrying a loss of function mutation in *Dh44*[43,46] were also long-lived compared to control animals, confirming the involvement of *Dh44* itself (Figure 5.2 C).

Manipulations that increase lifespan are often associated with changes in nutrient stores and/or increased stress resistance. Fat content was unaffected by either activation or

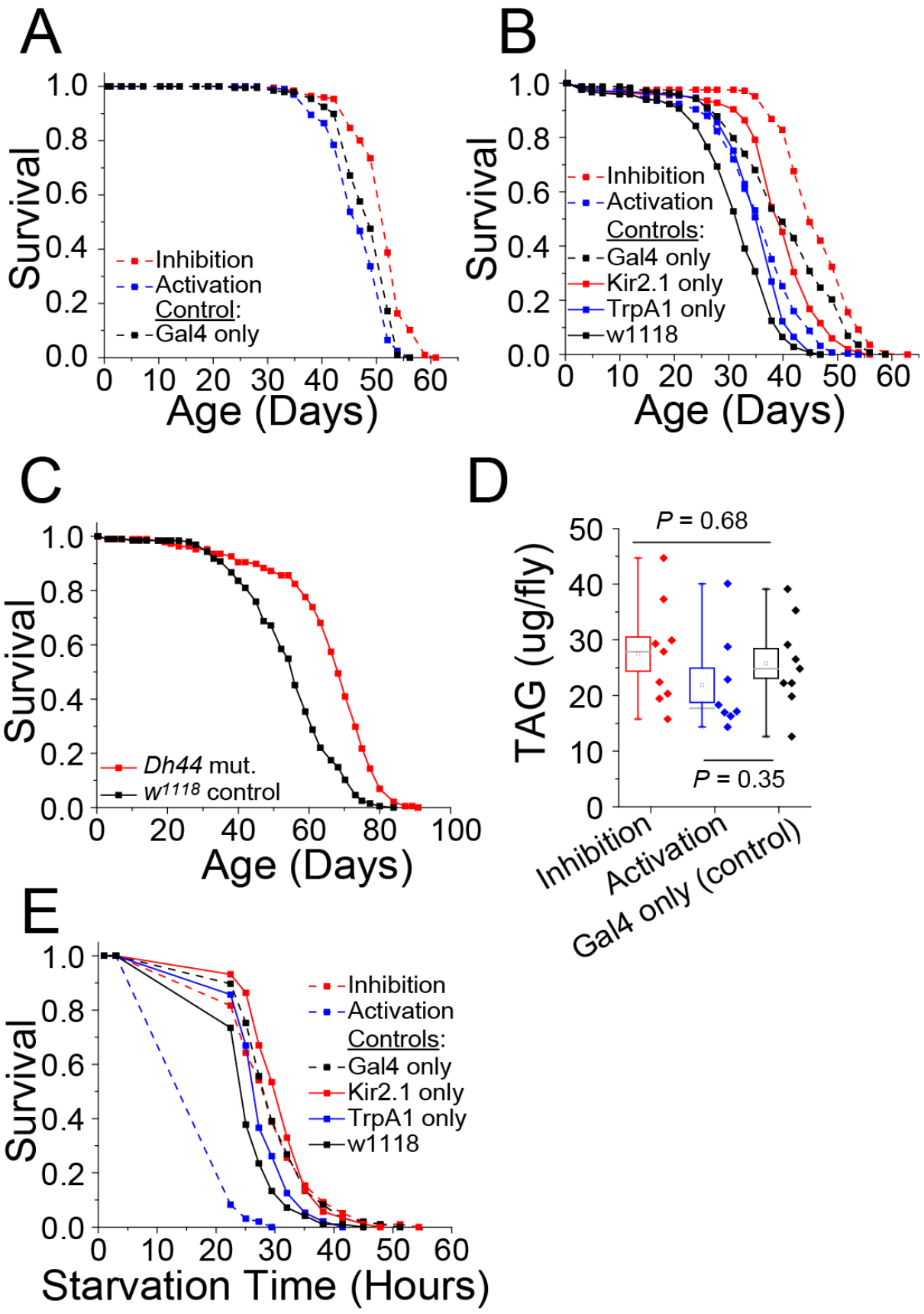

A
Survival
Age (Days)
Inhibition
Activation
Control:
Gal4 only
B
Survival
Age (Days)
Inhibition
Activation
Controls:
Gal4 only
Kir2.1 only
TrpA1 only
w1118
C
Survival
Age (Days)
Dh44 mut.
w1118 control
D
TAG (ug/fly)
P = 0.68
P = 0.35
Inhibition
Activation
Gal4 only (control)
E
Survival
Starvation Time (Hours)
Inhibition
Activation
Controls:
Gal4 only
Kir2.1 only
TrpA1 only
w1118

Figure 5.2: Inhibition or mutation of Dh44 increases lifespan. (A) A repeat assessment of lifespan finds that, similar to the results in the initial screen, inhibition *Dh44*-expressing neurons extends lifespan in females ($P << 0.0001$). Activation seems to have little if any negative effect ($P = 0.02$). (B) After backcrossing the *Dh44-Gal4* line, we see that inhibition of *Dh44*-expressing neurons still extends lifespan in females ($P << 0.0001$ comparing inhibition to any other group), and activation of these neurons may decrease lifespan ($P = 0.00086$ compared to Trp only, $P << 0.0001$ compared to Gal4 only). (C) Mutation of *Dh44* (backcrossed 8 generations) increases lifespan in females significantly compared to w^{1118} controls ($P << 0.0001$). (D) Neither activation nor inhibition of *Dh44*-expressing neurons alters fat stores in females. The small square represents the mean, with the box representing standard error of the mean. Whiskers define the 10%/90% marks, and the grey line is the median. (E) While there is little effect upon inhibition ($P = 0.94$ compared to gal4-only, $P = 0.43$ compared to kir only), activation of Dh44-expressing neurons significantly decreases starvation resistance in females ($P << 0.0001$ for all comparisons).

inhibition of *Dhh44*-expressing neurons (Figure 5.2 D). Previous findings have suggested that *Dh44* negatively impacts the organism's ability to cope with stress[47]. We found that activation of *Dh44*-expressing neurons led to a striking decrease in starvation resistance (Figure 5.2 E). Taken together, these results suggest that *Dh44* does not impact nutrient homeostasis *per se*, but may alter how flies respond to changes in the nutritional state of their environment.

Dh44 modulates feeding preference, but does not decrease food intake. As dietary restriction (DR) is the result of a change in the nutritional environment as well as the most commonly studied method of lifespan extension, it is possible that decreased DH44 signaling (through mutation or neuronal inhibition) might extend lifespan through behaviorally-induced DR. In an effort to determine whether the *Dh44* mutants feed similarly to their control animals, we measured food intake of flies when given a choice between protein and sugar sources. Under these conditions, the *Dh44* mutants consumed significantly more sugar than their wild-type controls (Figure 5.3 A), though protein consumption was not significantly different (Figure 5.3 B). These results suggest

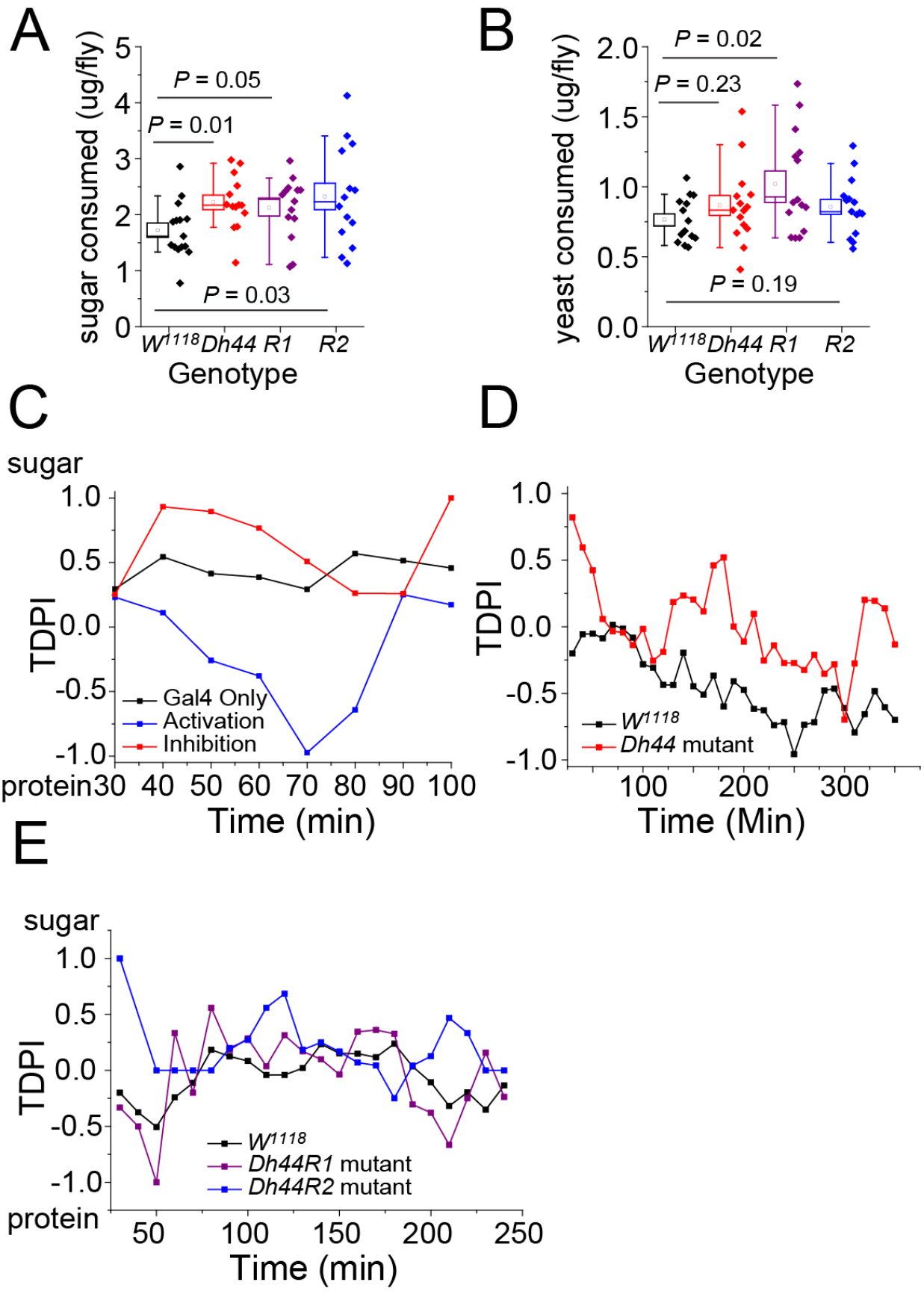

A
sugar consumed (ug/fly)
P = 0.05
P = 0.01
P = 0.03
W1118 Dh44 R1 R2
Genotype
B
yeast consumed (ug/fly)
P = 0.02
P = 0.23
P = 0.19
W1118 Dh44 R1 R2
Genotype
C
sugar
TDPI
Gal4 Only
Activation
Inhibition
protein
Time (min)
D
TDPI
W1118
Dh44 mutant
Time (Min)
E
sugar
TDPI
W1118
Dh44R1 mutant
Dh44R2 mutant
protein
Time (min)

Figure 5.3: *Dh44* alters feeding preference behavior specifically through the *Dh44R2* receptor. (A) When provided with a choice between solid sugar and solid yeast + sugar food, Both Dh44 and *Dh44R2* mutants significantly increase sugar consumption compared to wild-type controls, and *Dh44R1* mutants marginally increase sugar consumption. (B) Under the same conditions, only *Dh44R1* mutants eat significantly more of the yeast + sugar food than wild-type controls. (C) When provided the choice between liquid sugar and yeast + sugar food, activation of *Dh44*-expressing neurons leads to increased protein preference, whereas inhibition of *Dh44*-expressing neurons leads to increased sugar preference. (D) *Dh44* mutants prefer sugar over protein when compared with wild-type controls. (E) *Dh44R2* mutants prefer sugar over protein when compared with wild-type controls, whereas *Dh44R1* mutants do not show a difference in preference.

that *Dh44* mutants are not decreasing their feeding and thus the extended lifespan of *Dh44* mutants is unlikely to be a result of "self-imposed" DR. However, these results suggest that *Dh44* mutants might prefer to consume sugar over protein.

