Identification of Neurosensory Systems for Nutrient Evaluation and Their Regulatory Roles on Physiology and Lifespan

by

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Dedication

To all who taught me Passion

Acknowledgements

It was one of those late nights during my undergraduate years, I was watching an interview piece of Mr. Les Wexner, once a young man who borrowed $5,000 from his aunt to open a female clothing shop called “The Limited”, now one of the most influential American entrepreneurs who owns numerous leading brand retail shops world-wide. Mr. Wexner is also famous for his devoted philanthropic work through Wexner Foundation. One of the interviewer’s questions was, how come he had acquired such diversity in the foundation’s activity. He said, when he was still young in his business career, he once asked an established businessman the same question. The following answer captivated me since and became one of my life mottos; stay open-minded and follow your curiosity. That is what I wanted and still want for my life, constantly expanding horizon, guided by my own curiosity, and making positive contribution to the society with what I am good at. Looking back on the journey that I took to get where I am now, I realized that it is far from a straightforward path, yet it yielded countless lessons and fun that became basis for many branches of passion that I cultivated over the years. Not all girls who likes to sit in a wood to watch animals or wade through a river to spot a fish grow up to be a biologist who travels through different countries for studying eco-physiology of birds, given opportunities to learn mass spectrometry from industry leaders, be a part of a renown research community to study biology of aging as a PhD student, or founding a company to facilitate academic technology transfer. Indeed my scientific career, albeit still young, has been wide in breath, adventurous, and deeply rewarding. Pursuing my PhD in biomecial science has been large part of my life for last 5 years and I have many people to thank with my upmost gratitude, as without them I would not be where I am today.

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Preface

Nutrients: A major pillar of biology of aging

Aging is a process of changes that occur over the course of organisms' life. The rate of aging is influenced by both genetic and environmental factors and studying biology of aging brings us insight into why and how we age. After certain age, aging is associated with progression of physiological and behavioral decline, making an individual more vulnerable to disease and disability. Because aging is the largest underlying risk factor for most human diseases, uncovering mechanisms of aging can provide us with tools to combat aging process and thus prevent or mitigate age-associated diseases.

Although not intuitive, foods that keep us alive can have both positive and negative impact on organisms' lifespan. Eighty years ago McCay and colleagues first reported that one could restrict total food intake (dietary restriction, DR) of rats to influence maximum lifespan. This study spurred researchers to undertake many studies to investigate impact of nutrients on cellular and organismal senescence. Because consumption of food is essential for survival, an observation that restriction of food intake can extend organisms' lifespan revolutionized our thinking in what a food does to our body and how a food alters our physiology. We now understand, a process of extracting energy from consumed nutrients, a.k.a. metabolism, which is necessary for basic survival can act as a double edge sword to produce both beneficial and harmful effects to our body. We also learned that there is several evolutionarily conserved nutrient-sensing pathways, which are regulated by dietary composition or amount, are intimately associated with metabolism and can directly modulate organismal lifespan. Importantly, any factor that changes these pathways independent of food intake also impacts aging. For example, disruption in either insulin signaling or target of rapamycin (TOR) pathways through mutation in key genes alters cellular metabolism and lifespan in both invertebrates and vertebrates regardless of diet. During last twenty years, we began to understand various factors that can directly regulate nutrient-sensing pathways. Among those, one of the most exciting findings shown by invertebrate model organisms is that exposure to food odors is sufficient to limit the benefits of DR on lifespan. These studies suggest that central nervous system (CNS) must be involved as a master orchestrator of all physiological effects emanating from interaction between a food and

an animal. I hypothesized that neuronal processes that are involved in sensing and evaluating key longevity-modulating nutrients are potent regulator of organismal health and diet-mediated lifespan. My thesis aims to identify which neurosensory systems are involved in interacting with key-longevity modulating nutrients and how they regulate physiological effects. I describe my thesis in the following three parts.

Part I: Development of standardized methods for measuring lifespan and feeding behavior

The first aim of my thesis, corresponding to Chapter 2-3, was to develop a standardized and high-throughput way to measure lifespan and feeding in animals with minimal experimenter bias.

The vinegar fly, *Drosophila melanogaster*, is an attractive model organism for studying the mechanisms of aging due to its relatively short lifespan, convenient husbandry, and facile genetics. However, demographic measures of aging, including age-specific survival and mortality, are extraordinarily susceptible to even minor variations in an experimental design and environment. Therefore a well-defined laboratory protocol for survivorship experiments with a careful genetic background control is required for reliable measures of *Drosophila* lifespan. In Chapter 2, I described a standardized protocol for lifespan experiments that had been optimized for many years in the Pletcher lab and others. In addition, I introduce a computer interface that enabled high-throughput management of lifespan experiments called “dLife program”. The program accelerates throughput and promotes good practices by incorporating an optimal experimental design, simplifying fly handling and data collection, and standardizing data analysis.

The fruit fly is also one of the most powerful model systems to dissect neural mechanisms of complex behaviors. Unfortunately technical advances in neurobiology have outpaced those that facilitate basic observation. 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 and preference assays lack qualities appropriate for high-throughput analysis. This motivated me to invent a system that captures all tasting and feeding activities of an individual fly called Fly

Liquid-food Interaction Counter (FLIC). Using the FLIC system, I was able to obtain individual flies' continuous trajectories of what they eat, when they eat it, and how much they consume. In Chapter 3, I detailed how the FLIC system works and demonstrated that the FLIC not only can reproduce data from existing feeding assays, but also provides the power and flexibility to quantify many new aspects of feeding behavior, including temporal dynamics of food assessment and circadian feeding patterns.

Part II: Identification of neurosensory systems that modulate dietary protein selection and aging

Given tools to carry out high-throughput lifespan and feeding experiments, I was well equipped to study how process of nutrient assessment affects organismal aging in *Drosophila*. My second aim of the thesis, described in Chapter 4, was to understand how one of the key longevity modulating nutrients, dietary protein, is perceived and how it initiates widespread changes in physiology.

Dietary proteins in general and essential amino acids in particular, are potent modulators of lifespan. From previous work, dietary restriction (DR) is strongly mediated by changes in the availability of specific nutrients rather than by a reduction in caloric intake *per se.* For example, restricting only essential amino acid(s) is sufficient to extend lifespan in mice, rat, and fruit flies and supplementation of essential amino acids in the diet, but not other isocaloric nutrients, is sufficient to abolish lifespan-extension under DR in *Drosophila.* However, knowledge about how sensory information about dietary protein is processed or how it initiates widespread changes in physiology, including aging, remains rudimentary. Peripheral sensory signals are likely analyzed and interpreted by the CNS, which in turn alters physiological systems, but the neural circuits involved in this process are unknown.

In Chapter 4, I detailed underlying neurosensory mechanisms for protein-dependent feeding behavior in flies and how it affects lifespan. I first investigated whether flies are capable of sensing dietary amino acids. I found that adult flies restricted of protein or particular amino acids adjust their feeding preference to amend the protein deficiency, implying that neuronal mechanisms exist to sense amino acid availability both internally and externally. I then executed a screen for genes and neuronal cell populations that are required for this preference and identified serotonin, serotonin receptor 2a, and an amino acid transporter called JhI-21 as key players in protein valuation. Disruption of any one of these genes increased lifespan up to 90% independent of food intake suggesting the perceived value of dietary protein is a critical determinant of its effect on lifespan. My work suggests that evolutionarily conserved

neuromodulatory systems that define neural states of nutrient demand and reward are sufficient to control aging and physiology, independent of food consumption.

Part III: Identification of neurosensory systems that interact with nutrient stress and sleep

My last aim of the thesis work, described in Chapter 5, was to investigate what role nutrient perception plays in modulating other aspects of health. To study this, I leveraged on a long-recognized observation that under sever nutrient stress, flies will limit their sleep to extend their foraging effort.

Under starvation condition, organisms use multiple strategies to adjust resource allocation in order to maximize the chances of finding a food source, including longer foraging searches and limiting sleep behavior. Sleep loss in fruit fly is a characteristic response to nutrient deprivation. On the other hand, because sleep loss is also costly to the organism, mechanisms for evaluating the nutrient environment and quickly terminating sleep deprivation when food is available would likely to be beneficial. In Chapter 5, I have shown that the gustatory perception of sweetness is both necessary and sufficient to suppress starvation-induced sleep loss when animals encounter nutrient-poor food sources. In addition I found that dopaminergic neurons' activity mediates appropriate regulation of sleep behavior specifically under conditions of very low nutrient availability.

Summary

In summary, little is known about how a nutrient exerts their effect on aging and health. My thesis provides important insights into how nutrients interact with nutrient states of animals and how neurological systems are involved in such interactions. The idea that the sheer process of internal nutrient demand assessment and external nutrient valuation, independent of consumption, affects health and aging is a novel one. I believe results from my study will make positive contribution to both basic biology and clinical geriatric research. First, establishing connectivity between the CNS function and diet-dependent longevity in fruit flies provide basic framework to examine similar mechanisms in more complex organisms. Second, understanding how nutrient signals are processed can foster clinical research to target neurological regulation of nutrient intake or age-associated disease progression.

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Chapter 1: Introduction[1]

Food and Nutrient-sensing Pathways

An ability to recognize and assess different aspects of one's surroundings is a fundamental tool for survival of living beings. Indeed, such ability is found in a simple, single-celled organism to a complex species like human (Breslin, 2013; Kaeberlein et al., 2005; Renard et al., 2009). Although the level of sophistication is different across taxa, most organisms exhibit "sense organ(s)" that interact with both external and internal environments to receive information about surrounding conditions, further process, and produce biological responses (Jacobs et al., 2007). Among all elements of external environments, information regarding nutrients is perhaps one of the most important, and consequently, nutrient sensing systems share deep evolutionary roots across multiple species. Like all environmental sensing, the perception of nutrients requires two elements: sensory acuity and sensory processing. Sensory acuity entails the sensory organs' ability to receive inputs. Simultaneous stimulation of gustatory, olfactory, and somatosensory systems by food-derived stimuli comprise the sensation of nutrients to form a multi-modal food "experience" (Breslin, 2013). These first order sensory inputs about the food are then get integrated and processed for determining nutritional contents, taste, palatability, reward, value, and decision-making. Once sensory inputs are processed, coordinated behavioral or physiological responses follow. In this review, I will place the organisms' nutrient sensing systems in an evolutionary context to understand functional links between the neurosensory systems and physiology that provide insights into neurological modulation of organismal aging and health.

Research in model organisms revealed that nutrient sensing acuity and process (a.k.a. nutrient-sensing pathways) have remarkable conceptual and molecular similarities across invertebrates and vertebrates (Fontana et al., 2010). Specifics in chemosensory acuity perhaps are "private" to organisms occupying each ecological niche with their unique needs, yet processing of sensory inputs is likely to share common goals across species such as desires for food, mates, and avoidance of danger (Linford et al., 2011). For example, the molecular mechanisms of sugar-sensing systems maybe phylogeny-specific, but the facts that chemoreceptors exist for

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sweet-tasting sugars and that sugars are processed through insulin-like signaling are conserved from nematode worms to insects to mammals (Miyamoto et al., 2013; Yarmolinsky et al., 2009). Even single-celled organisms, brewer's yeast (Saccharomyces cerevisiae) for example, is equipped with a glucose-responsive pathway that is believed to be an evolutionary precursor to the insulin-like-signaling pathway in more complex organisms (Kaeberlein et al., 2005). Indeed investigation of "public" mechanisms of nutrient-sensing pathways in simple model organisms brought fundamental insights into understanding molecular mechanisms of food-related biology such as food searching, intake, and post-ingestive behaviors (Bjordal et al., 2014; Cunningham et al., 2012; Krashes et al., 2009; Linford et al., 2011; Stafford et al., 2012).

Nutrient-sensing Pathways and Aging

Availability of nutrients is one of the potent environmental factors that are known to modulate organismal lifespan. A correlation between food and aging has been long recognized since hundreds of years, yet it was McCay who first experimentally demonstrated that one could restrict food intake (dietary restriction, DR) of rats to increase their maximum lifespan about 80 years ago (McCay et al., 1935). Since then researchers have undertaken many studies to investigate the impact of nutrients on cellular and organismal senescence. Although there is no one mechanism that is believed to be entirely responsible for all of the effects of nutrients on aging, there are several metabolic pathways that are suggested as strong candidates for mediating diet-dependent lifespan or other phenotypes associated with effects of DR. These include insulin/insulin-like growth factor signaling (IIS), target of rapamycin (TOR) signaling, AMP-activated protein kinase (AMPK) pathway, and sirtuins-mediated pathways (Mair and Dillin, 2008). However, results from direct genetic manipulations of these pathways in various model organisms have been inconclusive at revealing these pathways' role in diet-dependent lifespan(Hansen et al., 2007; Houthoofd et al., 2003; Kaeberlein et al., 2004).

Although a requirement of metabolic pathways that are known to be important longevity modulators in diet-dependent lifespan is still under debate, components of nutrient-sensing pathways have been consistently implicated in DR-mediated lifespan effects across taxa. Loss-of-function mutation in a C. elegans transcription factor, PHA-4, completely abolished the life-extending effect of DR (Panowski et al., 2007). An adult-specific role of PHA-4 is not well understood, but it is notable that mammalian orthologue of PHA-4, Foxa family of transcription factors, are involved in glucose homeostasis (Friedman and Kaestner, 2006). The smell of food could also limited the full beneficial effect of DR on the lifespan of the fruit fly, Drosophila melanogaster, and C. elegans suggesting food perception through olfaction is required for diet-dependent lifespan (Libert et al., 2007; Smith et al., 2008). Based on these results, it is compelling to consider whether nutrient-sensing pathways or regulators of nutrient

homeostasis also function as modulators of diet-dependent lifespan in higher organisms.

Chemosensation of food-derived stimuli serves at least two functions: recognition of foods for nutritional quality or toxicity prior to ingestion and preparation of the body for post-ingestive metabolism. There is evidence suggesting that these two distinct processes occur even without complete ingestion of foods. Perhaps it may be due to the evolution of nutrient-sensing pathways has been driven by honest food-related stimuli rather than of fake or nonnutritive foods (Pavlov and Thompson, 1902; Powley, 1977; Wicks et al., 2005). Since pioneering work from Pavlov and colleagues which demonstrated that food-derived chemicals are sufficient to release “psychic secretions” in the stomach of a dog, researchers have uncovered many elements underpinning the concept of cephalic phase or preabsorptive reflexes (Pavlov and Thompson, 1902). Cephalic phase reflexes are anticipatory digestive and metabolic responses that are induced upon encountering sensory or learned cues of food. They are thought to effectively prime the body for digestion or enhancement of digestive capacity (Power and Schulkin, 2008). The best characterized cephalic phase response is insulin release in rodents and humans upon sensory perception of food (Ahrén and Holst, 2001; Powley, 1977). It has been documented numerous times that sham-feeding in humans, or sensation of sweet tastant, even without a nutritive value, in rodents are sufficient to stimulate pancreatic insulin release (Powley and Berthoud, 1985; Teff et al., 1995). Interestingly, even if the magnitude of insulin release during cephalic phase is lower than that involved in the postprandial response, cephalic phase insulin plays a significant role in enhancing post-absorptive glucose metabolism. It is also required for regulating lipoprotein lipase activity in peripheral tissues (Ahrén and Holst, 2001; Picard et al., 1999).

Such data suggest that physiological responses are not only reactive but also anticipative. Why would an anticipatory preparation for food be beneficial to animals? In what he coined “the paradox of feeding”, Woods (Woods, 1991) claimed that as much as the food is vital for survival, disturbance of internal homeostasis after sudden influx of nutrients after feeding is detrimental to viability of the organism. Perhaps it was this evolutionary arms race between acquisition of vital nutrients and maintenance of a stable internal milieu that necessitated a direct connection between the sensory perception of food and feeding-related physiology so that animals can quickly eliminate a disruption of internal milieu after feeding. Indeed, it has been shown that the chemosensation of food is required for efficient digestion and storage to quickly return to homeostatic set points of internal chemical balance after feeding (Ahrén and Holst, 2001). This supports the notion that hastened nutrient absorption after feeding must be desired by organisms to increase their fitness (Woods, 1991).