In an effort to more rigorously explore this possibility, we asked whether *Dh44* might drive preference for protein over sugar using the Fly Liquid-food Interaction Counter (FLIC)[48]. First, we observed the effects of activation or inhibition of *Dh44*-expressing neurons on feeding preference. We found that activation of *Dh44*-expressing neurons led to a transient (~60 minute) preference for protein, whereas inhibition of these neurons transiently increased the preference of flies for sugar (Figure 5.3 C). These results are not unexpected, as a recent study found that activation of *Dh44*-expressing neurons decreases the consumption of nutritive sugars[43]. Consistent with a role for DH44 signaling potentiating protein preference, we found loss of *Dh44* also demonstrated a preference for sugar over protein, though unlike the neuronal inhibition experiment this preference was maintained over the entire six hour experiment (Figure 5.3 D).

<u>*Dh44 extends lifespan independent of diet.*</u> While these results suggest loss of *Dh44* does not decrease nutritional intake, *Dh44* still might be downstream of diet, thereby

mediating the effects of DR on longevity. We measured lifespan in the *Dh44* mutant on a high-nutrient diet (15% sugar/yeast) and a low-nutrient diet (5% sugar/yeast)[49,50]. If DR regulates aging independent of *Dh44* then the beneficial effects of *Dh44* and DR on lifespan will be additive. Indeed, we found that *Dh44* mutant flies lived significantly longer than control animals on both diets (Figure 5.4 A). These results demonstrate that the beneficial effects of DR are independent of those of loss of *Dh44*.

However, nutrient dilution is a very specific form of dietary manipulation[51], and may not be sufficient to determine with *Dh44* regulates lifespan independent of diet. Several studies have demonstrated that restriction of specific nutrients, such as essential amino acids, can impose similar effects as DR[52,53]. However, as *Dh44* extends lifespan independently of DR, these are unlikely to lead to different results. As *Dh44* manipulations alter behavior when given a choice between protein and sugar food sources, such an environment could elucidate an interaction between *Dh44* and diet.

Thus, in an effort to determine if loss of *Dh44* extends lifespan regardless of diet, flies were provided with either the standard 10% yeast/sucrose mixture diet, or an alternative "choice" diet with two food sources: a 10% yeast food source, and a 10% sucrose food source. This "choice" diet, where flies construct their own yeast/sugar diet ratio, significantly decreases lifespan in wild-type flies (Jennifer Ro, unpublished data). If *Dh44* mutation extends lifespan independent of diet, *Dh44* mutants should be short-lived on the choice diet compared to the standard diet, while still maintaining a longer lifespan than controls also on the choice diet. When *Dh44* mutants were allowed to construct their own yeast/sugar diet ratio, they still exhibited a significant decrease in overall lifespan, though were still significantly longer-lived than control animals on the

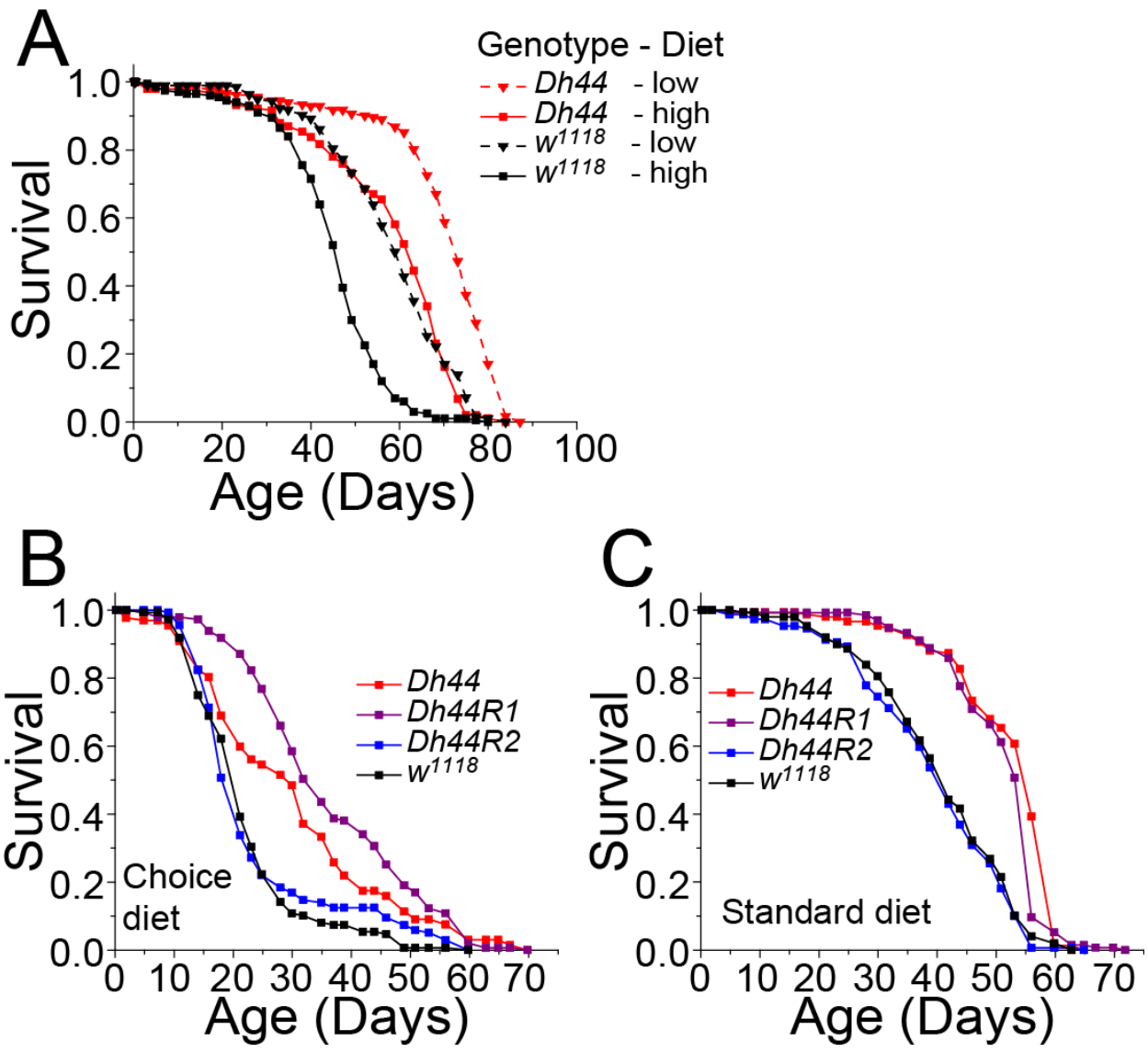


Figure 5.4: The effects of *Dh44* on lifespan work through *Dh44R1* and do not interact with diet. (A) *Dh44* mutants are long-lived under dietary restriction (5% sugar/yeast, a low nutrient diet, vs. 15% sugar/yeast food, a high nutrient diet), similar to wild-type controls(P << 0.0001). When compared to controls, mutation of *Dh44* extends lifespan to a similar extent on both high- and low-nutrient diets (P << 0.0001 for both comparisons, P = 0.43 for genotype:diet interaction by Cox regression), suggesting no interaction between dietary restriction and the lifespan-extending effects of *Dh44*. (B) *Dh44* (P << 0.0001) and *Dh44R1* (P << 0.0001) mutants are long-lived compared to wild-type controls and have similar lifespans to each other (P = 0.021) when allowed to construct their own diet from a 10% sugar food source and a 10% yeast food source. *Dh44R2* lives similarly to controls on this choice diet (P = 0.40). (C) In a control lifespan on 10% sugar/yeast food run alongside the choice lifespan, *Dh44* (P << 0.0001) and *Dh44R1* (P << 0.0001) mutants are again long-lived compared to wild-type controls and similarly lived to each other (P = 0.007), while *Dh44R2* has a similar lifespan to control flies (P = 0.43). It is notable that all four genotypes exhibit a similar decrease in lifespan when placed on a choice diet (P << 0.0001 for all genotypes), demonstrating that even in a more complex environment *Dh44* (and *Dh44R1*) extends lifespan regardless of diet.

same food type (Figure 5.4 B, C). These results demonstrate that the effects of *Dh44* on aging are not contingent on a specific diet. Furthermore, this lack of interaction between lifespan and diet under both DR and choice conditions suggests that the observed differences in feeding preference behavior are unlikely to be the driving force behind the role of *Dh44* on lifespan, as these differences in preference would be most evident in the choice diet where lifespan is still unaffected.

The Dh44 receptor Dh44R1 regulates lifespan, but another receptor, Dh44R2 does not.

We next sought to identify specific signaling pathways responsible for the effects of *Dh44* on aging. *Dh44* has two known receptors: *Dh44R1*, which is expressed primarily in the CNS and *Dh44R1*-expressing neurons have extensive arborizations with the PI (where both *Dh44* and insulin-producing cell bodies exist)[44], and *Dh44R2*, which is expressed in the gut and Malpighian Tubules in addition to the brain[47]. To determine whether either (or both) of these receptors are responsible for the extended lifespan and increased sugar feeding of *Dh44* mutants, we obtained loss of function alleles for each receptor[43,46]. Both mutants (similar to the *Dh44* mutant) are the result of MINOS insertions into intronic regions[43,46]. We found that *Dh44R1*, but not *Dh44R2*, leads to a lifespan extension similar to that seen in *Dh44* mutants (Figure 5.4 B, C). This suggests that the effects of *Dh44* on aging are the result of DH44 signaling specifically through DH44R1. However, when feeding preference is measured, *Dh44R2* mutants mimic the increased sugar preference observed in *Dh44* mutants, unlike *Dh44R1* mutants, which have similar preference as control animals (Figure 5.3 A, B, E). *Dh44* signaling through *Dh44R1* is therefore primarily responsible for its effects on aging, while feeding preference is mediated by *Dh44R2*.

Dh44R1-expressing neurons arborize to the PI, which is where not only *Dh44*-expressing neurons are found, but also the insulin producing cells[44]. As both *Dh44* and insulin-like peptides are responsive to sugar inputs and regulate lifespan, it is reasonable that *Dh44* might mediate longevity through dILP signaling. Preliminary data indicates that *Dh44* signaling may impact insulin signaling (Figure 5.5), suggesting the possibility that *Dh44* regulates lifespan through an insulin-dependent manner.

Beyond lifespan, an increase in healthspan would corroborate the beneficial effects of loss of *Dh44* on aging. In *Drosophila* the most common measure of health is climbing ability measured via negative geotaxis, which typically declines with age[54,55]. The climbing ability of *Dh44* mutants decreases at a significantly slower rate than that of control animals, further suggesting a slowed aging process (Supplementary Figure 5.2).

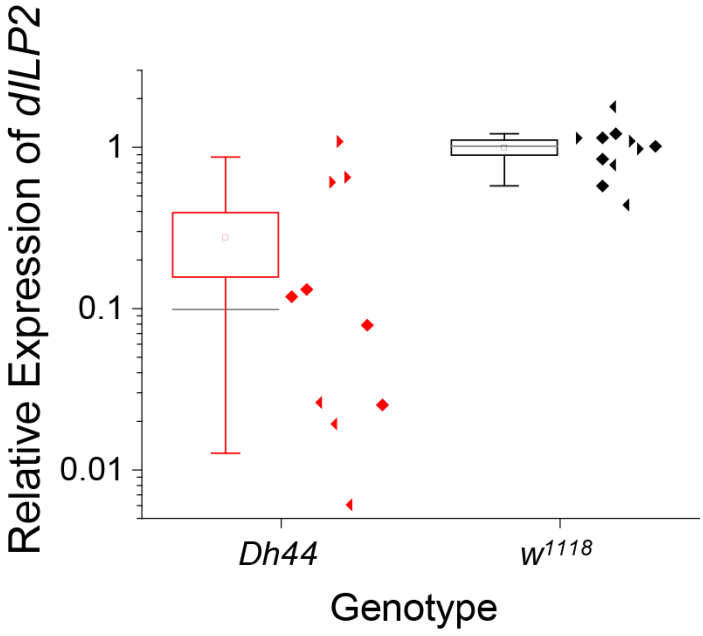


Figure 5.5: *Dh44* mutants have lower expression of *dILP2*. Using qPCR to examine transcripts of the insulin-like peptide *dILP2* we found that, over the course of three experiments (indicated by the diamond, left triangle, and right triangle, respectively), expression levels of *dILP2* were significantly lower in *Dh44* mutants than in wild-type controls (P = 0.00012 for genotypic differences by two-way ANOVA). No significant difference was found between experiments (P = 0.08) nor was there an interaction between genotype and experiments (P = 0.08). The small square represents the mean, with the box representing standard error of the mean. Whiskers define the 10%/90% marks, and the grey line is the median.

Discussion

While the traditional view of aging is that of the inevitable decay of many individual components over time, research over the past two decades has slowly brought to light the importance of central regulators of the aging process. Our initial screen emphasizes this point, as we found specific neuroanatomical areas that are important for regulating aging, just as other regions control homeostasis[41], circadian rhythms[45], or learning and memory[56]. The relatively large number of hits from our screen (greater than 8% of manipulations increased lifespan by over 10%) demonstrates that neuropeptides are an important component of how the brain mediates aging.

Discovering these methods of regulation is important, as finding central regulators of aging may allow for targeted anti-aging interventions with fewer side effects than treatments that directly affect cell signaling pathways throughout an organism, like Rapamycin. Focused interventions could stimulate a small number of cells which in turn regulate aging throughout the organism, like the ~6 neurons manipulated in our *Dh44* inhibition experiments. These interventions could then take advantage of the physiologic mechanisms through which organisms typically regulate aging to slow the aging process while potentially decreasing unwanted side effects.

The idea that these manipulations regulate aging not through alterations in behavior, but instead change some kind of fundamental state or "primitive emotion" within the brain is supported by these data. The effects of *Dh44* on lifespan appear to be independent of both diet and food preference behavior, suggesting *Dh44* regulates aging independent of its effects on behavior. This conclusion is supported by the fact that *Dh44R2* appears to be primarily responsible for the effects of *Dh44* on feeding

preference, whereas *Dh44R1* modulates aging. While one could assume *Dh44* alters other unknown behaviors, at this point Occam's Razor would suggest that *Dh44* alters the internal state of the fly which in turn directly modulates the aging process. This supports the idea that these "aging centers" in the brain are not regulating aging through alterations in behavior, but instead by changing the physiologic state of the organism. Considering that *Dh44*'s homolog, CRH, is commonly referred to as a "stress hormone" as well as *Dh44*'s role in the stress response, it is not unreasonable to consider that *Dh44* signaling might induce a state of stress, which in turn could be considered an "emotional" state.

Anderson and Adolphs define emotions as scalable, persistent, general changes in the central state that alter physiology and behavior with both positive and negative valence. We have established some, but not all, of these principles in our manipulations of *Dh44*. As activation and inhibition of *Dh44*-expressing neurons have opposite effects in terms of lifespan, feeding preference, and (in work done by others) excretion[43], *Dh44* manipulations exemplify generality and valence. However, the scalability and the persistence of *Dh44*'s effects have yet to be examined, so we cannot yet conclude that *Dh44* controls a primitive emotional state within the fly. That said, it could be argued that these manipulations of neuropeptidergic signaling are *directly* changing the internal state, and thus these emotional states. Further work in *Drosophila* could elucidate the exact mechanisms through which this occurs, which may reveal conserved mechanisms through which emotions influence aging, health, and physiology.

This will require identifying the targets downstream of DH44 and DH44R1 signaling that promote lifespan. Our preliminary evidence suggests *dILP2* may be downstream of

Dh44. This is a (theoretically) simple-to-test hypothesis through epistasis experiments, as mutants for the insulin-like peptides produced in the IPCs exist. Practically, such experiments are cumbersome due to genetic background issues, but the construction of lines combining these mutants and *Dh44* reagents are underway. Regardless of the outcome of this experiment, examining whether the effect of *Dh44* on lifespan requires *dFoxo* is also a prudent idea, as both insulin-dependent and –independent regulation pathways of FOXO proteins exist[57-59].

Corticotropin-releasing hormone (CRH), the mammalian homolog of *Dh44*, modulates insulin release from pancreatic beta-cells[60]. This relationship is not unidirectional, as insulin is also capable of modulating CRH release, albeit indirectly, through insulin-induced hypoglycemia[61]. CRH is also known to affect appetite[62] and bowel movement[63] as well as being involved in depression[64] and the general stress response[65], similar to the roles previously observed in *Dh44*. Thus, it is reasonable to ask whether CRH might influence aging and longevity in mammals. While a thorough examination of the relationship between CRH and longevity has not been performed, the CRH signaling pathway has been associated with aging-related issues such as working memory decline and Alzheimer's disease[66]. Ideally, as researchers continue to use simple model systems such as *Drosophila* to explore mechanisms underlying how peptidergic signaling mediates the aging process, mammalian scientists can press forward to determine if these relationships are conserved across taxa and ultimately in humans, where potential anti-aging interventions may be developed.

Methods

General fly husbandry: All flies used in this section were collected using the same method. Eggs were collected from yeasted grape juice agar plates, and 32 µl of eggs were placed onto bottles containing CT food. All flies except experiments involving either *UAS-TrpA1* or *UAS-kir2.1;tub5-Gal80^{ts}* were raised in a controlled humidity incubator at 25C in 12 hour light/dark conditions. Flies were collected into bottles containing 10% yeast/sucrose food within 24 hours of emergence, and were allowed to mate for 2-3 days, after which the flies were sorted using a light CO2 anesthetic and separated by sex into groups of 25 in vials containing the appropriate food source. Unless otherwise noted, this food source is 10% yeast/sucrose food. *Dh44*, *Dh44R1*, and *Dh44R2* mutants were graciously provided by Monica Dus.

Lifespan ("Fly Qi") screen: The screen took advantage of the FlyLight library of GAL4 lines[32], specifically selecting GAL4 lines using promoters from regions directly upstream of neuropeptide and neuropeptide receptor genes. These GAL4 lines were crossed to either w$^-$;*UAS-TrpA1*;+ (for activation) or *w*-; *UAS-kir2.1*;*tub5-Gal80^{ts}* (for inhibition), which were backcrossed for 8 generations into the *w^{1118}* background. These lines were compared to the GAL4 line crossed to the *w1118* genetic control for the *TrpA1* and *kir2.1;Gal80* lines. Lifespans were performed on both males and females, with a sample size of ~150 flies per genotype per sex using 12 chamber "hotels" to increase throughput.

Temperature-dependent neuronal manipulations: For temperature-dependent experiments (including the lifespan screen), experimental fly eggs were collected and raised in 18C, 12h:12h light:dark incubators. 2 days post-eclosion, flies were sexed and

sorted as described above. Following experimental set-up, flies were shifted to a 29C incubator for the remainder of the experiment.

Immunostaining: Immunostaining of adult *Drosophila* brains was performed using an adapted method described by Wu and Luo[67]. Adult brains were dissected and allowed to fix in phosphate-buffered saline (PBS), pH 7.4 containing 3.7% formaldehyde for approximately 1 hour. Fixed brains were transferred and washed in PBS with 0.1% Triton X-100 (PBS-T) to completely remove the fixation solution. Afterward, brains were blocked in 5% normal goat serum solution in PBS-T (NGS) for 30 minutes at room temperature, followed by incubation in a primary antibody diluted in 5% NGS for 2 nights at 4C on an orbital shaker. Following primary antibody incubation, brains were washed in PBS-T to completely remove the primary antibody and incubated in Alexa Fluor conjugated secondary antibodies diluted 1:500 in 5% NGS 1 day at 4C. After 3 quick and 3, 20-minute long washes in PBS-T, stained brains were transferred into Vectashield antifade mounting medium (Vector Laboratories) and mounted onto glass microscope slides. The rabbit-anti-DH44 primary antibody was kindly provided by Erik Johnson and used at a 1:500 dilution.

Composite brain images: Expression patterns of each GAL4 line was determined by either using the published expression patterns on the FlyLight website[32] or, if unavailable, crossing the GAL4 line to *UAS-mGFP* and immunostaining with anti-GFP to enhance the fluorescent signal. Once obtained, images were converted to a binary image using a brightness threshold of 20.0 using ImageJ and aligned to fit a reference brain. Using a custom MATLAB script, the binary images of lines that extended lifespan upon either activation or inhibition (or the no extension controls) were then overlaid,

and the number of images where expression occurred in a given pixel was counted. This count was then used to create a color heatmap of the brain, which is shown in Figure 5.1 C-E.

Measurement of fat content: Experimental flies were quickly frozen in a dry ice bath, then homogenized in 300 µl PBS/0.01% Triton-X in groups of 5 flies per sample, with 5-10 samples per treatment/genotype. Afterward, 5 µl of homogenate was added to 150 µl of Infinity Triglyceride Reagent (Thermo Electron Corp.) and incubated at 37°C for 10 minutes with constant agitation. Concentrations of Triacylglycerides (TAG) were determined by comparing the absorbance at 520 nm of experimental samples to known triglyceride standards.