Anticipatory physiological responses based on food perception would serve an advantageous role in wild animals or a billion humans that face low food security to help them identify and quickly absorb desired nutrients (Breslin, 2013). But for most modern humans, who are often surrounded by readily available energy-dense foods with high in sugar, salt, and fat, are up against intriguing biological problems: Over stimulation of nutrient sensing pathways that can lead to metabolic disorders (Einstein et al., 2008). Moreover research based on simple organisms suggests that over stimulating nutrient-sensing pathways can even influence health states and aging.

Considering the fact that evolutionary history equipped most animals' metabolic system to be responsive to environmental perception, could aging be directly modulated by sensory perception? The answer to this question appears to be 'yes'. When pioneering observations by Apfeld and Kenyon (Apfeld and Kenyon, 1999) discovered that sensory inputs is sufficient to regulate aging in C. elegans, it revolutionized the geroscience community and opened up new avenues of research into the neurological control of aging. Subsequent studies successfully established a direct link between neurosensory systems and lifespan across taxa to suggest that sensory perception is a "public" mechanism of aging (Gendron et al., 2014; Lee and Kenyon, 2009; Libert et al., 2007; Linford et al., 2011; Poon et al., 2010; Riera et al., 2014; Waterson et al., 2014). Pletcher and colleagues spearheaded a large effort directed toward discovering specific sensory inputs, sensors, and neural circuits that underlie regulation of aging. As a result, we now have a general idea which specific olfactory or gustatory inputs positively or negatively modulate lifespan of the fruit fly, as well as what sensors for those inputs and the particular neural circuits that connect them to aging-related metabolic pathways (Gendron et al., 2014; Poon et al., 2010; Waterson et al., 2014). Recently, for example, it has been shown that sensory inputs can not only impact aging, but also other aspects of physiology that is important for health, such as sleep (Linford et al., 2015).

Dietary Components and Aging

What components of the diet are relevant for influencing aging? DR was originally referred to as 'caloric restriction' because most of the early work implicated daily caloric intake regardless of the source of the calories. Subsequent research, however, has established that not all macronutrients are equally important in diet-dependent lifespan. Therefore, DR is now broadly defined as the reduction of intake of all or particular nutrients without malnutrition. In brewer's yeast and worms, adding glucose or genetically disrupting glucose metabolism have a profound effect on aging (Lee et al., 2009; Schulz et al., 2007). On the other hand, in D. melanogaster, dietary protein in general and essential amino acids, in particular, seem to be potent modulators of lifespan (Mair et al., 2005; Smith et al., 2008). Similarly, in rodents, restriction of methionine

or tryptophan intake is sufficient to extend lifespan of mice and rats (De Marte and Enesco, 1986; Sun et al., 2009; Zimmerman et al., 2003). Whether dietary protein has similar effects on lifespan of nematode worms or yeasts have yet to be determined. Alternatively, some argued that the ratio between protein to carbohydrate intake is more relevant for longevity instead of any one macronutrient intake (Lee et al., 2008; Skorupa et al., 2008). Although, details about which nutrient(s) affect which species' maybe different, these studies all point in the same direction that individual nutrients indeed need to be weighted differently when considering the impact of diet on organismal aging.

When and how much to reproduce for how long maybe private to each taxon or sex, but underlying trade-offs that shape each life-history strategy are likely to be universal across organisms: resources are finite and required by both survival and reproduction. It is possible that different life expectancies are shaped by differences in life-history traits of organisms. Moreover optimal foraging patterns for animals that adapted different life-history strategies are likely to be different based on the optimal foraging theory (Pulliam, 1974; Stephens and Krebs, 1986). Although most of the earlier theoretical work on optimal foraging behaviors only concerned maximizing total calorie acquisition in a given time (Stephens and Krebs, 1986), importance of obtaining optimal nutrients in addition to calories also had been recognized by few (Greenstone, 1979; Richter et al., 1938; Waldbauer and Friedman, 1991).

Some nutrients may have species or sex-specific effects on aging depending on their chosen life-history strategy. Dietary self-selection theory which claims that the availability of certain nutrients extrinsically (i.e., in the enviornment) can influence organisms to favor a specific life-history strategy (Waldbauer and Friedman, 1991). Indeed, there is empirical evidence that the nutritional requirements for the best survival or reproduction outcomes are different. For example, based on studies that allowed animals to feed on predetermined mixtures of fixed, but variable, protein to carbohydrate ratios (P:C), low P:C tends to maximize lifespan both in male and female fruit flies or field crickets (Jensen et al., 2015; Lee et al., 2008; Maklakov et al., 2008; Skorupa et al., 2008). However the P:C ratio that maximizes reproduction showed clear discrepancy between male and female animals. A high protein diet promotes female reproduction whereas males' reproduction is maximized at low P:C that is also known for promoting male longevity (Jensen et al., 2015; Maklakov et al., 2008). Perhaps this difference can be explained by inherited divergence in reproductive strategies between sexes. For instance, males' reproductive success is based on access to mating and hence consuming a calorically dense food such as a high carbohydrate diet is advantageous. However females' reproductive success is based on a number of offsprings produced, and, therefore consuming more protein, which is essential for oogenesis, is critical (Vanderzant, 1963; Wheeler, 1996).

The observation that male flies or crickets do not require different nutritional composition to maximize reproduction and longevity directly challenges the existence of trade-offs between these traits at least in males (Jensen et al., 2015). Alternatively providing the pre-determined P:C for the entire life of an animal and measuring lifespan and reproduction may not be the best approach to test the effects of nutrients on these two life-history traits. To date, there are two studies that directly addressed how sex-specific dietary self-selection influences reproduction and lifespan. In both studies, authors aged male or female fruit flies or field crickets in one of several choice diet pairs in which a pair of food differs in P:C as well as total density of calories (Jensen et al., 2015; Maklakov et al., 2008). Surprisingly both studies revealed that males and females rather preferred to feed on the same trajectories of P:C even if that particular diet was not optimal for neither of sexes' reproduction or longevity. Based on these data, feeding behavior may be qualified to be considered as a case of intralocus sexual conflict (ISC), which arise when shared genetic bases for a certain trait inhibits each sex to evolve independently to their optima (Chippindale et al., 2001; Rice and Chippindale, 2001).

Nevertheless providing independent sources of protein and carbohydrate as a choice has not been done in a context of aging research. Moreover providing multiple choices of premixed protein and carbohydrate diets still does not amend a scenario where animals are forced to ingest excess of certain nutrients to obtain a deficient nutrient (Simpson and Raubenheimer, 2007). Therefore we shall reserve our conclusions until we learn what animals choose to consume when individual nutrients are presented as a choice and how it affects their reproduction and survival.

Physiological States and Nutrient Balance

A complex organism has an internal agency that is prepared to operate when the normal state of the organism is disturbed. This idea has been first articulated by Claude Bernard and later further developed by Walter B. Cannon who coin the term homeostasis to explain the coordinated physiological reactions for maintenance of steady state of internal milieu (Cannon, 1929). Ironically an organism under homeostatic control rarely means a constant state of a body but rather implies that the state of the organism's internal system is under constant adjustments to accommodate ever-changing conditions. Consider an internal nutrient environment in an organism. The digestive system and associated organ systems need to repeatedly alternate their states to deal with surge of excess nutrients (feeding) and potential nutrient deficit (between feeding bouts) via constantly flowing nutrients into and out of storage pools so that an organism as a whole can maintain certain target levels of circulating nutrients.

Therefore feeding is a complex behavior that reflects and anticipates the current and future nutritional states of an individual and any minor error can bring detrimental to its vitality. So what influence nutrient requirements of an organism? There are distal and proximal factors influencing nutrient requirements of an organism. For example, optimal nutrients for different stages of life such as infanthood with exponential growth period, puberty with time of sexual maturity, and adulthood with active reproduction and post-reproductive stage are distinct. Even seasonal fluctuation of mate and food availability can shape different nutrient requirements. On the other hand, some factors act in a short range; animals adopted episodic instead of continuous feeding demonstrate circadian fluctuation of endocrines that puts them in appropriate physiological states for energy homeostasis. In addition, all animals experience cyclical hunger and sated states throughout the day and night periods. In addition to the factors listed above, there are other conditions such as infection or other pathologies and migratory behavior that could create unique nutrient demands.

How do organisms recognize internal nutrient demands? Such demands can be categorized into two; demand for a general energy source and demand for specific nutrients. ATP is a universal cellular energy currency in eukaryotes; hence the level of ATP is carefully regulated to avoid an interruption in normal cellular functions. In eukaryotic cells, AMP-activated protein kinase (AMPK) is thought to function as a fuel gauge because it is sensitive to rising AMP: ATP ratios, which indicate low cellular energy state (Hardie et al., 1998). In addition to acting as the general energy sensor, AMPK has been implicated in specific nutrient regulation such as glucose uptake in muscle cells, glycolysis, fatty acid uptake and synthesis, and protein synthesis (Steinberg and Kemp, 2009). Although not as extensively studied as a role of AMPK in nutrient homeostasis, there are other ways that animals recognize specific nutrient demand. Direct sensation of metabolic substrates, such as glucose, fatty acids, and amino acids, in the central nervous system or indirect sensation of circulating metabolites via hormones and peptides in orexigenic and anorexigenic neurons, have been speculated to be important for recognition of the specific nutrient demand (Zheng and Berthoud, 2008).

Once the nutrient demands are formed and recognized, there are two ways that the body can cope with the situation. One is to change feeding behavior to obtain appropriate nutrients, and another is to alternate physiology to compensate for nutrient imbalance. Some examples of compensatory physiological responses for imbalance nutrients include switch of metabolic substrates from carbohydrate to fat or protein, activation of autophagy, decreased rate of protein turnover and active reabsorption of salts in a renal system. These responses, however, tend to come as a second-line of defense with certain degree of metabolic reprogramming to cope with the lengthy nutrient demand. On the other hand, behavioral responses seem

to be organisms' first-line of defense to quickly respond and correct the nutrient demand in hand. Changes in appetite or aversion toward a certain food seem to be a common mode of behavioral switch to target desired nutrient intake. Sensitivity of odorant or gustatory receptors can change based on a hunger or satiety state of organisms and therefore often reported to be associated with changes in food intake (Simpson et al., 1991). Interestingly in addition to its role in sensing the current energy state, AMPK is known to activate effector pathways to coordinate whole-organism energy balance via neurological or hormonal means (Apfeld et al., 2004; Cunningham et al., 2012; Lam et al., 2011; Steinberg and Kemp, 2009). Indeed AMPK has been implicated in changes of appetite, feeding behavior, as well as selection of appropriate metabolic substrates to meet the nutrient requirements (Cunningham et al., 2012; Minokoshi et al., 2004; Steinberg and Kemp, 2009). Other well-known example of behavioral adjustments for nutrient demand is; foods missing essential amino acids can induce aversion in both invertebrates and vertebrates (Delaney and Gelperin, 1986; Koehnle et al., 2004). Later, researchers found that a protein kinase, GCN2, is responsible for recognizing the essential amino acid imbalance across taxa and in Drosophila larvae, dopaminergic circuitry is responsible for executing the behavioral response to ameliorate the demand (Bjordal et al., 2014; Maurin et al., 2012). Preferences for a high fat diet or state-dependent protein intake also had been observed in multiple species, yet comprehensive molecular mechanisms of such behaviors are still elusive (Lucas et al., 1998; Perello et al., 2010; Ribeiro and Dickson, 2010; Vargas et al., 2010). Besides, appropriate levels of micronutrients such as salts are also physiologically monitored and can affect trajectory of feeding behavior in insects, mammals, and humans (Epstein, 1982; Shepherd et al., 1984; Simpson et al., 2006; Trumper and Simpson, 1993; Watanabe et al., 2000). However molecular mechanisms on how animals determine the value of certain nutrients in a context-dependent manner and change food preference are not well understood.

Process of context-dependent valuation of nutrients must integrate sensory perception of food and internal nutrient demand, making the underlying mechanisms of nutrient valuation a good candidate for a modulator of diet-dependent aging. Many reported cases of active nutrient balance both in the wild and in the laboratory settings. For example, predatory spiders are known to select their preys depending on predictive nutrient composition (Mayntz et al., 2005), spider monkeys tightly regulate daily protein intake in the wild (Felton et al., 2009), and laboratory mice balance their macronutrient intake differently under influence of a drug (Shor-Posner et al., 1986). Extensive studies have been done both in insects and rodents to understand neural bases for protein to carbohydrate balance because these two macronutrients seem to form the largest bases of all biological activities (Cohen, 2001; LeBlanc and Thibault, 2003; Raubenheimer and Simpson, 2003; Thibault and Booth, 1999). Based on pharmacological

studies, serotonin, a neurotransmitter involved in a wide range of physiology, is believed to be important for carbohydrate satiety and possibly influence protein or lipid feeding (Anonymous, 1992; Johnston, 1992; LeBlanc and Thibault, 2003; Leibowitz et al., 1993; 1989; Magalhães et al., 2010). However our understanding on which part of the meal selection process serotonin is involved and how it is emanating its effect on behavior is rudimentary. A probable place of involvement is during integration of sensory perception of nutrients and evaluation of nutrients' value against animals' internal demand. State-dependent valuation and how it drives animals' behavior had been documented in both invertebrates and vertebrates (Pompilio et al., 2006; Tindell et al., 2006). Indeed, a neural mechanism that modulates context-dependent value of sugars has been established especially in fruit flies' oviposition preference (Yang et al., 2008; 2015). Therefore, it is likely that food preference behavior also includes a similar context-dependent signal processing. Because the valuation process determines the trajectory of future feeding behavior, it is compelling to consider a role it can play on organismal health and diet-dependent aging.

Outstanding Questions

The following unanswered questions became the fundamental motivation for this dissertation: How does internal nutrient state of organisms alter feeding behavior? What are the mechanisms for meal selection? Does a process of nutrient evaluation have profound effects on health and longevity? Addressing these questions comprise the subsequent chapters of this dissertation. First, my desire to study detailed feeding behavior prompted me to invent a new feeding monitor device, as described in Chapter 3; which is a significant advancement in assaying flies' feeding compared with conventional methods. Second, using the newly developed feeding monitor, I was able to capture transient protein-dependent feeding behavior in female flies and this became the bases for dissecting neural mechanisms of protein feeding behavior, as described in Chapter 4. Lastly, I answered the question of what role does nutrient perception play in determining major behavior trajectory such as choosing to sleep or eat? Chapter 5 describes how sweet-sensing receptors and subsequent processing of sweet taste through dopaminergic circuits modulate animals' decision to sleep or forage. In sum, my thesis work brings mechanistic insights into how nutrients interact with nutrient states of animals and how neurological systems are involved in such interactions. Moreover my study suggests that the neurological systems that integrate internal and external nutrient signals can modulate organismal health and longevity, which can foster new avenue of clinical research to target neurological regulation of nutrient intake or to reduce progression of aging through how we interact with foods.

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Chapter 2

Measurement of Lifespan in *Drosophila melanogaster*[1]

Abstract

Aging is a phenomenon that results in steady physiological deterioration in nearly all organisms in which it has been examined, leading to reduced physical performance and increased risk of disease. Individual aging is manifest at the population level as an increase in age-dependent mortality, which is often measured in the laboratory by observing lifespan in large cohorts of age-matched individuals. Experiments that seek to quantify the extent to which genetic or environmental manipulations impact lifespan in simple model organisms have been remarkably successful for understanding the aspects of aging that are conserved across taxa and for inspiring new strategies for extending lifespan and preventing age-associated disease in mammals. The vinegar fly, Drosophila melanogaster, is an attractive model organism for studying the mechanisms of aging due to its relatively short lifespan, convenient husbandry, and facile genetics. However, demographic measures of aging, including age-specific survival and mortality, are extraordinarily susceptible to even minor variations in experimental design and environment, and the maintenance of strict laboratory practices for the duration of aging experiments is required. These considerations, together with the need to practice careful control of genetic background, are essential for generating robust measurements. Indeed, there are many notable controversies surrounding inference from longevity experiments in yeast, worms, flies and mice that have been traced to environmental or genetic artifacts (Bokov et al., 2011; Burnett et al., 2011; Spencer et al., 2003; Toivonen et al., 2007). In this protocol, we describe a set of procedures that have been optimized over many years of measuring longevity in Drosophila using laboratory vials. We also describe the use of the dLife software, which was developed by our laboratory and is available for download (http://sitemaker.umich.edu/pletcherlab/software). dLife accelerates throughput and promotes good practices by incorporating optimal experimental design, simplifying fly handling and data collection, and standardizing data analysis. We will also discuss the many potential pitfalls in the design, collection, and interpretation of lifespan data, and we provide steps to avoid these dangers.

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Protocol[1]

We recommend storing experimental foods, yeast paste, and grape agar plates that appear in the protocol at 4 °C and using them within 1-2 months as long as mold and dryness have not set in. Standard environmental conditions for both the larval and adult stage involve maintenance of flies in an incubator at 25 °C with a 12:12 hr light dark cycle and 60% relative humidity.

1. Preparation of Experimental Food

   A. For larval growth, we use a modified Caltech Medium(Lewis, 1960), which is abbreviated in this protocol as CT.

   B. We recommend a diet for adult Drosophila (SY) that consists of sugar (sucrose) and yeast (lyophilized whole brewer's yeast) in a 2% agar base, that has been boiled, supplemented with antibiotics and anti-fungal agents, and distributed (10 ml per vial) (Skorupa et al., 2008). Food should be allowed to solidify and evaporate for 12-24 hr prior to storage. Because the nutrient environment can substantially impact longevity, consistency in cooking processes is essential both within an experiment and for comparison between experiments.

   C. If a pharmacological agent is to be added to the adult food, this drug can be mixed into a small batch of food and layered (2 ml) on the food surface, with control vials receiving layers containing vehicle alone.

2. Preparation of a Live Yeast Paste

   A. Combine 5-6 ml of water with 3 g of active dry yeast and mix well. The consistency of the yeast paste should be that of a smooth peanut butter.

3. Preparation of a Grape Agar Plate

   A. Add a packet of a grape agar premix in 500 ml of distilled water in a 1,000 ml flask and follow the instructions on the packet to dissolve the grape agar mix.

   B. Carefully pour a thick layer of the mixture into 100 mm Petri dishes while avoiding bubble formation. One packet of premix produces approximately 14 grape agar plates.

   C. Let the media cool and solidify in room temperature with lids on for 15 min. Plates can be kept at 4 °C, wrapped in plastic wrap, or used immediately. Collection of Synchronized Eggs

[1] Associated video of the protocol can be found at http://www.jove.com/video/50068/measurement-of-lifespan-in-drosophila-melanogaster

4. Collection of Synchronized Eggs

All media used in this protocol, i.e. yeast, grape agar plates, CT and 10% SY food, should be at room temperature.

A. Spread a 2-3 cm diameter layer of yeast paste on a grape agar plate and set aside.

B. Place a large egg collection cage on a CO2 pad with the mesh-side down to anesthetize the flies Nitrogen-based anesthesia is an alternative to CO2, used by some laboratories, that can produce high quality results (Rera et al., 2011).

C. Using a funnel, transfer 150-200 pairs of flies into the egg collection cage.

D. Place the grape agar plate to cover the open end of the cage and secure with an end cap.

E. Lay the cage on its side until flies wake up. Then, keep the cage, grape agar plate side down, in the incubator overnight.

F. On the next day, swap the grape plate with a newly yeasted grape plate. Only put about 1 cm diameter of yeast paste onto the surface of the plate. Discard the 1st day grape plate.

G. Allow embryos to collect on the surface of the grape agar plate for 16-22 hr. Once done, collect the grape agar plate and discard the parent flies.

H. Wash the surface of the grape agar plates with 1x Phosphate Buffered Saline (PBS). Eggs can be mobilized by gently scraping with a cotton swab. Take care not to scratch, damage, or scrape thin pieces of agar off the surface of the plate. With the aid of a funnel, pour the washed eggs into a 15 ml conical tube.

I. Let the eggs settle to the bottom of the tube and pour off the supernatant, being careful not to lose any eggs. The remaining volume should be around 2-3 ml.

J. Add 8-10 ml of PBS to the tube and repeat the above step 2-3 more times to thoroughly wash the eggs until the supernatant is clear. Getting rid of residual yeast is the key in this step.

K. After the wash, drain all supernatant until remaining volume is 2 ml. Aliquot 32 µl of eggs into CT bottles using a wide-bore pipette tip Eggs in the pipette tip should be compact, with little to no liquid aspirated. This can be achieved by inserting the pipette tip deep into the egg settlement and quickly releasing the plunger to aspirate.

L. Place seeded CT bottles back in the incubator throughout fly development.

5. Collection of Age-matched Adult Flies

Adults will typically eclose from day 9 onward. Discard the flies that emerged on the first day and place bottles back in the incubator overnight. This practice will avoid inadvertent selection for early emergents and allow for the collection of a maximum number of synchronized flies.

A. 16-22 hr later, transfer the day-old adult flies into 10% SY food bottles. If needed, another batch can be collected the next day.

B. Return flies back into the incubator and allow flies to reach sexual maturity and mate for two days. Record the day of transfer to 10% SY bottles as the first day of adulthood.

6. Sorting Flies and Setting up Longevity Experiment

A. Anesthetize small groups of flies on the anesthetic pad, then sort males and females into two groups using a paintbrush. If using CO2, it is critical to minimize exposure to prevent possible long-lasting health issues that can compromise the integrity of the longevity experiment.

B. Place 30 flies of the same gender into individual vials. Assuming no balancer chromosomes in the population and healthy progeny, each bottle should produce around 3-4 vials of each sex. Aliquoting flies into individual vials should take no more than 3-4 min, so that, in total, flies are only exposed to anesthesia for a maximum of 9-10 min.

C. Repeat steps 6.1-6.2 until there are 8-10 vial replicates for each gender and experimental treatment. Setting Up the Excel spreadsheet to Track the Longevity Experiment

7. Setting Up the Excel spreadsheet to Track the Longevity Experiment

A. We recommend randomizing vial position rather than grouping vials by experimental condition in order to avoid bias associated with the vial location in the incubator and to obscure the vial identity to the experimenter. To do this, first assign a randomized numerical ID to each vial in a spreadsheet program, then arrange the vials in trays by ID number. If you are using the dLife experiment management software, follow the tutorial on experiment setup to generate the ID number for each vial.

B. (Optional) If you are using an RFID reader or barcode reader in association with dLife, attach an RFID or barcode tag to each vial and associate the tag with the vial's numerical ID in dLife. The program will recognize each vial when scanned with a reader and guide one to record the data in the correct location in a spreadsheet. Usage of a tag reader in conjunction with dLife significantly reduces data collection time and recording error.

8. Maintaining the Longevity Experiment

The vials containing fresh food should be at room temperature for each transfer.

A. During the experimental period, transfer flies onto new vials containing fresh food every 2 days (young females), or 3 times a week (males or females >3 weeks of age). This step will ensure that the feeding environment for young females is not disrupted by the presence of larvae. This transfer should be completed without anesthesia, which can induce acute mortality, particularly in older flies (Pletcher, personal observations).

B. During each vial transfer, record the age, count the dead flies in the old vial, and the dead flies that are carried to the new vial. Record this information separately in two columns in a spreadsheet (either dLife or your own spreadsheet). This will ensure that the carried flies are not double-counted. The total number of deaths (dead + carried) should at least equal the number of carried flies from the previous transfer. Subtract the number of previously carried flies from the total number of deaths to determine the number of new deaths.

C. A fly is considered right-censored if it left the experiment prior to natural death through escape or accidental death. Animals exiting the experiment in this way should be entered into a separate column on the day that the fly exited the experiment. Censored flies are not recorded as dead (see below).

D. Continue to repeat steps 8.1-8.2 until the last survivor is dead. Be aware that as the flies age, some flies may lie on their back and appear dead due to their inactiveness. Therefore when counting carried (dead) flies, tap on the side of the vials to determine if there are leg movements. If so, these flies are still alive. In the case where flies remain stuck to the food in the old vial but alive, they should not be counted as dead and should be rescued by further tapping of the vial to dislodge the fly. Censoring such flies should be used with caution as it may result in experimental bias.

9. Data Analysis

A. The survivorship curve displays the probability that an individual survives to a given age and is typically calculated using a Kaplan-Meier approach (Figure 2.1) (Kaplan and Meier, 1992) In the absence of right-censored data, the formula can be simplified such that age-specific survivorship at age x (Sx) is determined by dividing the number of individuals alive at the start of a census time at age x (Nx) by the total number of flies in the experiment (N0); Sx=Nx/N0. 9.2) Survivorship curves are the most common form of data presentation, and they can be tested for equality between groups using a log-rank

test Reasonable inference can be drawn from as few as 50-100 individuals in a cohort. Survivorship is a cumulative measure, however, and therefore deaths that are not aging-related, such as those early in life, will depress survivorship throughout the lifespan making it difficult to ascertain effects specific to aging.

B. A second data visualization method is the age-specific mortality function, which displays the risk of dying through each age interval and presents a more nuanced description of survival data (Pletcher et al., 2000; Pletcher, 2002). Mortality measures are independent from one age to another, and the shape of the mortality curve is useful for inference about the dynamics of aging, particularly when treatments are adjusted during adult life. Estimates of age-specific mortality, however, lack both precision and accuracy with small sample sizes, and often require several-to-many hundreds of individuals per cohort for a reliable estimate (Promislow et al., 1999).

C. Other methods for estimating longevity differences include parametric (e.g. Gompertz) and semi-parametric (e.g. Cox regression) models. These models can be powerful but should be applied with caution because of the assumptions made about the shape of the mortality curves and the nature of treatment effects, which could lead to incorrect inference (Pletcher, 1999; Promislow et al., 1999).

Representative Results

A simplified scheme of the protocol is presented in Figure. 2.1, where key steps are outlined. The synchronization part of the protocol can be used for various assays that require age-matched adult flies.

Typical survivorship curves of wild-type flies are shown in Figure. 2a, using the dLife experiment management software (Figure. 2.2 b,c). Adult males usually live shorter, with both populations achieving a mean and median longevity of >50 days on a 10% SY food at 25 °C. Note that survivorship remains high in the early part of the experiment and then declines exponentially.

Drosophila lifespan is affected by environmental conditions, such as temperature and diet. Figure. 2.3a shows that adult males typically live markedly shorter as temperature is increased. Likewise, the effect of diet on lifespan is presented in Figure. 2.3b: Adult female flies on a less concentrated diet (5% SY) typically live significantly longer than those on a more concentrated diet (15% SY).

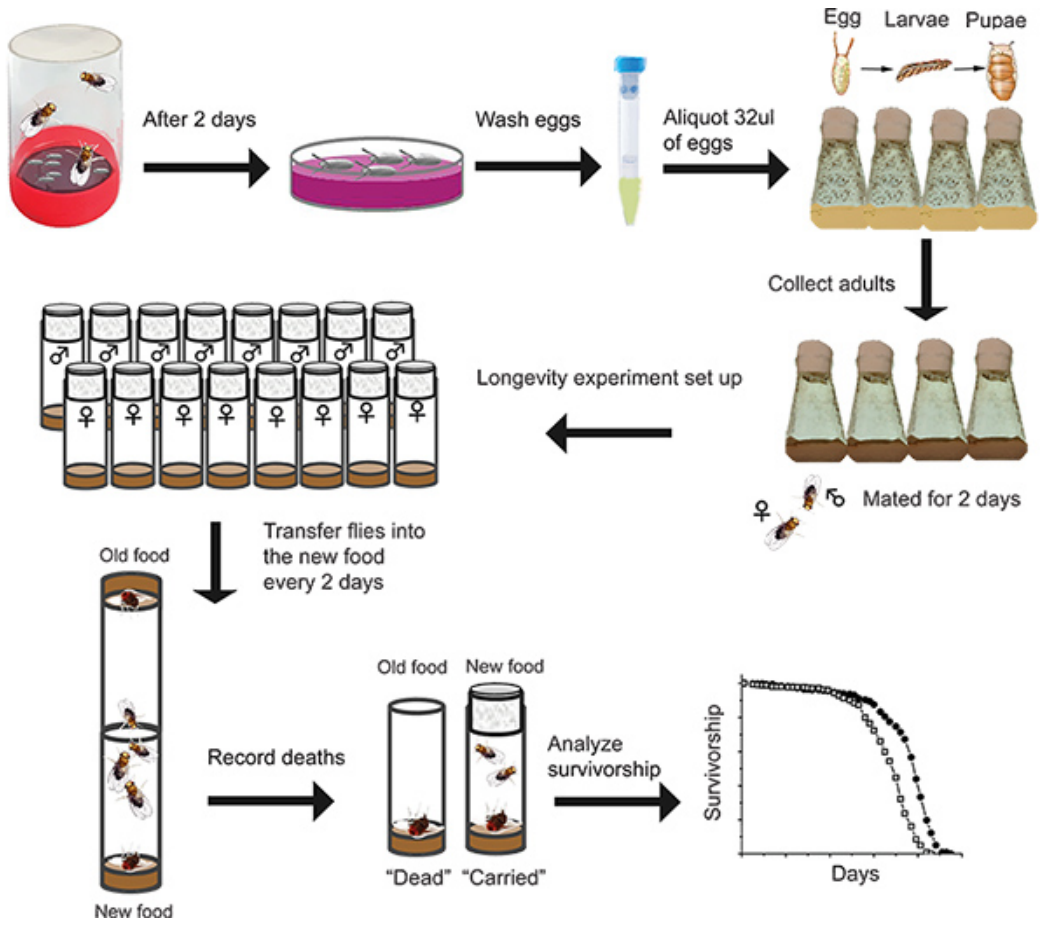

Figure. 2.1 Simplified schematic of a Drosophila lifespan Assay.