Measurement of starvation resistance: experimental flies were placed in fresh vials containing 1% agar (to serve as a water source). For each assay, 50-100 experimental flies (in vials of 10 flies/vial) were established for all treatment/genotype groups. The number of dead flies in each vial was recorded every 2-4 hours until no experimental flies remained alive.

Dietary restriction: The dietary restriction lifespan experiment used three distinct food types which are commonly used in the literature[49,50]. After aging adults for two days on 10% sugar/yeast (see "general fly husbandry" for details), the flies were placed in vials containing either a low-density food (5% sugar/yeast) or a high-density food (15% sugar/yeast) and maintained on that food for the remainder of the experiment. Other than the concentrations of sugar and yeast, there is no difference between the food types.

Choice diet environment: The choice diet lifespan experiment used two environments. In the choice environment, flies are provided two types of food to choose from: a 10% sugar food, and a 10% yeast food, from which they can construct their own carbohydrate/protein ratio. The non-choice environment uses the typical 10% sugar/yeast food. These experiments were performed in 12-chamber hotels, and both choice and non-choice diets were provided on plates with dividers to control for the presence of plastic.

Negative geotaxis assay: We measured vertical distance traveled by recording video footage of flies for 10 seconds after they were forcefully banged to the bottom of their lane, and then analyzing the number of vertical pixels traveled. We measured flies in cohorts of 14, with a single fly in a lane so distance could be tracked on a per-fly basis. We performed 5 replicates for each set of flies, giving 10 seconds of recovery time between each trial. Flies were discarded following analysis, and then we analyzed a new batch of flies from the same cohort at the next time point.

Food-dye assay: After aging the flies for 10 days, flies were placed in 12-chamber hotels (similar to the choice diet lifespans) and provided a choice between 1% sugar in agar and 2% yeast extract + 1% sugar in agar. On each plate, one of these food sources was spiked with blue dye (0.5% weight/volume). Each genotype was split into two groups, one with the dye in the sugar food source, and the other with the dye in the yeast extract. Flies were flipped onto the dyed food sources at lights-on (i.e. morning), and were left on the food for 6 hours before being frozen at -80C. Flies were then homogenized in 300 µl PBS/0.01% Triton-X in groups of 5 flies per sample, with 5-10 samples per treatment/genotype. These samples were then analyzed for blue dye using absorbance at

630 nm, and compared to a known standard curve of blue dye (starting at 10 mg/L) to determine the volume of food consumed.

FLIC feeding preference assay: After aging flies for 10 days, flies were placed in the Fly Liquid-food Interaction Counter (FLIC) and given a choice between 1% sugar in water and 2% yeast extract + 1% sugar in water. Flies were introduced into the arena using light CO2 anesthetic, and then were allowed to freely choose between the two liquid food sources for six hours. Following the experiment, the collected data was analyzed as discussed in the previous chapter on FLIC.

Quantitative PCR: experimental flies were quickly frozen in a dry ice bath. RNA was extracted using Trizol (Invitrogen) from 3 samples per genotype/treatment, with each sample containing at least 40 female fly heads. cDNA was then synthesized from equal amounts of RNA (after quantification with a NanoDrop system) using the Superscript III first strand synthesis kit (Invitrogen). Quantitative real-time PCR was performed with SYBR green from SA Biosciences. The following primers were used[41]:

Forward Dilp2 primer: TCTGCAGTGAAAAGCTCAACGA

Reverse Dilp2 primer: TCGGCACCGGGCATG

Statistics: Unless otherwise indicated, pairwise comparisons between both lifespan and starvation survivorship curves was performed using the DLife computer software[68]. P-values were obtained using cox-regression tests. Additionally, interaction terms between genotype and treatment were determined using the survival statistical package within the software program R (also using the cox-regression test).

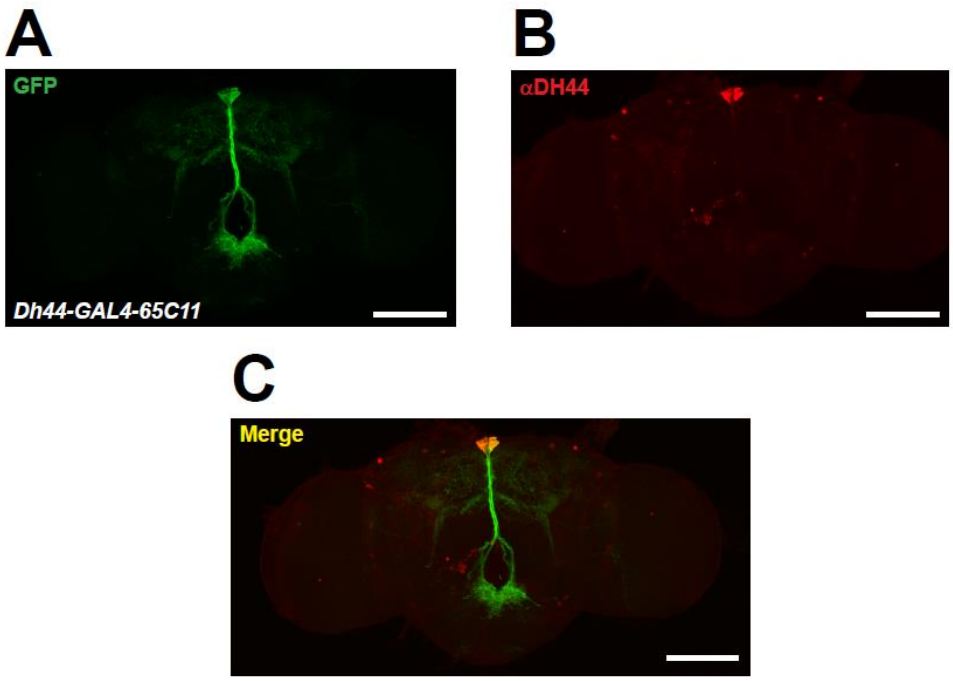
Supplementary Tables and Figures

Supplementary Table 5.1: Lifespan effects of all manipulations in the neuropeptidergic activation/inhibition screen. All genes are listed, in the order tested, along with average difference from the GAL4-only control specific to each driver. The first column identifies the driver lines included in our heatmap images (see Figure 5.1). Colored cells are included, cells with dashes indicate they are not suitable to any of the heatmap groups, and cells with uncolored labels indicate that the image has not yet been collected and processed for inclusion into the heatmaps.

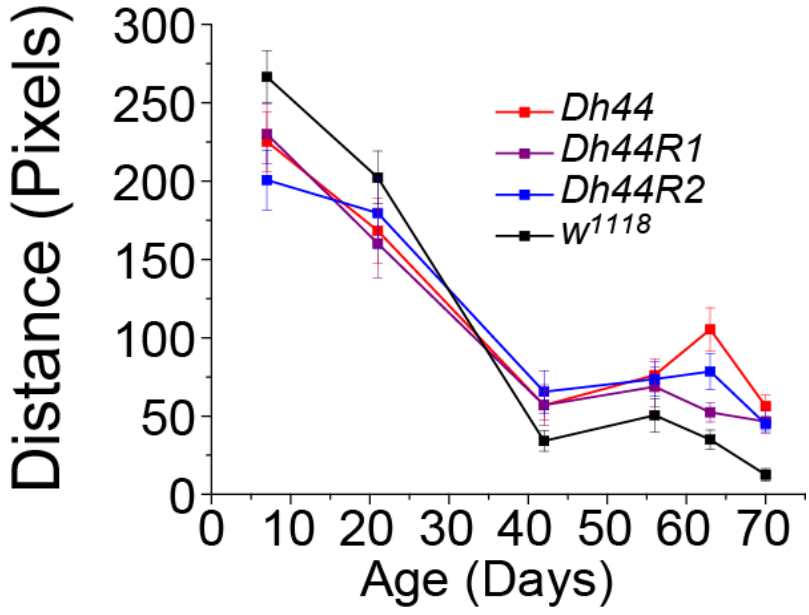
Which group? (colored if in image)	FlyLight Collection Identifier	Promoter	Activation Female Mean % Difference	Inhibition Female Mean % Difference	Activation Male Mean % Difference	Inhibition Male Mean % Difference
----------		**Group 1**	----------			
neutral	GMR65A01	amn	3.04074	-8.27235	4.83565	-6.13921
-----	GMR65D05	Ast	-8.80838	1.25176	8.85358	-24.28618
inhibit	GMR65D06	Ast	-9.67265	12.94633	-55.17587	7.52437
activate	GMR61H08	Ast-C	9.89118	-8.04427	-7.1775	-13.03794
-----	GMR67F03	Ast-C	-0.19648	8.25646	-38.18949	-20.95453
neutral	GMR60E07	capa	-2.03067	-7.16982	6.34753	-11.70082
activate	GMR18A04	CG12370	-15.26181	-88.98752	19.82776	-80.73746
-----	GMR19A04	CG12370	2.76522	-14.57734	-10.58718	-24.21039
inhibit	GMR65C11	Dh44	6.76159	**13.82693**	-61.57143	-26.12863
-----	GMR61D07	Dh44	-48.89179	-83.43912	-55.47597	-75.76082
-----	GMR57F07	Dh31	-1.39332	6.7927	-0.24149	-13.87724
neutral	GMR20D02	Dh31	-6.69909	3.18647	-5.07146	-6.39916
-----	GMR61H01	Dms	2.93283	13.59512	-50.16029	-5.56663
neut.	GMR63A04	Dsk	0.08721	3.3602	-3.93362	-5.49285
neutral	GMR60F12	Eh	-2.43886	-1.90028	4.18206	-0.36741
-----	GMR62A04	Eh	1.12461	0.22082	-15.82077	-3.9684
-----	GMR67E06	Fmrf	-0.26938	-22.83308	-15.46628	-8.52085
----------		**Group 2**	----------			
neut.	GMR61H10	hug	2.07069	-1.33523	2.63489	0.61772
activate	GMR65H11	hug	-0.53709	-1.77178	8.8345	-16.54048
inh.	GMR19E04	itp	5.6812	-5.30653	3.13244	11.40222
-----	GMR18F10	itp	0.26921	-64.23367	-20.20527	-90.02347
activate	GMR20G03	Leucokinin	4.76154	-27.41462	1.94024	-24.34784
activate	GMR19H09	Leucokinin	13.904	-12.12152	15.93018	-18.70423