Density of cohorts during development can influence adult lifespan and alter developmental timing. Here we show an example of how different densities of synchronized eggs affect larval development. As shown in Figure 2.4, adult fly yield is poor and the food surface is susceptible to drying when the number of eggs is too low. On the other end of the spectrum, larval development is retarded in over-crowded bottles, and the yield of adult flies is reduced. The survivorship curve of the cohort as a whole can be influenced significantly by anomalous vial effects, as shown in Figure 2.5. Irregular survival data for individual vials may have several causes, such as poor food quality or bacterial/fungal accumulation and infection. While such anomalous deaths can skew the population survivorship measure, there is not a straightforward metric for appropriately determining that a vial should be excluded from the experiment. These situations are therefore best avoided by good handling practices and mitigated by using a large sample size. Figure 2.6 shows examples of vial conditions that can lead to anomalous deaths. In general, any condition that can lead to small crevasses where flies may get stuck and die should be avoided. Examples include: bubbles in the food, dryness in the food that leads to shrinking

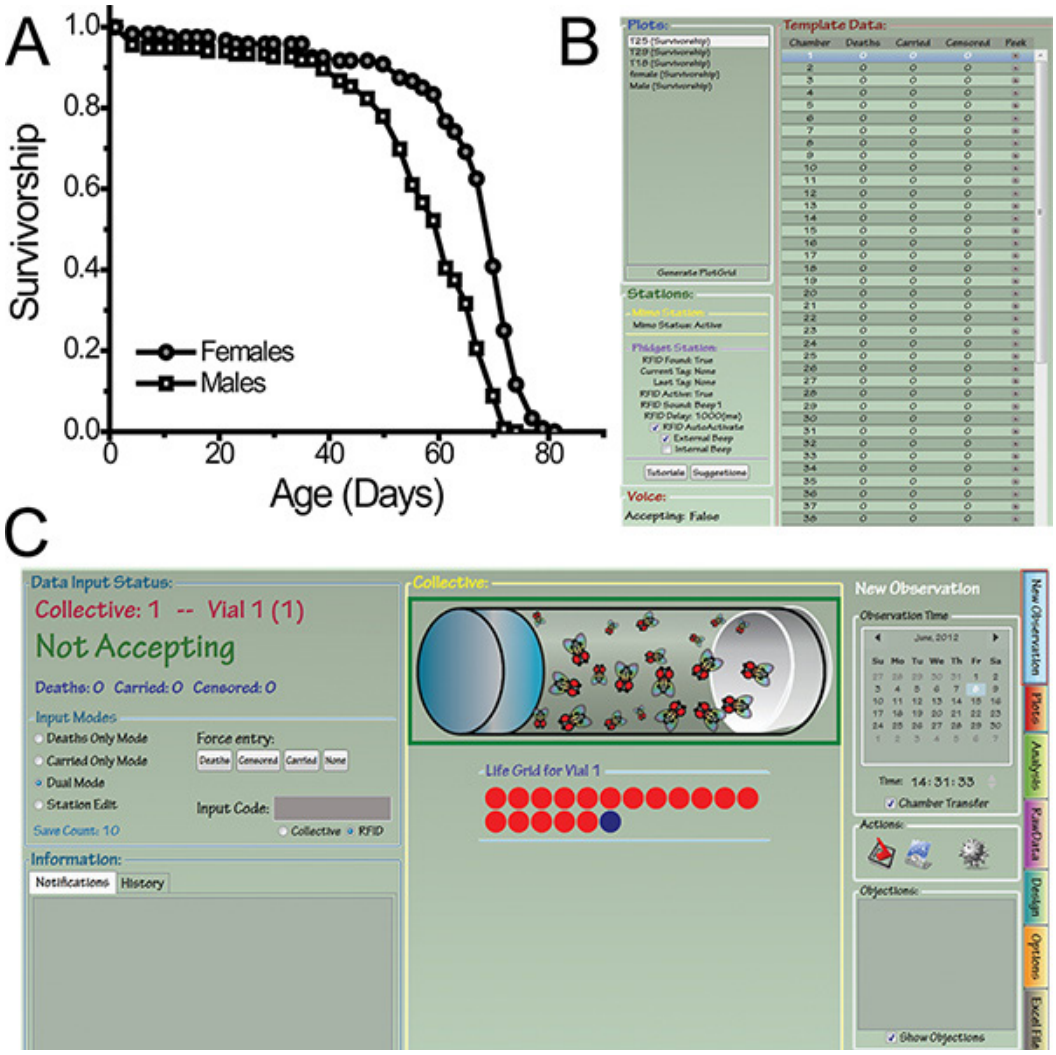

Figure. 2.2 Representative fly survivorship curve in dLife software interace
(A) Representative lifespan curves of w^{1118} control female (circles) and male (squares) adult flies at 25°C on a SY10% food. (B,C) Representative screen shots of the dLife software.

away from the vial wall, and cracking in the food are shown in Figure 2.6a (a mild example with a single bubble), Figure 2.6b, and Figure 2.6c, respectively. Excessively dry food, as shown in Figure 2.6b and Figure 2.6c should be carefully flipped during transfers, as the food can dislodge and collapse onto the flies in the new vial. Bacterial growth on the surface of the food can lead to infection or physical entrapment and thus may increase mortality. Some bacteria on the surface of the food appear transparent and shiny as if there is perspiration from the food (not shown), while other types of bacteria will manifest as white colonies (Figure 2.6d). Vials exhibiting any of these conditions should be noted and further considered when the data are interpreted. In general, we emphasize that careful attention to husbandry in both the larval and adult stages can support the longevity and health of adult flies and reduce the occurrence of

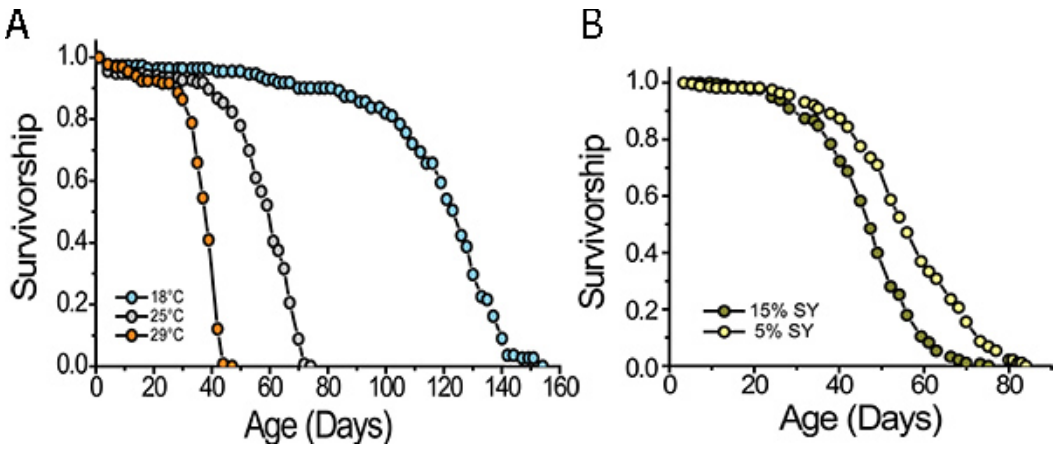

Figure. 2.3 Effects of temperature and diet on adult lifespan.
(A) Adult control (Canton S.) male flies Ire maintained through adulthood at 18°C, 25°C, or 29°C. (B) Adult control (w^{1118}) female flies Ire exposed to either a 15% SY or 5% SY diet.

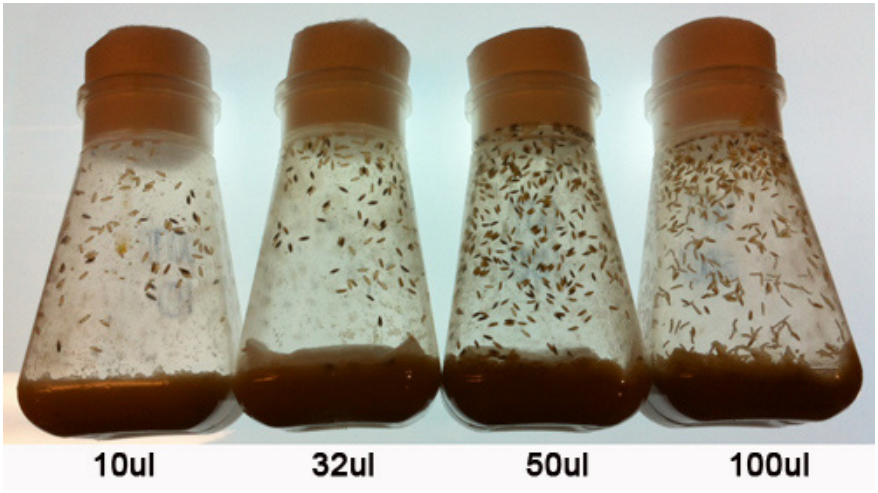

Figure. 2.4. Day 9 of development (at 25°C) of synchronized eggs, aliquoted in CT food bottles.
Volume of the embryo-containing aliquot is as shown beneath each bottle.

problems leading to ambiguous causes of death in later life.

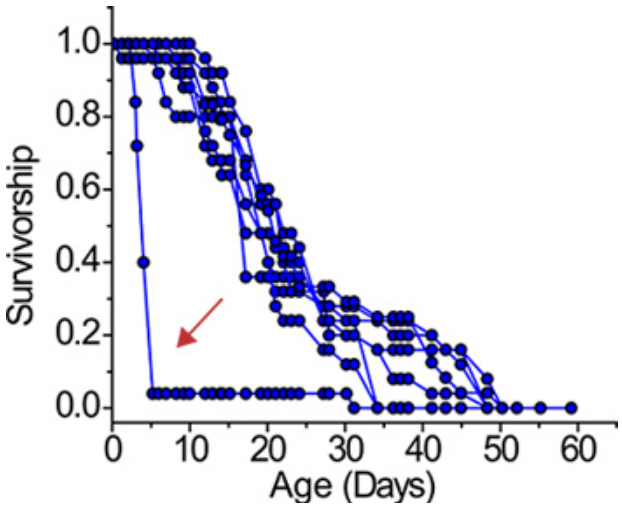

Figure. 2.5. Representative plot from the dLife software showing survivorship by vial.
The arrow indicates a single anomalous vial within a group.

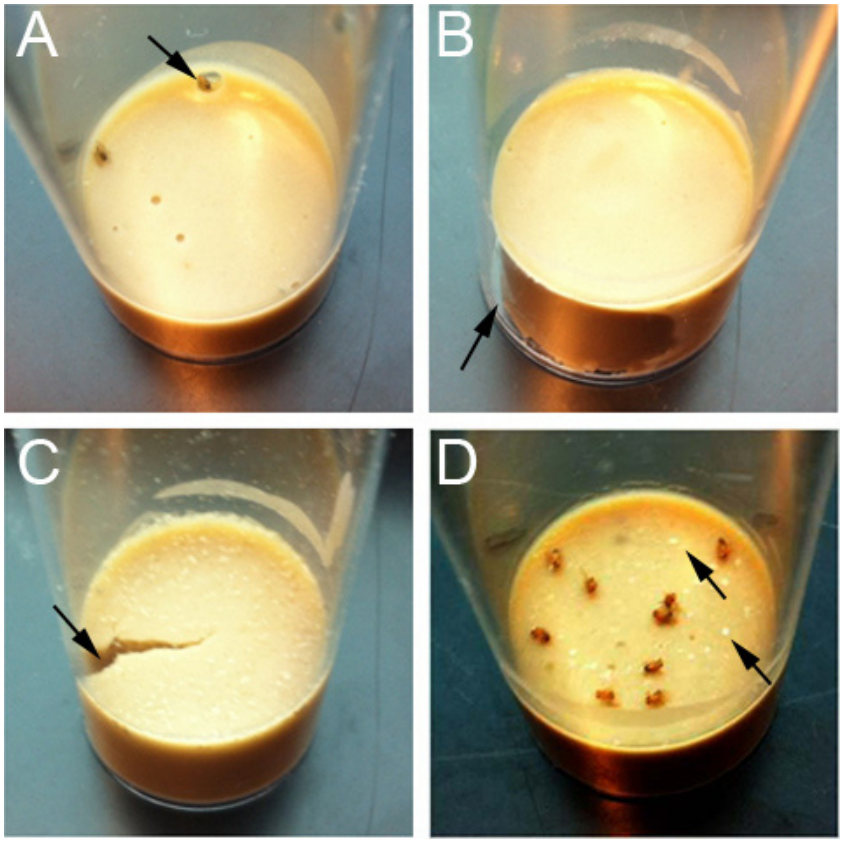

Figure. 2.6. Examples of suboptimal food quality.
(A) Bubbles on the food surface. (B) Food shrunk away from the edge of the vial. (C) Cracks of the food. (D) Bacteria accumulation of the surface of the food.

Discussion

The protocol presented here describes a method for producing reproducible measurements of adult longevity in Drosophila that is adaptable for assessment of genetic, pharmacological, and environmental interventions. Crucial aspects of the protocol include carefully controlling the larval development environment, minimizing adult stress, and minimizing bias across experimental groups and controls. We also present the use of the dLife lifespan experiment management software. By simply attaching a bar code or RFID tag to each vial, the dLife program will assist in data acquisition for each measurement and in plotting the survivorship curve. While it is currently most suitable for studies of fly lifespan using vials, this experiment management tool could be easily adapted for use in other organisms, with different types of population chambers, or for additional measures of survival, including stress resistance and drug toxicity.

Prior to commencing any longevity assessment, one must first carefully control the production of parental stocks. Genetic variability is an important factor, as is the health of parental strains. These factors have led to considerable public controversy associated with early studies that reported putative longevity regulators (Partridge and Gems, 2007). The researcher has several options to minimize effects attributable to variability in genetic background. For genetic manipulations, all genetically altered strains should be background-controlled through either backcrossing to a control strain (at least 6 times) or using inducible systems (e.g., temperature sensitive alleles or drug-inducible transgene expression). The GeneSwitch and Tet-on systems (Ford et al., 2007; Roman et al., 2001) are popular and allow direct comparison of flies with the same genetic background in which the transgene is either induced or not induced. For all inducible systems, appropriate contemporaneous controls are required to avoid confounds associated with the inducer. Failure to control for genetic background nearly always results in hybrid vigor, which can extend lifespan in the F1 generation of a cross between two different strains for reasons unrelated to the intended manipulation (Partridge and Gems, 2007). This factor is of particular concern when using the GAL4-UAS system. For environmental interventions (e.g., drug treatment, diet, temperature, etc.), it is advisable to study the effects of the intervention on more than one strain. Finally, both parental age(Priest et al., 2002) and stress (Smith et al., 2007) can influence the longevity of the F1 generation, and for this reason, healthy young adults should be chosen for egg production.

Important aspects of controlling the larval/pupal environment include the prevention of overcrowding and the maintenance of a controlled environment with strict regulation of light-dark periods, humidity, and temperature. These factors will affect both the timing of development and the physical quality of the resulting adults. Adverse larval environments, such

as high larval density, may lead to activation of stress-inducible factors (e.g., heat shock protein expression) that are known to influence adult longevity (Sørensen and Loeschcke, 2001).

During the adult phase, careful attention to the environment remains essential. The choice of diet alone can greatly influence lifespan and can interact with genetic factors to produce diet-specific effects on longevity. Furthermore, some common food base alternatives (yeast extract instead of lyophilized whole brewer's yeast) can dramatically shorten lifespan (Bass et al., 2007), leaving open the possibility that the food itself is causing organismal stress that may impair the assessment of longevity. In this article, we have highlighted potential sources of stress in the dietary environment including physical anomalies in the food (e.g., bubbles, foam, cracks, bacteria, etc.) that can physically entrap the animals. Regular replacement of old food with fresh food (at least three times each week) can overcome many of these difficulties. Furthermore, temperature(Miquel et al., 1976), humidity (personal observations), lighting (Pittendrigh and Minis, 1972), and the presence of conspecifics (social environment)(Joshi and Mueller, 1997) can all modulate lifespan, and attention to controlling these factors within the experiment is important to avoid bias in the results. While the number of flies within a vial decreases with time, we have found that the use of anesthesia to adjust the number of flies can increase mortality in an age-dependent manner (Pletcher, personal observations), and we do not recommended this procedure. The precise position of the vials in an incubator is also a factor, even in a seemingly controlled environment. Randomized physical distribution of experimental groups with controls can mitigate bias associated with vial placement and is required for proper statistical inference. Even with carefully controlled conditions, slight differences can be observed between experiments and the use of within-experiment controls is essential.

Alternative feeding approaches have been proposed for lifespan analysis including a capillary feeder approach (CAFE method) (Ja et al., 2007). This method excels in the ability to provide precise measurements of food consumption, but it results in markedly short-lived flies (Lee et al., 2008). Potential stresses associated with the feeding environment must be considered when assessing the combined relationship between diet and genetic factors in overall longevity.

Demographic analysis, including the calculation of survivorship and mortality curves can reveal much information about the dynamics of the aging population. A typical survivorship curve will remain relatively flat for a long period early in life and increase its rate of decline at older ages, which corresponds to a period of low mortality followed by a period of an exponential increase in mortality. A stressful environment will usually manifest as an excess of early deaths in the population and an anomalous dip in the survivorship curve. While such a result may indicate significant differences between treatments, it will not normally be robust to replication. We therefore recommend at least two independent (i.e., non-contemporaneous) replicate

experiments be executed before any firm conclusion are drawn. It may be that additional effort toward increasing the sample size, controlling the husbandry conditions, and improving the health of parental stocks is required.

Right-censoring (removing animals from the experiment that escape or are presumed dead from accidental causes) of animals that die from stressful environmental conditions should be applied with extreme caution. Strictly speaking, censoring must occur randomly across experimental treatments, and if the experimental intervention modulates stress sensitivity, one could inadvertently apply treatment-level selection to the population. As a general rule, avoiding the presence of factors that could produce ambiguous early death (primarily associated with the food source) is better than censoring, and censoring should only be applied to organisms that were observed to die or escape during physical handling.

A final consideration is the assessment of statistical significance. While large cohort sample sizes provide impressive power to distinguish small differences between treatments, the potential biological significance of such a difference must also be considered. With reasonably sized longevity experiments, differences as low as 1-2% are often highly statistically significant, but the overall impact of the intervention on health status may be minor. Therefore, both statistical and biological significance must be considered when interpreting the overall results of the experiment. Inference about the aging process from survival experiments can be augmented by measures of age-related declines in behavioral or physiological health measures, including climbing ability(Gargano et al., 2005) and gastrointestinal wall integrity (Rera et al., 2011).

In summary, the Drosophila model organism an appealing choice for studying mechanisms of aging. With careful experimental technique, robust demographic analysis can provide insight into the impact of pharmacological and genetic factors on the aging process.

Materials

Name	Company	Catalog Number	Comments
Active Dry Yeast	Fleishmann's Yeast	2192	
Grape Agar Powder Premix	Genesee Scientific	47-102	
Large Embryo Collection Cages	Genesee Scientific	59-101	
Large Replacement End Caps	Genesee Scientific	59-103	

6 oz Square Bottom Bottles, polypropylene	Genesee Scientific	32-130	
Flugs Closures for Stock Bottles	Genesee Scientific	49-100	
Drosophila Vials, Wide, Polystrene	Genesee Scientific	32-117	
Flugs Closures for Wide Vials	Genesee Scientific	49-101	
Wide Orifice Aardvark Pipet Tips, 200 ul	Denville Scientific	P1105-CP	
Flystuff Flypad, Standard Size	Genesee Scientific	59-114	
BD Falcon 15 ml Conical Centrifuge Tubes	Fisher Scientific	14-959-70C	
Fisherbrand Petri Dishes with Clear Lids, Raised Ridge; 100 O.D. x 15 mm H;	Fisher Scientific	08-757-12	
Kimax* Colorware Flasks 1,000 ml yellow	Fisher Scientific	10-200-47	
PBS pH 7.4 10x	Invitrogen	70011044	
Gelidium Agar	Mooragar	n/a	
BreIr's Yeast	MP Biomedicals	0290331280	
Granulated Sugar	Kroger	n/a	
Tegosept	Genesee Scientific	20-266	Fly Food Preservative
Propionic Acid, 99%	Acros Organics	149300025	Fly Food Preservative
Kanamycin Sulfate	ISC BioExpress	0408-10G	
Tetracycline HCl	VWR	80058-724	

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Chapter 3

FLIC: High-throughput, Continuous Analysis of Feeding Behaviors in *Drosphila*[1]

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(Venken et al., 2011). 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

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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 (Carvalho et al., 2005; Geer et al., 1970; Min and Tatar, 2006; Wong et al., 2008) and preference assays lack qualities appropriate for high-throughput analysis (Itskov and Ribeiro, 2013). 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 (Gordesky-Gold et al., 2008; Skorupa et al., 2008). Tracer methods are most effective when exposure periods are short, otherwise they report gut size rather than feeding rate (Wong et al., 2008). 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 (Ja et al., 2007). 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(Mair et al., 2005; Wong et al., 2009), 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 two 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 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.

Materials and Methods

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 (Dahanukar et al., 2001). 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 (Hardin et al., 1990).

FLIC system details

The FLIC system is comprised of four components (Figure 3.1a). 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 3.1a; Supplemental Figure 3.1). 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 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 (e.g., Figure 3.1a) 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 provided by the authors.

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 (Devineni and Heberlein, 2009). 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 Kaplain-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 (Fig. S2a; Fig. S2b). 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 (Pfeiffenberger et al., 2010). 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

Automated Feeding Behavior Data

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 3.1b). 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 3.1b, 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 3.1c). 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 3.1d). Among individuals, the average duration of an event in our experiment was 1.5sec, while the average time between events was 11.3min (Figure 3.1e). Finally, the average wait time from a randomized point between 12pm-2pm until the next interaction with the food was quite long (197min; Figure 3.1f), although it should be noted that this analysis spanned the time of day when feeding behavior is thought to be least frequent (see below).

The FLIC system vs. standard methods

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 3.2a).

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 (Devineni and Heberlein, 2009) .When identical foods were placed in both wells the average PI among male and female flies was near zero, indicating that there is no significant bias inherent in the FLIC design (Figure 3.2b). 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.

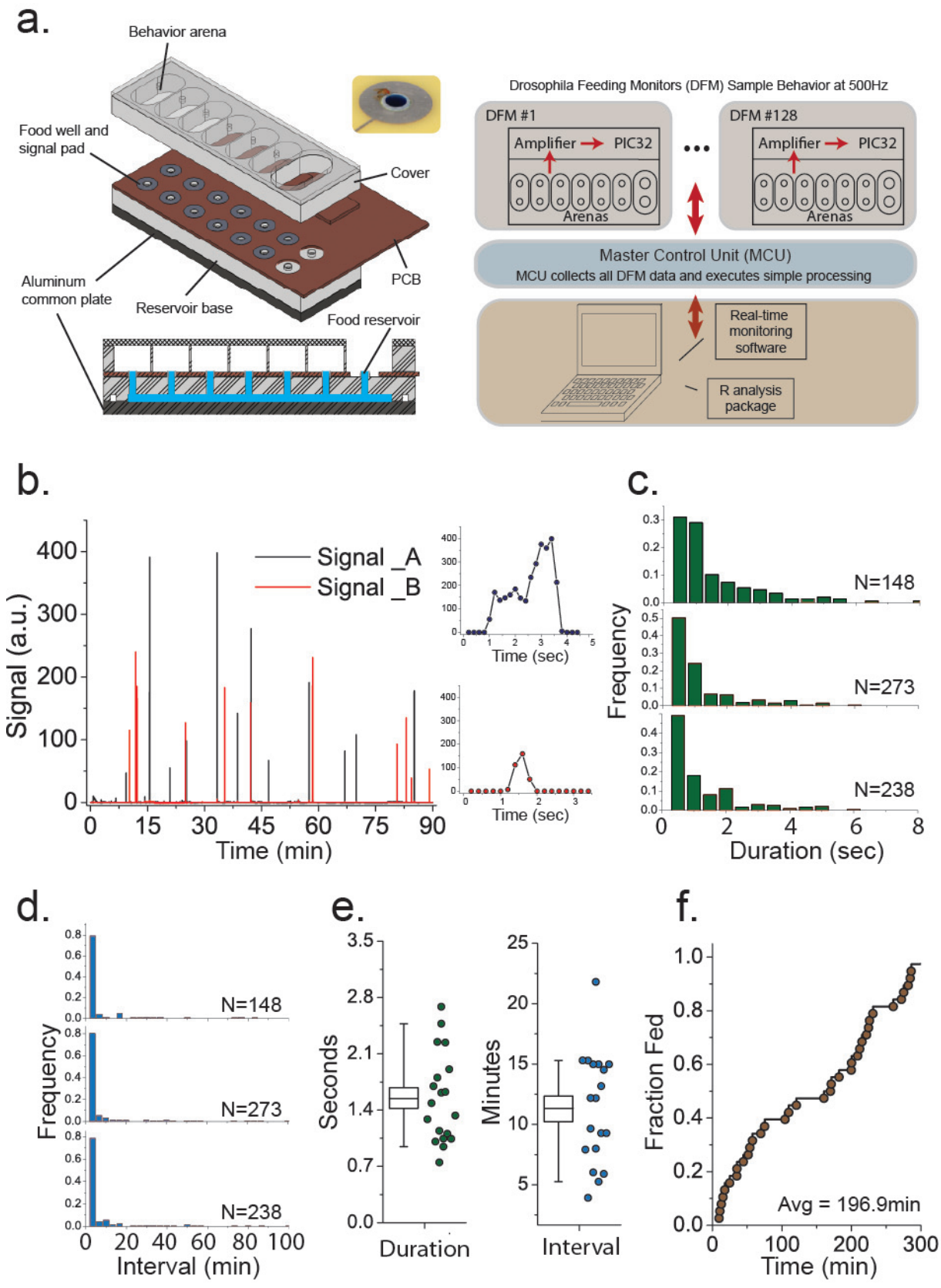

a.
Behavior arena
Food well and signal pad
Cover
Aluminum common plate
Reservoir base
PCB
Food reservoir
Drosophila Feeding Monitors (DFM) Sample Behavior at 500Hz
DFM #1
Amplifier → PIC32
Arenas
DFM #128
Amplifier → PIC32
Arenas
Master Control Unit (MCU)
MCU collects all DFM data and executes simple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

Figure 3.1. 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.

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 3.2c), 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 denatomium-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 (Gordesky-Gold et al., 2008). 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 3.2d).

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

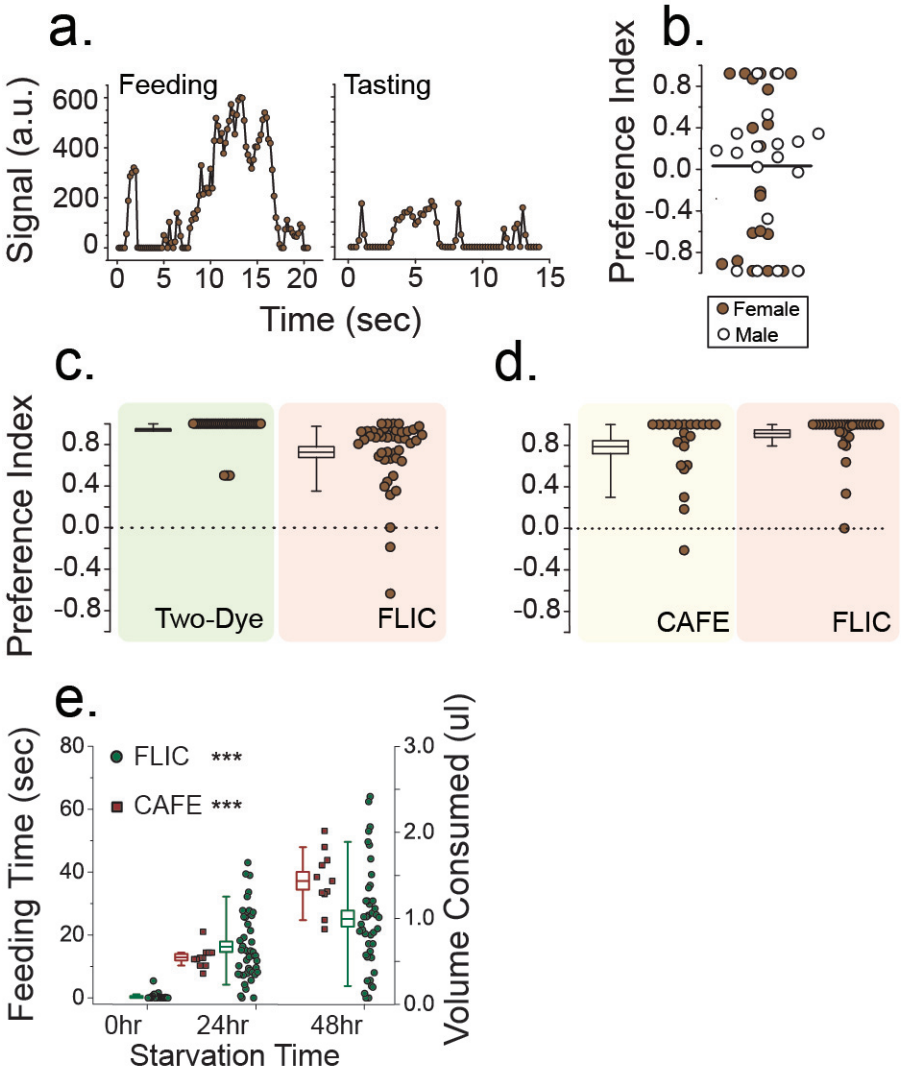


Figure 3.2. Comparison between traditional food choice assays and the FLIC system.
(A) The analog signals from feeding (left) and tasting (right) behaviors have distinct characteristics. (B) When presented with identical food in both food wells, male and female flies do not exhibit a preference, which 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≤0.05; **P≤0.01; ***P≤0.001.

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 3.2e; 0hr). Following 24hrs and 48hrs of starvation, a significant increase in feeding could be measured in both assays (Figure 3.2e), 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).

New dimensions of feeding behaviors

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 (Dethier, 1976). 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 3.3a). 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 3.3b). 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 (Jacobs and Sharma, 1969), a method that provides a continuous estimate of preference is desirable. In such cases it is possible to calculate a time--dependent preference index, 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 3.3c, left panel), the time-dependent PI measures revealed that preference for 10% sucrose changed during the experiment (Figure 3c, 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 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 3c, 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 3. 3d). 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 3.3e).

an 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 (Dus et al., 2011). 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 3.3f) (Dus et al., 2011). 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.

Circadian Feeding Behavior

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 (Supplemental Figure 3.2a,3.2b). 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 (e.g., Figure 3.4a). Indeed, 100% of the flies exhibited rhythmic feeding, with an average period of 23.3 hours and a combined power value of 64.6 ((Pfeiffenberger et al., 2010) and Methods and Materials). Much like general activity, feeding behaviors were concentrated near lights-on and lights-off (Figure 3.4b). The periodicity remained through constant darkness, although feeding appeared to coalesce around the subjective evening at the 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 3.4c).

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 3.4d, 3.4e). 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

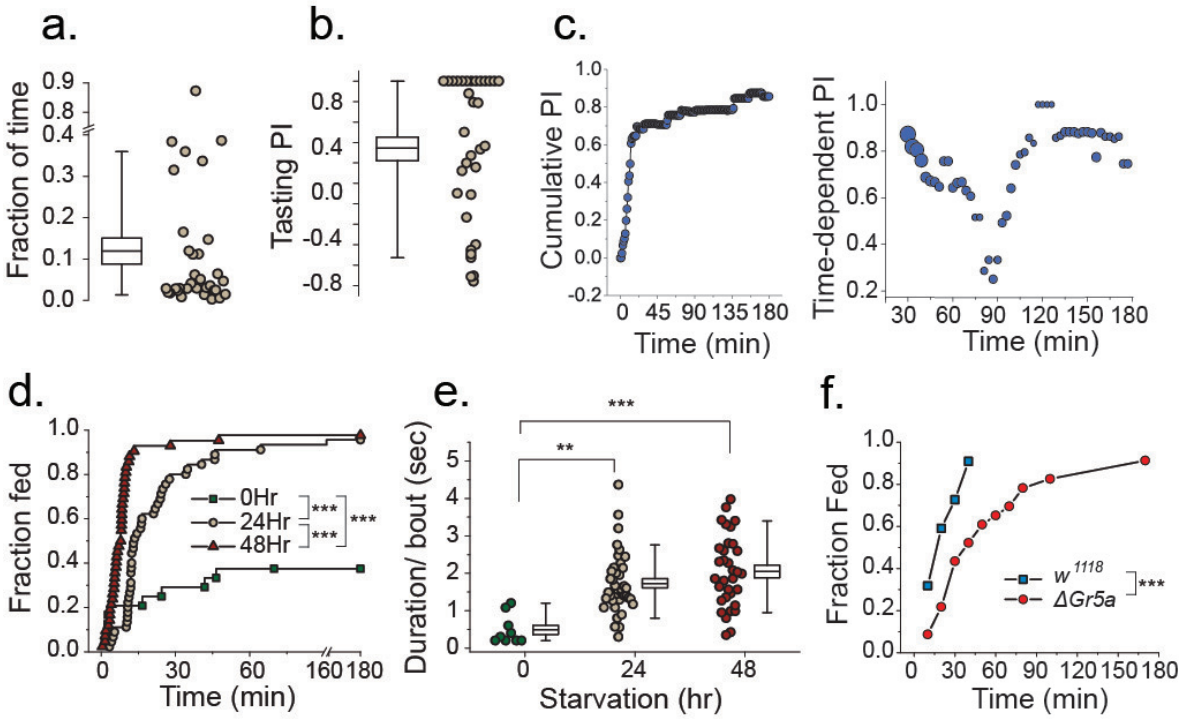


Figure 3.3. New types of behavioral inference from the FLIC system.