Which group? (colored if in image)	FlyLight Collection Identifier	Promoter	Activation Female Mean % Difference	Inhibition Female Mean % Difference	Activation Male Mean % Difference	Inhibition Male Mean % Difference
inh.	GMR21A04	Leucokinin	-15.88431	-22.81572	-9.80957	10.40497
-----	GMR61A01	Mip	9.20723	3.71445	-31.82513	-3.45566
neutral	GMR22E12	Mip	5.27353	-5.93845	-1.30734	-2.73639
-----	GMR67D02	npf	-9.64087	-27.3871	-14.05536	-32.02156
-----	GMR57F05	Nplp1	-50.85126	-87.37808	-73.21536	-92.312
neut.	GMR61G12	Pdf	-0.86223	-6.13618	-2.30917	9.21337
-----	GMR61H02	Proct	-39.29201	-29.68943	-52.05242	-32.88791
neutral	GMR67D03	Proct	7.55874	Lethal	4.06305	Lethal
-----	GMR57E07	sNPF	-82.39372	-39.51725	-81.54311	-33.13944
neutral	GMR19H12	sNPF	-6.68517	-6.75382	-1.58597	-5.60659
neutral	GMR20B12	sNPF	5.10791	-6.88871	-5.47125	-9.26983
----------		**Group 3**	----------			
act/inh	GMR61H07	Tk	27.98335	12.35661	3.78587	5.66392
-----	GMR11A12	AlCR2	-10.78933	-22.16242	-35.07823	-24.02975
inh.	GMR57D01	AlstR	-3.22973	6.18426	-1.6772	11.01228
-----	GMR67E10	AR-2	-58.35492	-92.31837	-79.48705	-89.6266
-----	GMR64C11	CcapR	-18.74349	-27.14793	-44.27417	-42.00758
activate	GMR25G04	CCKLR-17D1	27.07528	4.28804	0.26755	-11.51725
inhibit	GMR24B06	CCKLR-17D3	-1.31599	10.36821	-44.20793	-12.73364
-----	GMR25C03	CCKLR-17D3	4.93428	-1.3147	-16.86326	-26.00881
inh.	GMR96B07	CG10738	-10.39347	20.24425	-6.22447	4.40168
-----	GMR57G09	CG14593	-7.96587	0.54638	-12.75171	-23.65624
neutral	GMR23E03	CG4395	-1.52108	-12.74552	-3.6168	-10.50455
neutral	GMR20C04	CG8784	6.54976	7.47205	-9.6338	0.65782
activate	GMR21B09	CG8795	13.83014	-1.88989	-3.72864	-12.31442
-----	GMR57E02	Dh31-R1	Lethal	Lethal	-38.47213	-27.64667
-----	GMR64C06	DmsR-1	-15.43492	-25.89288	Lethal	Lethal
----------		**Group 4**	----------			
-----	GMR64G11	DMSR-1	Lethal	Lethal	-64.44941	Lethal
activate	GMR64F10	DMSR-2	-41.52727	-87.05647	11.37118	-83.50815
neut.	GMR57E10	ETHR	9.08477	9.8182	5.84317	1.49033
-----	GMR65E08	FR	Lethal	-78.54903	-83.48338	-81.77981

Which group? (colored if in image)	FlyLight Collection Identifier	Promoter	Activation Female Mean % Difference	Inhibition Female Mean % Difference	Activation Male Mean % Difference	Inhibition Male Mean % Difference
neut.	GMR57B01	Fsh	-2.64619	6.6129	-6.57914	0.76232
inh.	GMR57E05	GRHR	-5.42074	3.03473	-2.47389	14.74144
activate	GMR24H05	GRHRII	15.11255	-85.05882	0.02399	-82.53228
inhibit	GMR28H01	InR	-3.93641	12.90235	-16.05358	-3.83691
inhibit	GMR17G11	Lgr3	4.12341	11.67928	-0.03132	4.57754
-----	GMR65C07	Lkr	-19.26436	4.53945	3.63135	-0.08685
inhibit	GMR19G10	NepYr	-12.1421	17.25903	2.86535	10.01382
inh.	GMR61H06	NPFR1	-6.27692	3.88219	-11.35676	12.94031
neut.	GMR61G05	Proc-R	-4.10013	9.19427	-4.97198	6.08777
activate	GMR78E04	rk	17.07582	-64.66963	-10.6483	-16.00081
-----	GMR57F10	SIFR	-79.28252	-85.05367	-84.52831	-86.0768
-----	GMR64G06	sNPF-R	-54.92399	-14.64882	-25.64585	-26.09556
neut.	GMR62A02	Takr86C	-2.93552	2.46136	-7.70612	-3.75031
----------		**Group 5**	----------			
neut.	GMR67D03	Proct	-2.84962	Lethal	-5.38576	Lethal
-----	GMR20D06	sNPF	-14.38415	-6.65866	-9.8158	7.48598
neut.	GMR19H06	sNPF	6.36466	5.57105	2.26821	-2.4557
-----	GMR23H07	CG30106	1.95868	-51.57086	-39.32763	-14.79315
-----	GMR57F03	CG9918	-9.08235	-13.13111	-8.08924	-46.73424
-----	GMR57E02	Dh31-R1	-13.00517	-16.5055	-13.05422	-9.24194
-----	GMR66B09	DmsR-1	-21.98292	Lethal	-27.21819	Lethal
neut.	GMR18D01	pdfr	Lethal	-1.78454	Lethal	-5.44995
-----	GMR19A03	pdfr	-22.48134	-27.87892	7.0877	-21.10129
-----	GMR64D03	Takr99D	-12.02186	0.40619	0.49847	0.70234



Supplementary Figure 5.1: *Dh44-Gal4* expression pattern is similar to DH44 immunostaining. (A) *Dh44-Gal4* driving *UAS-mGFP*, stained with anti-GFP. (B) Staining of the same brain in S.1A with anti-DH44. (C) Merged image showing overlap between GAL4 expression and immunostaining demonstrates that our *Dh44-Gal4* drives expression in a subset of neurons that produce DH44, specifically those within the Pars Intercerebralis. Staining protocols are discussed in the immunostaining subsection.



Supplementary Figure 5.2: Climbing ability of *Dh44* mutants decreases at a slower rate than in controls. The climbing ability of *Dh44*, *Dh44R1*, and *Dh44R2* mutants declines more slowly over time than the climbing ability of wild-type controls (comparing to w^{1118} by two factor ANOVA, P = 0.027 for *Dh44*, P = 0.035 for *Dh44R1*, P = 0.00081 for *Dh44R2*). This could indicate a slower rate of aging in these flies, though this effect would not have been expected in the *Dh44R2* mutants.

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Chapter VI: Discussion of Implications and Future Directions[6]

The results presented in this thesis demonstrate the importance of the brain in general, and neuropeptidergic signaling in particular, in the aging process. We have identified specific external stimuli, receptor neurons, neuropeptidergic molecules, and downstream targets that regulate aging in flies. Furthermore, new techniques for both the collection of individual feeding data and the analysis of mortality patterns will hopefully allow the field to be pushed further toward understanding how aging occurs in individuals. While there is still much to be done in *Drosophila* as well, it is our hope that the conserved pathways found to regulate aging in this work will be studied in higher organisms to determine if their effects on aging are conserved. We have preliminary evidence that at least one of these pathways may be suitable for pharmacologic interventions to slow aging. Notably, both the pathway and drug have clear ties to emotions, suggesting that further research is needed specifically in the interaction between aging and emotions.

[6] This chapter was written and edited by Zachary Harvanek (ZMH). Dr. Scott Pletcher (SDP) provided comments. Undergraduate students assisted greatly in counting flies for the experiment in figure 6.2. Daniel Promislow (DEP) performed the analysis of the neurometabolomics and created the plot in figure 6.3.

The brain regulates aging

Since the discovery that sensory input can regulate longevity, the aging community has been slowly realizing that the brain is not just a victim of aging, but a perpetrator as well. Previous studies on the interplay between the nervous system and aging typically focused on inputs related to finding food (i.e., gustation or olfaction)[1-4]. However, we demonstrated that more complex inputs, such as perception of and interaction with the social environment, are also capable of regulating longevity through specific receptors and signaling pathways. Specifically, these findings upend the traditional view of costs of reproduction[5,6], as they demonstrate that, at least for male flies, reproduction is not costly in itself, but instead that these costs result from a regulated response to perception of the opposite sex. Instead of being costly, mating is actually beneficial, opposing the costs accumulated by pheromone perception.

Beyond the sensory inputs, we discovered a number of neuropeptides that may be capable of regulating aging. Many of these neuropeptides are released from the Subesophageal Ganglion (SOG), Mushroom Bodies (MB), or Pars Intercerebralis (PI). The PI is the homeostatic center of the fly similar in function to the mammalian hypothalamus[7-9], and contains neurons expressing *Diuretic hormone 44* (*Dh44*), the homolog of mammalian corticotropin-releasing hormone (CRH)[10]. While previous works have identified a role for *Dh44* in feeding preference[11], we found that *Dh44* and one of its receptors, *Dh44R1*, regulate lifespan independently of diet. This rules out trivial explanations such as *Dh44* mutants imposing dietary restriction (DR) on themselves, and strongly suggests a direct role of the brain in general and the PI in particular in regulating the aging process. This conclusion is supported by previous

work identifying insulin-like peptides (dILPs), which are also expressed in the PI, as regulators of aging as well[12]. In both the cases presented here there are changes occurring to the state of the organism's brain. The aging effects of both pheromones and mating require specific neuropeptidergic pathways (NPF, CRZ, and AKH) and lead to measureable changes in the general neurometabolome of the brain, providing a direct measurement of at least one facet of the brain's internal state and, though correlative, a link between the internal state and aging. In the case of *Dh44* and other neuropeptides identified in the screen, we are directly manipulating the state of the CNS through altered neuropeptidergic signaling pathways. Combined, these results demonstrate that several types of neurons in multiple brain regions that respond to various inputs can regulate the aging process, not just insulin producing cells.

The power of model systems in the study of emotions

Manipulating the release of neuropeptides would be expected to impact the state of the brain, and in turn potentially to alter the primitive emotions of the fly. These primitive emotions are defined by Anderson and Adolphs as changes in the state of the brain that result from a stimulus (internal or external) and are persistent, scalable, valent, and general[13]. While such primitive emotions may not directly correlate to human emotions, their underlying mechanisms could certainly be conserved. Thus, through studying primitive emotions in model systems the processes underlying complex human emotions may be revealed.

Generally, emotions and feelings would be considered nearly impossible to study in model systems, where surveys and communication is not as easy as with humans, but this is largely the result of a lack of a scientific framework to avoid the "feelings" aspect

of emotions, and instead focuses on how emotions regulate behavior and physiology. For example, a loud noise that startles a woman awake might cause her pupils to dilate and increase both her heart rate and sensory acuity (i.e., a generalized response due to a stimulus). Further noises may lead to tachycardia and a cautious search for the origin of the noise (i.e., scaling up of the response). After finding it was just her cat, she might be unable to fall asleep until this state of increased arousal has passed (i.e., persistence of fear beyond the stimulus). This relaxation may be aided by petting the cat (i.e., valence, with calm opposing fear). Most would agree that the woman's changes in both physiology and behavior are a result of emotion. Now, if instead the positions between the cat and human are exchanged, and it is the cat being startled by their owner, these same physiologic and behavioral changes would likely occur. A layman could explain these results by saying the cat was "scared", just as they would for the human. On the other hand, scientists observing a stimuli that produces the same results in different species would likely suggest that the underlying mechanism is conserved. Thus, by viewing emotional states not as an ill-defined feeling but instead as a complex set of physiologic and behavioral responses, it becomes reasonable to study these states using model systems.