(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≤0.01; ***P≤0.001

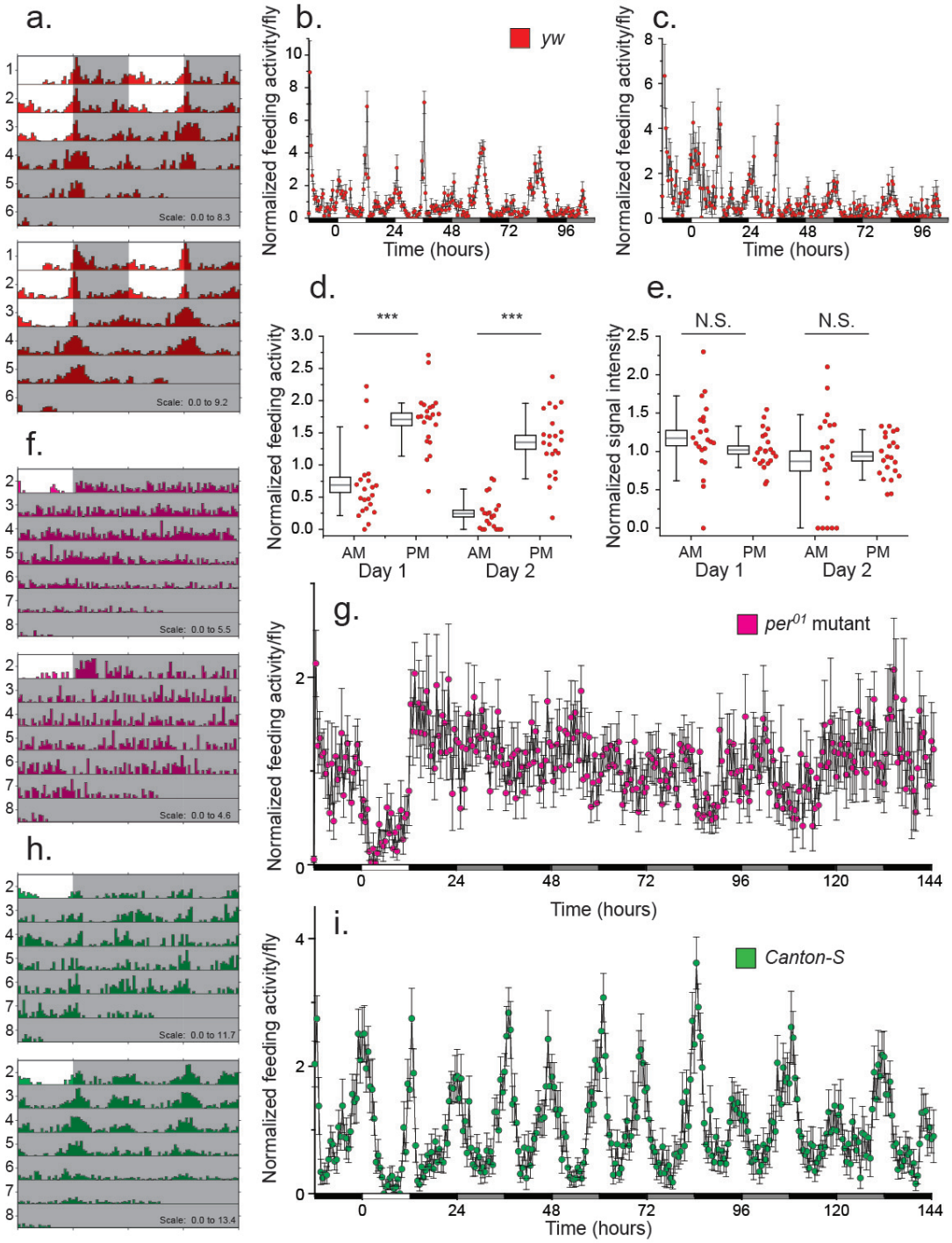

a.
Scale: 0.0 to 8.3
Scale: 0.0 to 9.2
b.
yw
Normalized feeding activity/fly
Time (hours)
c.
Normalized feeding activity/fly
Time (hours)
d.
***
***
Normalized feeding activity
AM
PM
AM
PM
Day 1
Day 2
e.
N.S.
N.S.
Normalized signal intensity
AM
PM
AM
PM
Day 1
Day 2
f.
Scale: 0.0 to 5.5
Scale: 0.0 to 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 3.4. 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≤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 Per01 mutant flies. (G) Averaged normalized feeding activity as a function of time for male Per01 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).

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 3.4f), and the population as a whole was highly arrhythmic (Figure 3.4g). 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 (e.g., Figure 3.4b); 100% exhibited rhythmic feeding with an average period of 23.4 hours (SEM = .042 h) and a power of 67.2 (Figure 3.4h, 3.4i) (Pfeiffenberger et al., 2010). Similar to *yw* males (e.g., Figure 3.4d), 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 (Supplemental Figure 3.3). 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

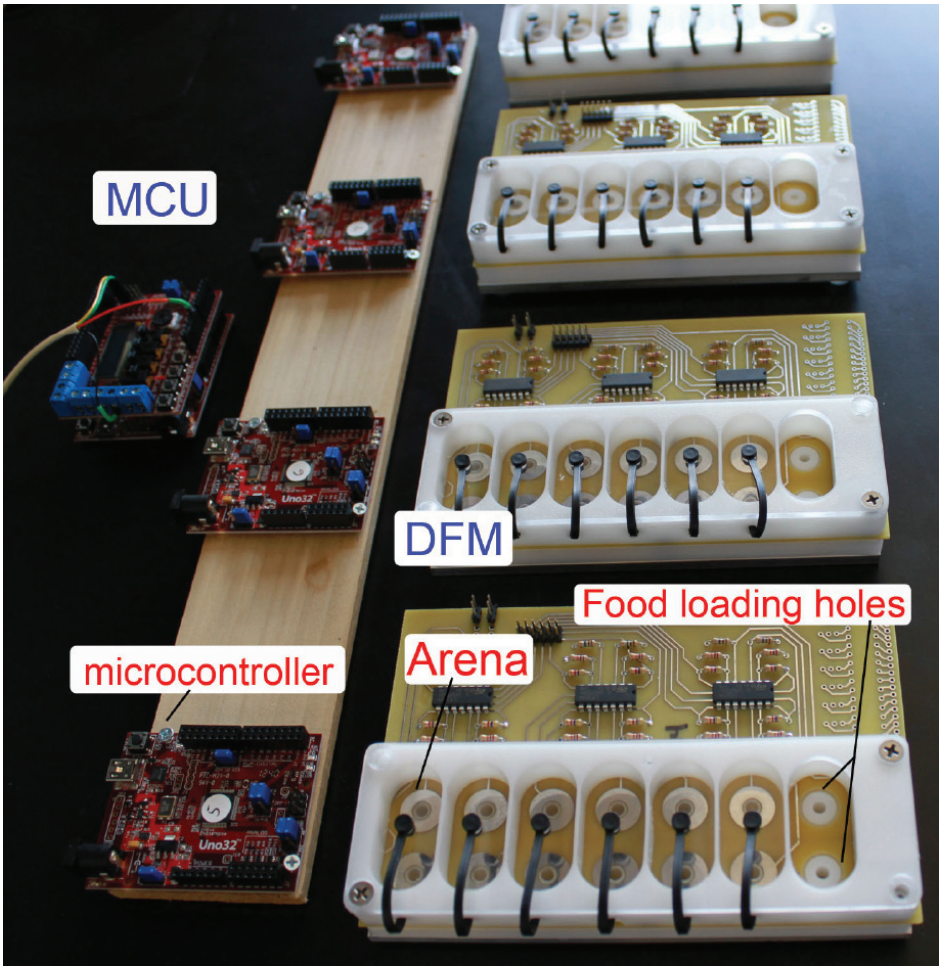
The FLIC system provides a precise and continuous quantification of the number and duration of interactions a fly has with food. It complements conventional methods of analysis, such as the CAFE assay and tracer dye approaches, by allowing comprehensive long-term studies of new and subtle aspects of 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 , 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 (Chatterjee and Hardin, 2010; Tanoue et al., 2008) feeding activity in the morning(Xu et al., 2008). 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(Tully and Quinn, 1985). 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 (Atkinson, 2009; Devineni and Heberlein, 2009; Venken et al., 2011) . 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.

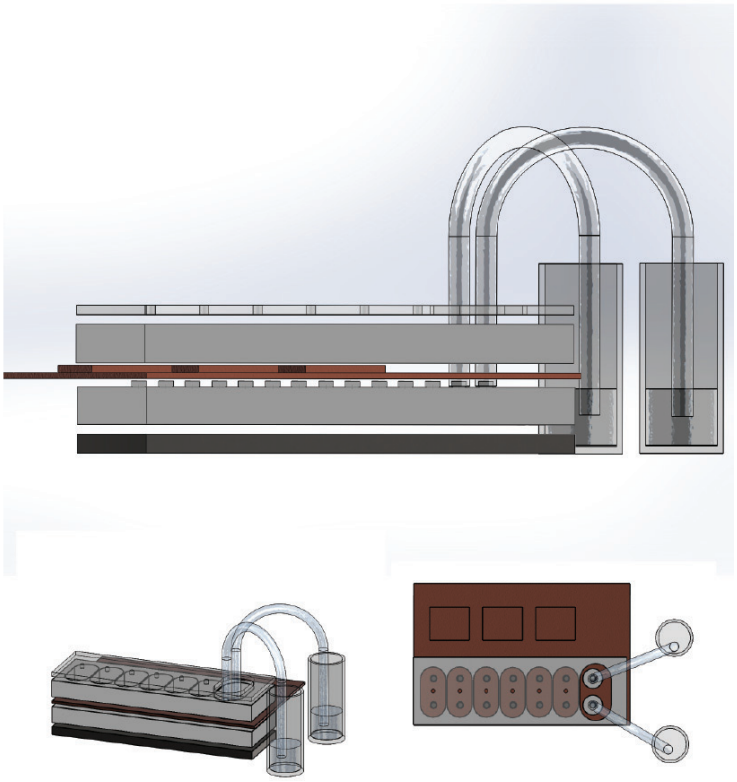
In summary, the FLIC system is a powerful tool for dissecting context-based feeding behaviors that encompass complex interactions among the characteristics of the food and the physiological drives of the animal. By combining the capabilities of the FLIC system with genetic tools available in Drosophila 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 Information

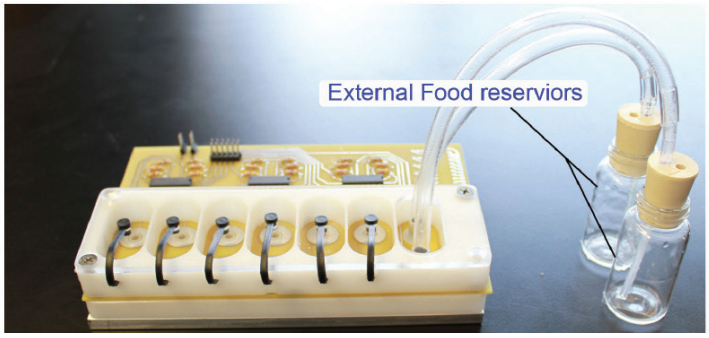


Supplementary Figure 3.1. An image of the FLIC system.
A picture of the FLIC system showing a master controller unit (MCU), four microcontrollers, and four Drosophila feeding monitors (DFM) that consist of six behavioral arenas and a pair of food loading holes per DFM.

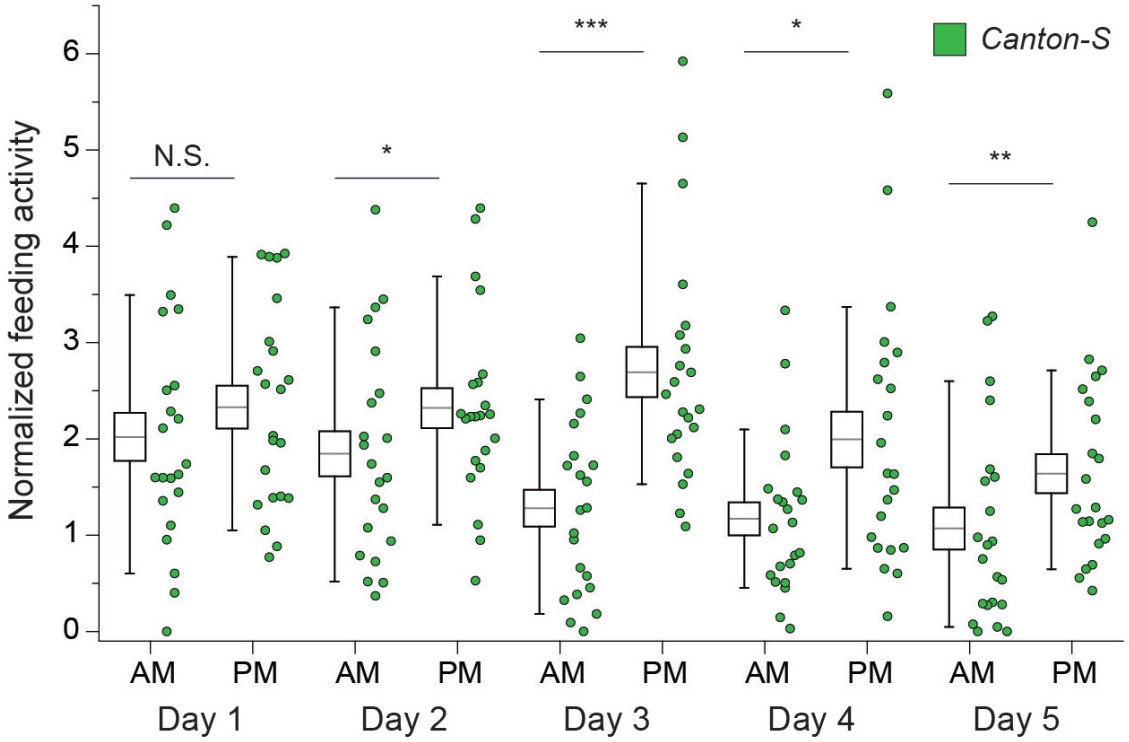
a.



b.



Supplementary Figure 3.2. 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 3.3. 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 4

Serotonin Signaling Mediates Protein Valuation and Aging[1]

Abstract

Research into how protein restriction (PR) improves organismal health and lengthens lifespan has largely focused on cell-autonomous processes. In certain instances, however, nutrient effects on lifespan are independent of consumption, leading us to test the hypothesis that central, cell non-autonomous processes are important PR regulators. We characterized a transient feeding preference for dietary protein after modest starvation in the fruit fly, *Drosophila melanogaster*, and identified tryptophan hydroxylase (*Trh*), serotonin receptor 2a (5HT2a), and the solute carrier 7-family amino acid transporter, *JhI-21*, as required for this preference through their role in establishing protein value. Disruption of any one of these genes increased lifespan up to 90% independent of food intake suggesting the perceived value of dietary protein is a critical determinant of its effect on lifespan. Evolutionarily conserved neuromodulatory systems that define neural states of nutrient demand and reward are therefore sufficient to control aging and physiology independent of food consumption.

Introduction

The availability of dietary protein elicits rapid and significant effects on behavior and lifespan across taxa (Kamata et al., 2014; Mair et al., 2005; Mayntz et al., 2005). Availability of specific nutrients, rather than overall caloric value, may be the driving force for this effect under some circumstances, and dietary protein is particularly important (Kamata et al., 2014; Mair et al., 2005; Mayntz et al., 2005). Protein restriction extends lifespan in crickets (Reddiex et al., 2013), flies (Mair et al., 2005), mice (Solon-Biet et al., 2014), and probably humans (Levine et al., 2014). Nearly all research into the mechanisms of this phenomenon has focused on the consequences of amino acid imbalance within cells, mostly through investigation of the TOR pathway and its effectors (Efeyan et al., 2015). Remarkably, however, many of the effects of diet manifest independently of food consumption, likely through global integration of

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nutrient signals and cell non-autonomous regulation of nutrient signals by the nervous systems (Linford et al., 2011; Taylor et al., 2014). Indeed, sensory neurons in *Drosophila melanogaster* and *Caenorhabditis elegans* can promote or limit lifespan depending on the specific neurons involved (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999; Libert et al., 2007), and the first instance of sensory modulation of lifespan in mice was recently reported (Riera et al., 2014).

We postulated that cell non-autonomous responses to protein availability might be important determinants of aging. There is evidence that organisms forage to balance their intake of specific nutrients rather than merely to meet energetic requirements, and even humans are known to make feeding decisions based on dietary protein (Felton et al., 2009; Griffioen-Roose et al., 2011; Mayntz et al., 2005). Although the molecular mechanisms for such choices are not well understood, important components of the process must include the ability to sense protein, to assess the value of protein relative to demand, and to execute behavioral and physiological responses that maintain protein homeostasis. Here, we present that serotonin signaling through receptor 2a are required for protein preference by determining value of protein at the time of physiological demand. Moreover we describe the first incidence of functional connection between amino acid transporter, JhI-21, and serotonin signaling in a context of macronutrient selection. We further demonstrate that modulators of dietary protein selection also mediate diet-dependent lifespan when animals are exposed to a complex nutrient environment where they have to constantly evaluate internal nutritional state and available nutrients from environment. These results highlight how the macronutrient valuation process itself, in the context of perceived availability and demand, can influence the aging process independent of food consumption.

Methods and Materials

Fly stocks

The following stocks were obtained from Bloomington Stock center; *Canton S., w^{1118}*, *Trh^{c1440}* (Neckameyer et al., 2007), *$5HT2a^{PL00052}$* (Nichols, 2007), *$JhI\text{-}21^{GE15185}$* (Jin et al., 2008), *CCKLR-$17D1^{MB02688}$, $Ir64a^{MB05283}$, $S6KII^{G1845}$*, P{TRiP.GL00267}attP2, *$Gr64f^{MB12243}$*, *$Gr66a^{\Delta 83}$*, *$Gr93a^{3}$*, *$DopR1^{attp}$*. For RNAi-mediated knock-down of *5HT2a,* we used P{KK110704}VIE-260B from Vienna Drosophila Resource Center. *UAS-dTrpA1* (Hamada et al., 2008), *UAS-shi ^{ts1}* (Kitamoto, 2001), *Trh-GAL4* (Daubert et al., 2010), *S6KII $^{ign\text{-}\Delta 58\text{-}1}$* , *$4EBP^{\Delta}$*, *$ppk28^{\Delta}$, Chico, $dFoxO^{\Delta 94}$, $NPFR^{c01896}$* , $Orco^{2}$, *$Gr5a^{\Delta 5}$, Gr32a* were gift from P. Garrity (Brandeis University, Waltham, MA), T.Kitamoto (U of Iowa, Iowa city, IA), B.G. Condron (U of Virginia, Charlottville, VA), R. Jackson (Tufts

University, Boston, MA), R.J. Wssells (Wayne State University, Detroit, MI), K. Scott (U of California Berkeley, Berkeley, CA), L. Partridge (U college of London, London, UK), S. Waddell (U of Oxford, Oxford, UK), L. Vosshall (The Rockefeller University, New York city, NY), J. Carlson (Yale University, New Haven, NJ), and H. Amrein (Texas A&M, College Station, TX), respectively. All 5HT receptor mutants (*5HT1a* ^{MB9812}, *5HT1a*^{MB9978}, *5HT1b*^{MB5181}, *5HT1b*^{MB5999}, *5HT2b*^{MB06501}, *5HT2a*^{li3299}, *5HT2a*$^{MI00459\text{-}GAL4}$) were gifts from H. Dierick (Baylor College of Medicine, Houston, TX). G.W. Roman shared *Tdc2-GAL4* and *Th-GAL4* as gifts. A novel *Ast-C* deletion alleles was created using a FLP-FRT recombination mediated strategy described previously (Parks et al., 2004) using d00174 and f00146 (Harvard-Exelixis stock center). *Trh*^{c1440}, *5HT2a*^{PL00052}, and *JhI-21*^{GE15185} mutants were backcrossed at least 8 generations to *w*^{1118} prior to any followed up experiments after the candidate screens.

Husbandry

All fly stocks Ire maintained on a standard cornmeal-based larval growth medium and in a controlled environment (25 °C, 60% humidity) with a 12 Light: 12 dark cycles. If flies contained temperature-sensitive transgenes, they Ire reared in 23°C and maintained at this temperature as adults until the experiments. We controlled the developmental larval density by manually aliquoting 32uL of collected eggs into individual bottles containing 25ml of food.(Linford et al., 2013) Following eclosion, mixed sex flies Ire kept on SY10 medium for 4-10 days until they Ire used for experiments. Unless otherwise noted, We used mated female flies that aged between 5-14 days old for the choice experiments. When starvation was required for the feeding assay, We used 1% agar medium to deprive food but prevent dehydration.

Food intake and choice measurements using the CAFE assay

We used a modified CAFE assay (Ja et al., 2007) to measure food intake or food choice as described previously (Ro et al., 2014). All choice experiments using the CAFE assay lasted 3h in 25°C, 60% relative humidity, and uniform lighting. The Preference Index (PI) was calculated as ‘‘[(Volume of protein+sugar consumed/fly)-(Volume of sugar consumed/fly)]/ [Total volume of food consumed/fly]’’. We used either bovine albumin serum (BSA; Fisher Scientific) or autolyzed yeast (Bacto™ yeast extract, BD) as protein sources and 1% sucrose as sugar in the choice experiments. The candidate screen was done using the CAFE assay and 45mg/mL of BSA as a protein source. For the screen, I added $10^{-5.5}$µM of denatonium benzoate (Sigma-Aldrich) in the protein-supplemented food to reduce the frequency of false positives.

Food Choice measurement using the FLIC assay

Details of the feeding experiments using the Fly Liquid-food Interaction Counter (FLIC) system

can be found in (Ro et al., 2014) and wikiflic.com. Briefly, we filled one side of the food trough with a 1% sucrose+ protein solution and the other trough with a solution of 1% sucrose-only. To avoid positional bias, we alternated the side of each food type across different *Drosophila* feeding monitors (DFMs). After loading the foods,we introduced an individual fly into each behavioral arena using an aspirator. We began the FLIC monitor software (V. 2.0-2.1) before flies were loaded to ensure that no feeding signals were lost during the fly-loading time. Unless otherwise noted, choice experiments lasted 3h. A Cumulative PI of an individual was calculated as ‘‘[(Total feeding interaction time on protein+sucrose) - (Total feeding interaction time on sucrose)]/Total feeding interaction time’’. We computed a time-dependent feeding PI from an individual fly by calculating fixed feeding PI within a 30min time-window every 10 min. Flies that did not generate any feeding signals in a given 30min window Ire treated as missing data for that period. Average time-dependent PI values and their SEM across biological replicates Ire therefore calculated based only on flies that exhibited at least one feeding event during the period in question. Initial characterization of the protein feeding behavior when starved and fully-fed Ire determined by the FLIC system as Ill as all subsequent follow-up experiments after the candidate screen.

Total food intake measurement using tracer dye

Fifteen day old, age-matched female flies Ire kept on SY10% food, then transferred to test foods mixed with 0.5% blue dye (FD&C Blue no. 1; Spectrum Chemical). We let flies feed on dyed food between 4pm till 1am the next day (8h), capturing one period of “light-off” when flies normally consuming more than half of their daily food intake (Ro et al., 2014). We then froze flies at −20 °C to stop the feeding experiment and homogenized flies in 150 µL PBS + 0.1% Triton X-100 (IBI Scientific). Homogenates Ire centrifuged at 3,750 × g for 10 min to settle debris, filtered through a 0.4µm filter, and read absorbance at 630 λ, with 670 λ as a reference wavelength. Absorbance values from 670 λ readings Ire subtracted from 630 λ readings to correct for background from fly homogenate. For measuring flies’ proportional food intake within a choice environment, We had three diet groups where I dyed either 10% sucrose only, or 10% yeast only, or both. We used 10 female flies per sample and 8 biological replicates per genotype and treatment group.

Quantification of protein, fat, and carbohydrate from fly homogenates

For quantifying total protein, fat, and carbohydrates from fly homogenates, we froze flies after experimental treatment at −20 °C, then homogenized in groups of five in 500 µL PBS + 0.1% Triton X-100 (IBI Scientific). Samples were centrifuged at 3,750 × g for 1 min to settle debris. All measurements were based on colorimetric assays that Ire carried out using a Synergy2 plate reader (BioTek). For triacylglyceride (TAG) measurement, 5 µL homogenate was mixed with 150

µL of 37 °C Infinity Triglycerides reagent (Thermo Scientific) and incubated at 37 °C for 10min. Absorbance was measured at 520 λ. A serial dilution of 2mg/mL glycerol was used as a standard. For glucose+trehelose measurement, I mixed 10 µL of homogenate with 100 µL of 37 °C Infinity Glucose reagent (Thermo Scientific), followed by 30 min incubation at 37 °C. Absorbance was measured at 340 λ. We created a calibration curve from 2mg/mL glucose standard. For glycogen measurement, we followed the same protocol as the glucose+trehelose measurement, except that we used 10 µL of homogenates that were treated with 0.5 µL of amyloglucosidase (0.1U/µL), an enzyme that breaks glycogen down to glucose, for 30min at 37 °C. We then subtracted total free-glucose concentrations that were obtained from the initial glucose measurements to compute concentrations of glycogen. For protein measurement, 2 µL of fly homogenate was incubated with 200 µL of (1:50) 4% (w/v) cupric sulfate/BCA Solution (Novagen) at room temperature for 30min. Absorbance was measured at 562λ. A serial dilution of 2mg/mL BSA standard were used to construct the calibration curve. For assessing nutrient stores in fully-fed or starved flies we used 10 female files per sample and 10 biological replicates. To calculate total stored calories, we assumed 1g of protein or carbohydrate generate 4kcal of energy and 1g of fat generate 9.1kcal of energy then converted amount of protein, carbohydrates, and fat found in a fly homogenate into kcal of energy. For assessing metabolites in flies between fixed and choice-diet conditions, we aged 8-day-old female flies in respective diet conditions for 8 days (6 biological replicates).

Quantification of serotonin using UPLC-MS

Sample preparation for the UPLC-MS

To extract serotonin and its related metabolites, female flies we snap frozen in liquid nitrogen and then vigorously vortexted to remove heads. The heads were then homogenized with ice-cold 3x volume of acetonitrile (we I assumed a single head is equal to 1µL) using a pestle grinder. We centrifuged the homogenates at 18,000 x g for 5 min and collected the organic phase as a tissue extract. To derivatize our samples prior to the UPLC-MS analysis, 12 µL of each tissue extract sample were benzoylated by the sequential addition of 6 µL of carbonate buffer (sodium carbonate, 100 mM), 6 µL of benzoyl chloride (2% in acetonitrile, v/v), and 6 µL of an internal standard solution (1% H_2SO_4 in 20% acetonitrile, v/v)(Song et al., 2012). Internal standards contained analytes that had been labeled with C^{13}-benzoyl chloride.

Liquid chromatography

To separate analytes, we used a Waters nanoAcquity UPLC system fitted with an Acquity HSS T3 C18 column (1 mm x 100 mm, 1.8 µm, 100 Å pore size). Mobile phase A was 10 mM ammonium formate with 0.15% (v/v) formic acid in water. Mobile phase B was acetonitrile. The gradient

used was: initial, 0% B; 0.01 min, 17% B; 0.5 min, 17% B; 3 min, 25% B; 3.3 min, 56% B; 4.99 min, 70% B; 5 min, 100% B; 6.5 min; 100% B; 6.51 min, 0% B; 8.5 min, 0% B. We used a total flow rate of 100 µL/min and 5 µL of sample injection in partial loop injection mode. The auto-sampler was kept at ambient temperature whereas the column was held at 27°C.

Mass spectrometry

An Agilent 6410 triple quadrupole mass spectrometer was used for analyte detection. We used electrospray ionization in positive mode at 4 kV. The gas temperature was 350°C, gas flow was 11 L/min, and the nebulizer was at 15 psi. Benzoylated 5HT was detected by tandem mass spectrometry at a precursor mass to charge ratio (m/z) of 385 and product m/z of 264 using a fragmenter voltage of 140, collision energy of 20 V, and accelerator voltage of 4 CAV. C^{13}-labeled internal standard was detected the same except percusor m/z was 397 and product m/z was 270. After the MS analysis, We performed automated peak integration Agilent MassHunter Workstation Quantitative Analysis for QQQ, version B.05.00. All peaks were visually inspected to ensure proper integration. We used 0.5 - 100 nM synthetic serotonin (5HT; Sigma) diluted in water as a standard to construct a calibration curve. The standard curve was prepared based on the peak area ratio of the standard to the internal standard by linear regression.

RNA extraction and quantitative PCR

For serotonin receptor mRNA expression analysis, the heads of flies were removed following diet treatments and then frozen at −80 °C. I then extracted RNA using TRIzol reagent (Invitrogen) following the manufacturer's protocol. We diluted extracted RNA samples with RNase-free water to an equal concentration and then performed RT-PCR using SuperScript III First Strand cDNA Synthesis (Invitrogen) to generate cDNA. Real-time PCR analysis used PoIr SYBR Green PCR Master Mix and a StepOne Plus Real-time PCR system (Applied Biosystems). We pulled heads from 50 females per sample and had 3 biological replicates per treatment group.

I used following primers:

5HT1a_F: AATAATCAGCCGGACGGAGG
5HT1a_R: GGTGTTGACCGTGTTCGTTG

5HT1b_F: CAGCGATGCGGATGATTA
5HT1b_R: CGAGGCTATCAGATGGTGCT

5HT2a_F: GGCTCGAGGCATCGATCTAC
5HT2a_R: ACGCATATGTTAGGCTCGGG

5HT2b_F: ACTCCAAGAATCACGCCTCG
5HT2b_R: TCGGACGGTCAGGCAATATG

5HT7_F: TTTTGTGCGACACTTGCCAC
5HT7_R: TTCAGCGCGTTTACTGGGT

RP49_F: ACTCAATGGATACTGCCAG
RP49_R: CAAGGTGTCCCACTAATGCAT

Survival analysis

Flies were prepared for survival experiments as previously described (Linford et al., 2013), with a slight modification. Briefly, 2-3 day old adult female flies Ire transferred onto test food medium. For each genotype per treatment, we put 25 flies per vial with 8-10 vial replicates. Flies were transferred to new food three times per week at which time survival was recorded and dead flies removed. For the protein-restriction experiment, we used a fixed mixture of 5% sucrose + 5% yeast as the protein-restricted diet, and a fixed mixture of 5% sucrose +15% yeast as the high protein diet. For the lifespan experiments comparing “Fixed food” vs “Choice food” environments, we created inserts that fit into individual vials. These inserts allowed us to expose the flies to two separate sources of food simultaneously. For these experiments we either loaded the same foods on both sides (no choice diets) or a different food on each side (choice diet). Specifically, the no choice diets contained either 10% sucrose in each well, 10% yeast in each well, or a fixed mixture of 10% yeast and 10% sucrose in each well. The choice diet entailed 10% sucrose on one side of the insert and 10% yeast on the other side of the insert. To test effects of a sugar-rich diet on longevity, we provided flies a fixed mixture of 30% sucrose + 5% yeast as the sugar-rich food, or a fixed mixture of 5% sucrose + 5% yeast as the sugar-restricted food.

Egg lying assay

Seven day old female and male flies that were kept on SY10% food since eclosion and were transferred to five different egg laying media: 10% sucrose mixed with either 1%, 2.5%, 5%, 10%, or 20% yeast. We provided fresh medium every 24h for 4 days and counted the number of eggs laid each day. We reported the eggs laid on 4th day when control flies’ reproductive output was fully equilibrated to the concentration of dietary protein. We measured 8 biological replicate per genotype and treatment group.