Some might argue that even if emotions can be studied in model systems, invertebrates such as *Drosophila* are not suitable for such applications. However, there are multiple examples of fly "emotion primitives" where a stimulus, such as a shadow or image of a potential predator[14,15], causes persistent, scalable changes in behavior and physiology. While the cases laid out here also show these characteristics, the advantage of using invertebrate systems is the ability to investigate conserved mechanisms through which

primitive emotions might regulate aging. In this work, we have demonstrated that a primitive emotion (reproductive drive) in male flies regulates aging through NPF, AKH, and FOXO signaling pathways, all of which have homologs in mammals. These studies would have been nearly impossible to do in vertebrate systems like mice due to cost, time, and the tools available to manipulate gene expression.

In fact, the available genetic tools could make *Drosophila* an ideal model system to begin dissecting the interaction between emotional neural circuits and their effects on aging. The "Fly Qi" neuropeptidergic screen for longevity is only possible because of the plentiful genetic tools available in *Drosophila*, which allow us to manipulate the neural state of the fly by easily activating or inhibiting specific subsets of neurons in a temporally-controlled fashion[16-19]. As many circuits and signaling molecules are highly conserved from *Drosophila* all the way to mammals[7-10,20], using a cheap, genetically tractable organism with a relatively simple CNS is likely to be the most effective method to identify the neuronal circuits and downstream mechanisms that underlie emotions and their physiologic and behavioral effects.

New methods allow for investigation of temporal dynamics of mortality and behavior

Given the fleeting and personal nature of emotions, behaviors, and environments, understanding the temporal dynamics of how they affect health and aging on an individual level will be all the more important. It is currently believed that some changes, like smoking cessation, can quickly and dramatically decrease an individuals' chance of death[21]. Similar (though slower) dynamic effects have been found for social support systems' effects on mortality[22]. Unfortunately, the primary method for

measuring aging in humans, flies and most other organisms is to analyze mortality rates or survivorship, which cannot provide an individual's probability of death at a given time or even determine if heterogeneity exists within a population[23]. Thus, it is impossible to confirm if these changes can decrease probability of death in an *individual*, as opposed to simply decreasing the probability of death across a population.

Thus, to address the problem we developed a method combining computational and wet-lab techniques to determine the temporal dynamics of aging in individuals given population measures. This technique allowed identification of the duration of the effects of pheromone exposure on lifespan (five weeks), while simultaneously demonstrating that pheromone exposure significantly increased heterogeneity in exposed flies. These results reinforce the idea that investigators studying reversible or middle-aged aging interventions must consider and account for heterogeneity to draw accurate conclusions regarding individual aging from their studies.

As with methods for survival analysis, behavioral assays in *Drosophila* also tend to lag behind (when compared to *Drosophila* genetics), which led to the development of the Fly Liquid-food Interaction Counter (FLIC). Diet, taste, and hunger all seem to have a role in regulating aging, but prior to the development of the FLIC, all existing assays were lacking in some combination of sensitivity, scalability, or temporal resolution. FLIC is a semi-automated method that can detect slight feeding preferences with multiple measurements per second for up to a week with almost no researcher intervention after setup. Improved temporal resolution is important, as Jennifer Ro, a co-developer of FLIC, has discovered that some feeding behaviors like protein

preference are transient in nature and are difficult to identify using traditional methods (Jennifer Ro, unpublished data).

Future directions

Although we have identified multiple genes that are involved in mediating the costs of reproduction in male *Drosophila*, there are some relatively straightforward follow-up questions that will help clarify the underlying circuitry:

Are ppk23, npf, and dFoxo directly connected, or are there intermediary signaling neurons/molecules? This question can be broken into three parts: first, do *ppk23*- and *npf*-expressing neurons directly interact, second, is NPF itself involved in regulating the pheromone response, and third, does NPF signaling directly modulate *dFoxo*. Regarding the former question, based on known expression patterns of *ppk23* and *npf*, it would be surprising if no interneurons were mediated this connection[24,25]. Assuming information travels from *ppk23* sensory neurons to *npf* neurons, the most likely locations for interneurons connecting these subsets include the subesophageal ganglion and the ventral nerve cord. While traditional screening experiments using output phenotypes such as stress resistance or fat stores would be viable methods to identify these neuronal subsets, new genetic tools that label active neurons might speed up this process[26,27].

Thus far, we have demonstrated that neurons expressing *npf* (homolog of mammalian NPY) [20] are involved in the pheromone response, but have not yet confirmed involvement of NPF itself, as other neurotransmitters expressed in this neurons could also be responsible for these effects[28]. In favor of direct NPF involvement, *npf* transcription is regulated by pheromone exposure, and preliminary evidence using a

putative *npf* hypomorph suggests that NPF signaling is at least partially required for pheromone effects (Figure 6.1). However, these results must be confirmed, perhaps using additional methods such as *npf* RNAi or a complete *npf* knockout.

To date, there is there evidence for a direct link between NPF signaling and *dFoxo*. Examining the connection between NPF and *dFoxo* could be done through activation of *npf*-expressing neurons and observing outputs of FOXO signaling, such as *4EBP* (*thor*) transcription. However, this result would not rule out potential intermediaries.

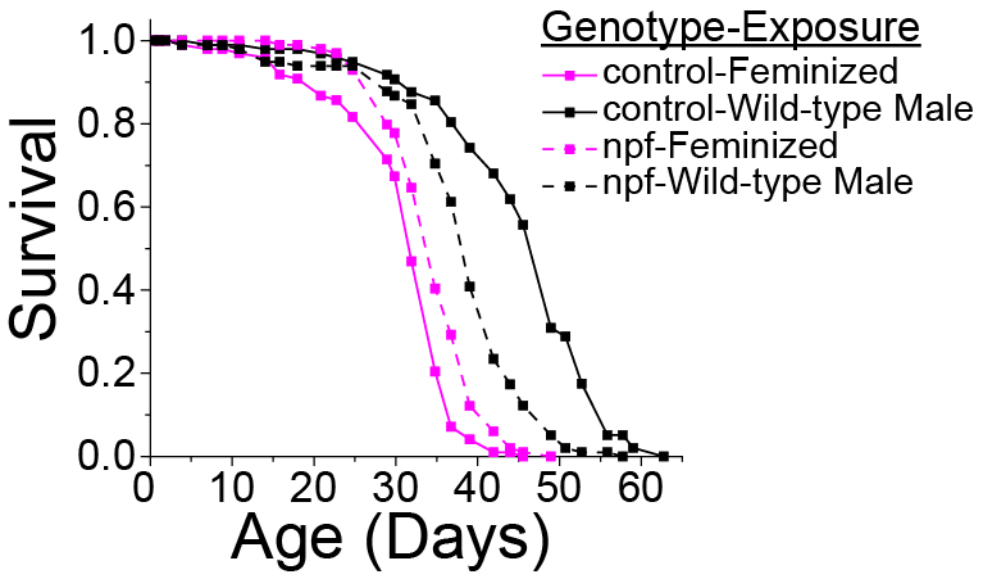

Figure 6.1: *npf* mutation may prevent the effect of pheromones on lifespan. A putative *npf* hypomorph decreases the impact of pheromone exposure on lifespan (Cox interaction term $P = 4.0 \times 10^{-13}$). However, there is still a significant effect of pheromones on the putative *npf* hypomorph ($P = 1.7 \times 10^{-7}$). Additionally, the hypomorphic nature of these mutants has only been demonstrated phenotypically, and the mutants are yet to be backcrossed, though the control is from the same general background ($y^{-}w^{-}$). For all groups N = 100, and experiments were conducted as described in chapter 2.

Ultimately, proving a direct connection will require demonstrating that an NPF receptor can directly modulate *dFoxo*, likely through cell culture-based assays. Such experiments are still worth pursuing, if only because connecting *npf* and *dFoxo* would provide a potentially conserved mechanism through which NPF (and NPY) regulates the aging process.

How does this ppk23-npf-dFoxo pathway interact with AkhR, which is required only for short-term effects? We have demonstrated that decreases in fat stores caused by pheromone exposure can be prevented by disrupting AKH signaling, and despite this pheromone exposure still decreases lifespan in *akhR* mutants. However, it is possible that this phenotype is due to pheromone exposure being unable to counteract the fattening effect of disrupting AKH signaling. Although the transcriptome analysis suggests that pheromone exposure alters the expression of lipases that may be targeted by AKH signaling as well as the *akhR* gene itself[28], direct evidence linking changes in AKH signaling following pheromone exposure is lacking. Connecting *ppk23*- or *npf*-expressing neurons to AKH signaling would be one way to address this question. Alternatively, altered AKH release (or *akh* expression levels) upon pheromone exposure would also suggest a causative role in the short-term phenotypes. While it may seem a subtle difference, distinguishing these hypotheses would determine if such changes in metabolism and stress resistance are linked to the mechanisms underlying aging, or simply occurring through a parallel pathway.

How does mating oppose the negative consequences of pheromone exposure? Further dissecting the mechanisms through which mating opposes the negative consequences of pheromone exposure will be necessary to determine whether they occur through

opposition within the same pathway (e.g., opposing effects on NPF signaling), or through distinct mechanisms. While *crz*-expressing neurons are required for and potentiate the beneficial effects of mating, activating these neurons is not sufficient, suggesting there are other components of the mechanism. Beyond this, how mating and *crz*-expressing neurons interact with pheromones and modulate aging is also unknown. Past research suggests that CRZ peptide can enter in the hemolymph and may act directly on peripheral tissues[29], which could be a potential mechanism to alter *dFoxo* activity. Alternatively, CRZ may be capable of regulating both AKH and insulin-like peptide (dILP) signaling, though these connections are not completely clear[30]. CRZ signaling alters transcription of *dILP* genes, but the connection to dILP signaling is questionable. The link between CRZ and AKH signaling is interesting, as the CRZ receptor, *CrzR* (also known as *GRHRII*), is highly similar to the AKH receptor, *GRHR*, and thus their ligands may be able to interact with both receptors[31]. Another potential mechanism is through modulation of NPF signaling, as mating and failure to mate have opposing effects on *npf* transcription[32]. Identifying how mating interacts with pheromone exposure will provide useful information not only on the mechanisms of aging, but also on how primitive emotions might valently oppose each other (e.g. if both have opposite effects on NPF signaling). With regards to this question, further work on how the neurometabolome is affected by various socio-sexual environments could elucidate how changes in the brain's state could influence aging and physiology.

Do more attractive females have a more severe effect on health and aging? While the approach used here generally assumed that any exposure to female pheromones was equivalent, it is possible that pheromone profiles of varied attractiveness may have

stronger or weaker effects. A key mediator of attractiveness in *Drosophila* females is insulin signaling[33]. If this hypothesis is true, it would suggest that insulin signaling in one individual can affect aging and physiology in a different individual. An endocrine pathway in one organism altering physiology in a second organism would provide both a novel concept of signaling between organisms as well as a potential molecular mechanism for diseases such as obesity that spread through social networks[34]. Thus far, attempts to identify if attractiveness of females affects male lifespan have been inconclusive, as the females were not differentially attractive in our experiments. However, future experiments can use more reliable manipulations to properly address this question.

Are female costs of reproduction regulated through similar neural and downstream pathways? Beyond the previously described experiments elaborating upon these findings in males, the mechanisms underlying the costs of reproduction should be closely analyzed in females as well. Our lab has shown pheromones have a smaller (but significant) effect on aging in females[28]. Research from other labs has found a significant role for a signaling peptide transmitted in sperm known Sex Peptide[35], but the underlying mechanisms are largely unknown. Sex Peptide is thought to act in the genital tract and directly in the CNS, and therefore the costs of reproduction in females may also be mediated through changes in the state of the CNS, similar to the effects observed in males. Thus, experiments designed to identify the effects of Sex Peptide on the neurometabolome and to map the neural circuits and downstream mechanisms responsible for these effects should be pursued. Such experiments could determine if

the mechanisms underlying the costs of reproduction are similar between sexes, or work through fundamentally distinct pathways.

Is Dh44's role in regulating aging related to the costs of reproduction? A peculiar aspect of the effects of *Dh44* on lifespan are that mutation of *Dh44* and inhibition of *Dh44*-expressing neurons extends lifespan specifically in females but not in males. This is particularly interesting in light of the fact that these effects apparently work through the *Dh44R1* receptor. Some neurons expressing *Dh44R1* are known to also be either *fruitless*-positive or *doublesex*-positive neurons, which marks them as being sexually dimorphic[36]. While the effects of *Dh44* on lifespan do not require co-housing of females with males, it is notable that these *Dh44R1* neurons are thought to be involved in controlling sperm ejection in females, similar to known effects of Sex Peptide signaling[36]. Both *Dh44R1* and Sex Peptide regulate the post-mating response in females[36,37]. As females are mated for two days prior to lifespan experiments, it is possible the *Dh44* mutation extends lifespan in females by eliminating the negative effects of this mating on longevity. This hypothesis could be tested by determining if virgin *Dh44* females are long-lived compared to controls.

Does Dh44 mediate the change in feeding preference that results from mating? Mating is known to drive changes in feeding preference by increasing the desire for protein, which requires both Sex Peptide Receptor and TOR activity[38]. Based on our knowledge of *Dh44* and feeding preference, these results could be explained by altered DH44 signaling. While *Dh44* regulates aging independent of diet, these experiments might also implicate *Dh44* to the costs of reproduction. This potential relationship between *Dh44* and the costs of reproduction in females is fertile ground for future study.

Does pheromone perception increase reproductive success early in life at the expense of late life reproduction? As the costs of reproduction are typically considered from an ecological or evolutionary perspective, it may be worthwhile to attempt to understand the implications of our findings on evolutionary and sexual selection theories. Preliminary studies suggest that, in competitive laboratory conditions, loss of pheromone perception (by *ppk23* mutation) decreases reproductive output of males early in life, but late in life *ppk23* mutant males out-compete wild-type controls (Figure 6.2). These results suggest that pheromone perception may increase early-life reproductive output. However, the alternative explanation is that the *ppk23* mutants simply outlive their wild-type controls due to an inability to perceive pheromones.

Thus, the underlying question is whether the physiologic state resulting from pheromone perception improves reproductive output. To disentangle finding a mate from pheromone perception, a potential experiment is to activate *ppk23* neurons directly (using *TrpA1*) and determine whether activation improves reproductive output compared to wild-type controls when exposed to masculinized females (i.e., females that will not activate *ppk23*-expressing neurons in the wild-type males). Further experiments could ask whether *dFoxo* or *npf* mutants exhibit similar reproductive output phenotypes. Such findings would help to determine if the physiologic changes induced by the reproductive emotional state are evolutionarily beneficial responses to improve the chances at reproductive success, or maladaptive byproducts of a system to motivate flies to mate and reproduce.

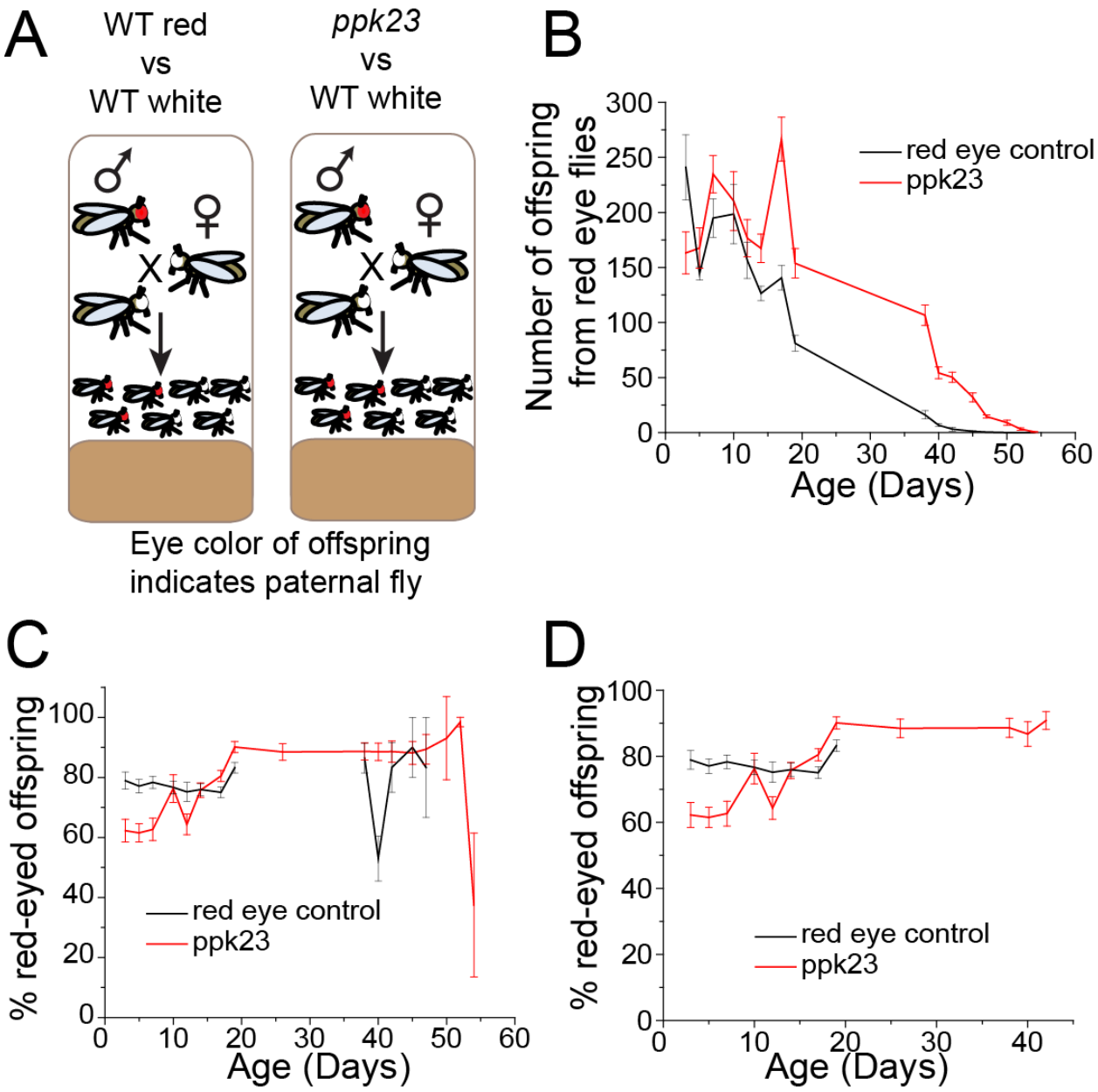

Figure 6.2: Pheromone perception decreases early-life reproduction and increases late life reproduction. (A) Schematic of experiment, where in control bottles 50 WT males with red eyes were placed in competition with 50 WT males with white eyes (to which the red-eyed line was backcrossed) for 100 WT females. In experimental vials, 50 ppk23 males (with red eyes) were placed in competition with 50 WT males with white eyes (to which the ppk23 mutant line was backcrossed) for 100 WT females. Parentage was then determined by the eye color of the offspring. (B) Total number of offspring from red-eyed parents. (C) Percentage of offspring from red-eyed parents in each type of bottle when all bottles are counted and (D) when only vials with more than 50 offspring are counted. Two-way ANOVA from days 0 – 19 (when all flies are still producing offspring) indicates a clear interaction between genotype and age (interaction term: $P = 1.3 \times 10^{-6}$). Note that there are still bottles of offspring to be counted from middle age (days 20 – 37), and thus these values are not final.

Do other environmental modulators of aging have similar temporal dynamics as the effects of pheromones? The combination of computational and wet-lab methods we developed to analyze the effects of pheromone perception on the dynamics of individual aging could also be adapted for a wide range of other aging inputs, which might help determine if similar mechanisms underlie their effects. Over the years, our lab has discovered that, besides pheromones, detection of yeast, carbon dioxide, water, sugar, and bitter substances are all capable of regulating longevity, as are many other manipulations found by other research groups[1-4,39-41]. However, many of these regulators of aging have only been studied with life-long manipulations primarily using population measures, often due to the nature of the manipulations (e.g., genetic mutants). Adapting our combinatorial approach along with current genetic techniques to provide temporal specificity to manipulations of these regulators of aging could elucidate both the individual reversibility and the range of time scales on which aging might be regulated. This in turn could provide evidence either for or against a single united pathway through which such manipulations alter the aging process. If a large number of manipulations show similar dynamics, then it would be reasonable to assume they work through similar mechanisms. While the use of time scales in this manner may seem a bit far-fetched, the data indicating that the short- and long-term effects of pheromone exposure are operating on far different time scales ultimately led to the finding that the effects of pheromones work through multiple mechanisms (i.e., AKH-dependent and –independent pathways).

Can FLIC help determine how the diet of an individual correlates with aging over time? Taking advantage of improvements in technology will be particularly important

for understanding behavior, physiology, and aging on an individual level. FLIC allows for analysis of feeding and preference behavior on an individual level with high temporal fidelity over long periods of time. For example, using FLIC it may be possible to measure the total amount of food consumed by individuals over a lifespan, and determine (1) if food consumption affects longevity and (2) if there are changes in eating patterns that correlate with increased mortality. While the answer to the former question would likely be yes on the basis of known DR effects, this hypothesis has never been testable in flies before. The latter question could identify changes in eating patterns that either predict or bring on death, which could then be untangled in future experiments.

Can FLIC identify the effects of varying individual "doses" of pheromone exposure?

Beyond feeding analysis, FLIC could be adopted for other uses. One potential example is using FLIC to examine pheromone exposure in males. By planting females (or feminized males) in agar in place of the liquid food, exact measurements of the contact duration and timing between males and females could be obtained and correlated to another measure such as starvation resistance, which could be measured directly within the FLIC apparatus. This might help improve understanding of the dose-response to pheromone exposure. Our simulations assume that exposure linearly altered frailty to alter mortality rates. However, most dose-response curves have both upper and lower plateaus, and experiments that can correlate an individual's pheromone exposure and response will have significantly more power than population measures. Such experiments would have been completely impractical a decade ago, and even now further advances upon FLIC and similar technologies will be needed to increase

throughput and scalability, making measures on individuals simpler to obtain and more powerful to analyze.

What brain regions, and which connecting sets of interneurons, are important for regulating aging? Our laboratory continuously works to improve scalability and throughput of all experiments, but the efficiency of lifespan analysis is particularly important given the quantity of flies needed for powerful experiments and the nature of the lab's interests. The "Fly Qi" project, which attempts to identify regions of the brain and specific neuronal subsets that can modulate aging, is a product of these efforts. Dr. Scott Pletcher has developed software to improve the speed, reliability, and functionality of performing lifespan experiments[42], and the use of twelve-chamber "hotels" increases the number of flies that can be handled at once. However, the current state of the Fly Qi project is only the beginning, as these results are being followed up with analysis of the effects of activating or inhibiting additional neuronal subsets beyond the ~80 lines screened here. Ideally, Fly Qi will identify specific regions of the brain that regulate aging through specific changes in the internal state of the organism.

Thus far, the approach of screening various neuronal subsets has proven successful, as three distinct brain regions have been identified. These include the PI, similar to the mammalian hypothalamus, and the SOG, which handles sensory inputs, as important regions for regulating aging[43,44]. The Mushroom Bodies (MB) also seem to be involved in regulating aging. The MB are known to be involved in higher sensory processing and are known to receive inputs from the SOG, implying that the distinct regions discovered in our analysis thus far may communicate with each other[45,46]. Further experiments may help define not only important regions, but could help identify the connections between

these brain regions. Finding such connections would strengthen the idea that specific neural states, with groups of neurons becoming more/less active, are powerful regulators of lifespan and aging. Thus, the Fly Qi project may be better served by directing future additions toward defining these connections, perhaps by focusing on interneurons or neuropeptidergic targets.

Are specific neurometabolomic changes associated with increased longevity? Beyond mapping specific regions of the brain that are important for regulating aging, neurometabolomics could be used to identify a potential "fingerprint" of long life. Upon analyzing the changes in the neurometabolome created by manipulating *Dh44*-expressing neurons, we found that both inhibition and activation substantially alter the metabolomic state of the brain (Figure 6.3). While the *Dh44* neurometabolome is in females and the pheromone exposure neurometabolome is in males, the fact that both manipulations show clear changes in the state of the brain suggests that such changes may be present in a wide array of treatments that alter the aging process. While it would be financially prohibitive to screen all the lines in the Fly Qi project for neurometabolomic alterations, such experiments in treatments that extend lifespan may help reveal whether multiple lifespan-extending manipulations lead to similar neurometabolomic states. The existence of similar neurometabolomes across lifespan-extending phenotypes might suggest a causative effect for some of these metabolites, whereas the existence of a diverse range of neural states would suggest that the changes in the neurometabolome is more likely to be a side effect to the neural signaling pathways driving changes in aging and longevity.

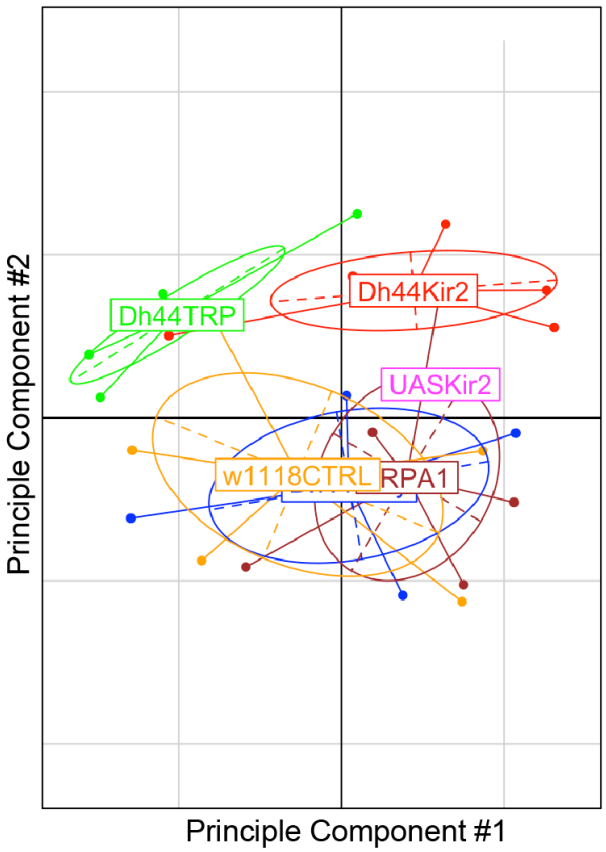

Figure 6.3: Manipulation of *Dh44*-expressing neurons alters the neurometabolome. Both activation (Dh44TRP, green) and inhibition (Dh44Kir2, red) lead to changes in the neurometabolome when compared to controls (yellow, blue, brown, pink). Activation and inhibition appear to have opposing effects along the axis of the 1st principle component, whereas their effects are similar along the axis of the 2nd principle component. Further analysis of these data sets along with metabolomics from *Dh44* mutants may provide further insight into which metabolites are relevant to aging.

Implications for evolutionary biology

When considering the potential implications of the internal state or "emotions" regulating the aging process, it is important to recognize how conservation of emotions across taxa would force evolutionary biologists to think about emotions in the context of natural selection. Evolution is driven by natural selection, and individuals with traits that improve the chances of their offspring surviving and reproducing will typically outcompete other, less-adept individuals. If emotional states are evolutionarily conserved, then emotions must be considered as a mechanism to improve reproduction and survival, not just as a side-product of advanced, self-conscious minds. While we may not say a fly is "afraid", in the presence of predators they do alter neuropeptidergic

signaling and change their behavior to avoid being eaten (or their offspring being eaten), a response that seems to have obvious evolutionary benefits[14,15].

Investigation of the molecular mechanisms underlying these "emotional" responses across a wide range of taxa will help determine whether these mechanisms developed independently multiple times or have been conserved since first developing. By discovering the signaling pathways responsible for these changes in the internal state, it will be possible to begin to map the evolution of emotions. Furthermore, if different emotions map to different signaling pathways, it might even be possible to identify environments where specific types of internal states are more or less advantageous. This in turn could help both evolutionary biologists considering the "purpose" behind these emotions as well as the potential for understanding the origins of emotional disorders in humans.

Perhaps the most obvious implications of our findings on evolution result from the risk/reward of pheromone perception and mating. Taken at face value, our results suggest that an encounter with the opposite sex induces the costs of reproduction, but a *successful* encounter largely negates these consequences. Based on our preliminary results suggesting that pheromone perception increases early reproduction at the expense of late reproduction, we might conclude that these costs are the result of a "bet" by the male that he will successfully mate. A failed bet leads to severe negative effects, whereas successful mating avoids them. From a natural selection perspective, this wager will push evolution to move at a more rapid pace, as losers suffer from decreased survival as well as a failure to reproduce, whereas winners both mate and avoid the brunt of the other costs of reproduction. While we don't know the exact nature of

mechanisms underlying these benefits, the fact that *crz*-expressing neurons are required and potentiate the effect suggests it is a regulated process. Thus, the beneficial effects of mating act as a positive feedback loop in terms of reproductive fitness, and could amplify other selective pressures. Such mechanisms could play an important role in increasing the speed at which populations adapt to changing environments, as it will amplify the rate at which beneficial mutations can proliferate.

Implications for human health

Determining whether the effects of these studies, particularly the sexier ones, carry over to humans will be a long road, but conservation in the underlying molecular mechanisms provides hope that the effects on health and aging will also be conserved. Of the numerous studies on the effects of sex, stress, and emotions on human health, the vast majority are correlational with at best tenuous or trivial mechanisms[47-52], and thus the identification of candidate genes to regulate these effects is an important step. We have discovered three conserved neuropeptides that were previously not known to regulate lifespan in flies: *Crz*, homolog of gonadotropin-releasing hormone (GnRH), *npf*, homolog of NPY, and *Dh44*, homolog of corticotropin-releasing hormone (CRH).

GnRH and *Crz* are involved in similar phenotypes and have similar relationships with other signaling pathways, suggesting a high level of conservation. *Crz* is known to regulate reproductive physiology in male *Drosophila*[53] and reproductive selection in female *Drosophila*[54], while GnRH has similar functions, as it is known to regulate sexual behavior in addition to reproductive physiology and GnRH neurons are even anatomically altered by social interactions in Cichlid fish[55,56]. In flies, *Crz*-expressing neurons also express the receptor *Dh44R1*, which we demonstrated can regulate

longevity[57], and *npf* and *crz* have both been implicated in similar physiologic effects[58,59]. Notably, both CRH and NPY are known to interact with GnRH neurons in mammalian systems[60-63], suggesting a high level of conservation in these signaling pathways. Our finding that *crz* modulates aging in response to mating is corroborated by recent studies providing direct evidence that GnRH can affect aging, and perhaps even longevity, in mice[64]. Thus, it is reasonable to consider whether interventions involving GnRH might influence aging and longevity in humans as well.

As CRH and NPY can modulate GnRH signaling and their homologs were identified here as regulating aging, they are also potential targets for intervention. Interest in NPY has grown significantly within the aging community recently[65], based on results implicating NPY in both hypothalamic regulation (the home of GnRH neurons) and dietary restriction. However, CRH has not been studied in the context of regulating aging. Instead, it is mainly thought to be involved in the stress response, with CRH acting to induce phenotypes such as appetite suppression, local inflammation in diseases like rheumatoid arthritis, and increased anxiety[66]. Interestingly, the effects of CRH are sexually dimorphic in mammals, with an increase in female sensitivity mirroring our female-specific phenotype[67]. Currently multiple CRH receptor antagonists are under development, with the goal being to treat both anxiety and the long-term manifestations of chronic stress. Our lab has preliminary evidence collected by Drs. Tuhin Chakraborty and Christi Gendron suggesting that Antalarmin, one of these antagonists, may increase lifespan in *Drosophila*. This supports our findings that mutations in *Dh44* and *Dh44R1* extend lifespan, and we are currently collaborating to determine if Antalarmin inhibits DH44 signaling in flies. This development would

provide not only a drug target but also a specific candidate for anti-aging interventions in mammalian model systems and ultimately humans.

Beyond the development of interventions, this work points toward a significant role for emotional stress in regulating the aging process. Both DH44 and CRZ signaling pathways have previously been implicated in the "stress response" in flies[68,69], and CRH has been labeled with the moniker of a "stress hormone" in humans[70], but it is worthwhile to consider what is meant by these labels. In invertebrates, "stress" from the laboratory is often physical in nature, be it induced by starvation, temperature, or toxins. However, the "stress" of pheromone exposure would not fit in with this group, and would point toward a more emotional stress: the flies sense something attractive, but are unable to obtain the expected reward. If the reward is obtained, the stress is lessened, though some effects may remain. This sort of emotional stress is more familiar to humans, who, at least in developed countries, are less likely to encounter the more physical varieties of stressors. While there is certainly evidence suggesting hormesis (minor stressors increasing overall stress resistance) play a role in aging, these stressors are almost always physical in nature and the benefits typically result from increased cellular repair activity[71]. These findings provide a signaling pathway for something most of us know innately: stress is harmful to our health. So instead of spending our time stressing about chores, finances, and writing 200 page research documents, we may be better served by relaxing, bonding with our loved ones, and pondering how to best focus our (Fly) Qi.

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