Statistics

For Cumulative PIs between two genotypes, we used Student's t-test. For comparison involving food preference, gene expression, metabolite amount, serotonin amount, and food consumption with more than two genotypes or treatment groups, we performed one-way ANOVA followed by post-hoc significance test. We took linear regression approach to model the relationship between time spent feeding on either protein or sugar as dependent variable of total feeding time. To test the effects of diet and genotype in flies' food intake of the fixed vs choice diets, we used Two-way ANOVA. Unless otherwise indicated, pairwise comparisons between different treatment survivorship curves Ire carried out using the statistical package R with DLife, a survival analysis package developed in the Pletcher Laboratory (Linford et al., 2013). P-values for survivorship comparisons were obtained using log-rank test. For testing interaction between genotypes and diets, we used cox-regression analysis to report P-value for the interaction term. In all cases, two-tailed P-values are reported. We also calculated P-values for aging experiments using a mixed effects modeling approach where standard linear models were applied using survival time as an outcome and vial within genotype as a random effect. In all cases, the P-values for the genotype effect on mean lifespan was significantly lower than we obtained using non-parametric log-rank statistics. Therefore, for all analyses, we report the P-values from the log-rank analyses because they are more conservative.

Results and Discussion

Drosophila develop a preference for protein under mild starvation

We hypothesized that mechanisms underlying behavioral responses to protein availability would also be important determinants of lifespan and therefore sought first to identify central mechanisms involved in protein-dependent feeding decisions. We characterized a dynamic and transient protein-seeking behavior in *Drosophila* in response to nutrient demand. Using a new real-time feeding monitoring system called FLIC, which quantifies all contact interactions an individual fly has with food (Ro et al., 2014) (http://www.wikiflic.com), we found that after mild starvation both male and female flies preferred a sugar diet supplemented with autolyzed yeast (the major protein source in the fly diet) over a diet composed exclusively of sugar (Figure 4.1a, 24h starvation). This preference persisted for just over one day, with longer food deprivation increasing protein preference (Supplementary Figure 4.1a). On the other hand, fully-fed animals consumed a sugar-only diet more often throughout the experiment (Figure 4.1a, fully-fed). Food choice was not based on caloric content because starved flies also chose a 1% protein diet over an isocaloric 1% sugar diet and an increase in protein concentration to a hypercaloric 5% did not affect this preference (Figure 4.1b). The FLIC system quantifies which food each individual

fly interacts with, together with when and for how long. Using this information we found that total feeding time was a much stronger predictor of total protein feeding time than it was of sugar feeding time (Figure 4.1c and Supplementary Figure 4.1b). Moreover, flies with more total feeding time were more likely to choose a protein containing food as their first meal and to have a stronger protein preference over the course of the experiment (Figure 4.1c).

Flies develop a specific preference for dietary protein independent of the source or type of proteins after starvation because we observed that flies preferred autolyzed yeast, which is primarily composed of short peptide/single amino acids (Figure 4.1a; Supplementary Figure4.1c), or a pure complex protein such as bovine serum albumin (BSA) (Figure 4.1d) over a wide range of concentrations. Twenty-four hour starvation had no detectable effect on internal protein levels (Figure 4.1e), suggesting that preference was independent of gross protein stores. Our results demonstrate that individual flies are affected differently by 24h starvation and that those under high nutrient demand choose to eat protein as their first meal and continue to consume greater amounts of protein to ameliorate their deficit. Protein preference is, therefore, not limited to reproductively active female flies (Ribeiro and Dickson, 2010; Vargas et al., 2010) and is more dynamic than previously suspected.

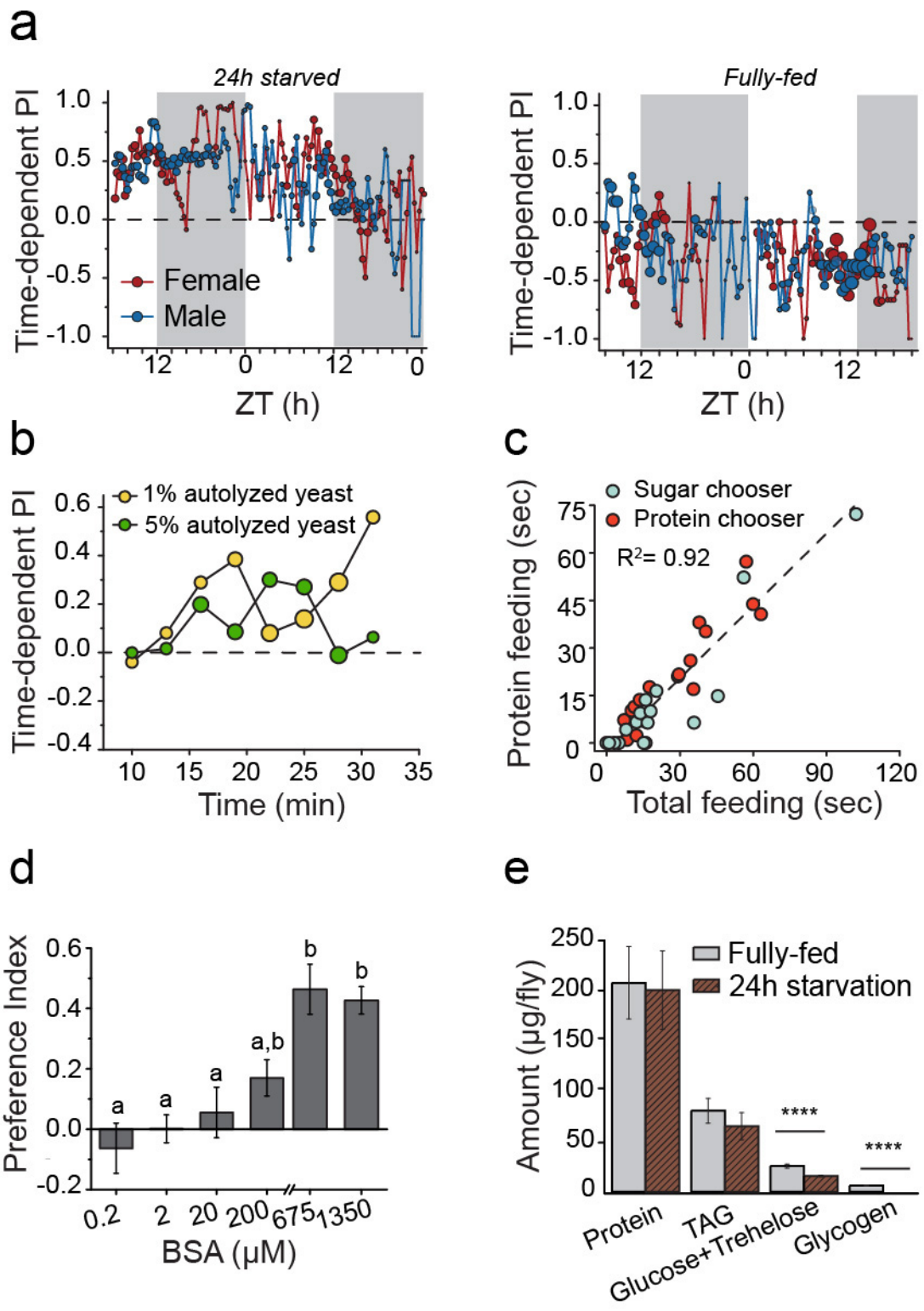

a
24h starved
Fully-fed
Time-dependent PI
1.0
0.5
0.0
-0.5
-1.0
Female
Male
12
0
12
0
ZT (h)
b
1% autolyzed yeast
5% autolyzed yeast
0.6
0.4
0.2
0.0
-0.2
-0.4
10
15
20
25
30
35
Time (min)
c
Sugar chooser
Protein chooser
R²= 0.92
Protein feeding (sec)
75
60
45
30
15
0
0
30
60
90
120
Total feeding (sec)
d
Preference Index
0.6
0.4
0.2
0.0
-0.2
a
a
a
a,b
b
b
0.2
2
20
200
675
1350
BSA (μM)
e
Amount (μg/fly)
250
200
150
100
50
0
Fully-fed
24h starvation
****
****
Protein
TAG
Glucose+Trehelose
Glycogen

Figure 4.1. Drosophila demonstrates energy-state dependent protein feeding preference.
(A) Male and female Canton-S flies' real-time feeding preference over 24h. The choice was given as 2% autolyzed yeast (w/v) +1% sucrose (w/v) vs. 1% sucrose (w/v). Gray shades on the graphs indicate "light-off" periods. The size of the symbols is proportional to the number of flies that were feeding during the given time period (fully-fed female flies N=17; fully-fed male flies N= 21; starved female flies N=10; starved male flies N=11). A Preference Index (PI) = 1 indicates complete preference for the yeast-containing food. (B) Time-dependent PI plot from flies given a choice between 1% sucrose vs. 1% autolyzed yeast or 1% yeast vs. 5% autolyzed yeast. Similar yeast preference is observed in both experiments, and in the first experiment the diets are isocaloric. (C) Flies that had increased total food consumption during the choice assay were likely to eat more protein meal. From the continuous FLIC data, we identified, for each fly, its first meal choice, time spent on protein feeding, and total feeding time at the end of a 30min choice experiment. Flies were given a choice between isocaloric 1% sucrose vs 1% autolyzed yeast. Linear regression analysis revealed that total feeding time positively correlated with the protein feeding time ($F(1,32)= 173.7$, $P <1.8E-14$). (D) 24h-starved female flies' BSA preference was dose-dependent. Bars indicate the mean and the standard error of the mean (SEM). (N= 8-14 per each concentration treatment. Letters differentiate groups that are significantly different from one another as determined by Tukey's multiple-comparison at $\alpha=0.05$) (E) Quantification of stored nutrient levels in fully-fed or 24h-starved female flies. Flies lost a significant amount of carbohydrate reserves after 24h of starvation. (P values determined by two-way ANOVA, followed by Tukey's multiple-comparison test. *** $P \leq 0.001$).

Serotonin signaling through 5HT2a mediates protein preference

To identify mechanisms underlying protein-feeding preference, we performed a candidate screen designed to disrupt putative nutrient sensing pathways, sensory systems, and reward circuits (Figure 4.2a; Supplementary Table 4.1). Among all manipulations, disruption in serotonin signaling repeatedly reduced protein preference in starved flies (Figfure 4.2a blue bars; Supplementary Table 4.1 candidate # 63, 65, 67, and 68). Silencing of serotonergic neurons and treatment of flies with a serotonin receptor 2a (*5HT2a*) antagonist, ketanserin (Colas et al., 1995), eliminated preference entirely, such that starved animals exhibited an aversion to dietary protein that mimicked what we routinely observed from fully-fed, control animals.

We verified a role for serotonin signaling in protein feeding decisions using additional genetic manipulations. Flies lacking tryptophan hydroxylase (*Trh*), a rate-limiting enzyme for neuronal serotonin synthesis (Neckameyer et al., 2007) that is the *Drosophila* homologue of TPH2 (Bao et al., 2010), exhibited a reduction in preference for both autolyzed yeast and BSA compared with control animals (Fig. 2b and c). In *Drosophila*, there are five known serotonin receptors, each of which is evolutionarily conserved: *5HT1a, 5HT1b, 5HT2a, 5HT2b, and 5HT7* (Gasque et al., 2013). We verified a role for *5HT2a* by examining flies that carried two independent *5HT2a* mutant alleles (Figure 4.2d, Supplemental Figure 4.2a) as well as with RNAi-mediated knock-down of *5HT2a* transcript (Supplemental Figure 4.2b). All of the *5HT2a* manipulations abrogated the preference for protein following mild starvation, effectively recapitulating *Trh* mutation.

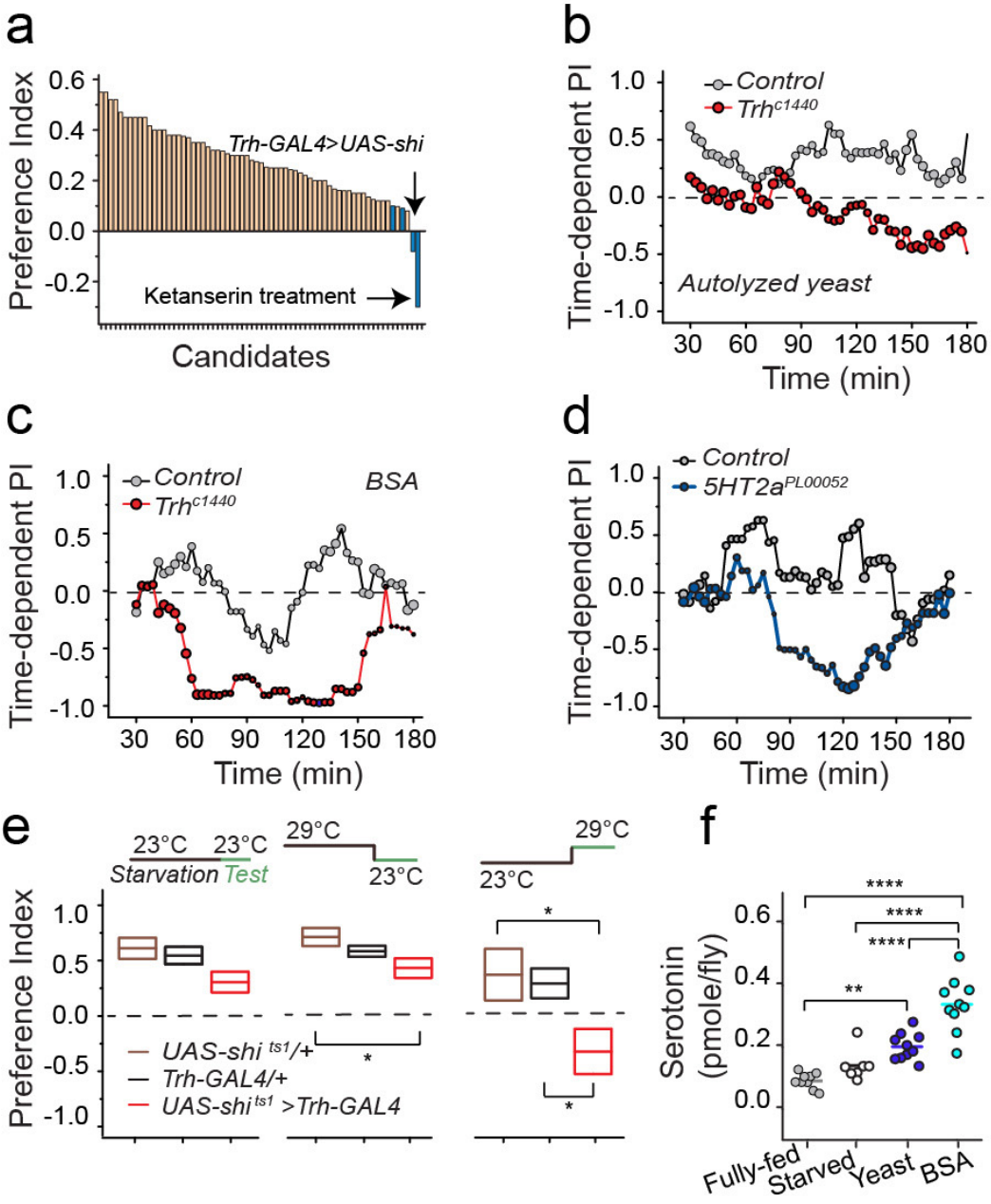

a
Preference Index
0.6
0.4
0.2
0.0
-0.2
Trh-GAL4>UAS-shi
Ketanserin treatment
Candidates
b
Time-dependent PI
1.0
0.5
0.0
-0.5
-1.0
Control
Trh^c1440
Autolyzed yeast
30 60 90 120 150 180
Time (min)
c
Time-dependent PI
Control
Trh^c1440
BSA
Time (min)
d
Time-dependent PI
Control
5HT2a^PL00052
Time (min)
e
23°C 23°C
Starvation Test
29°C
23°C
29°C
23°C
Preference Index
UAS-shi ts1/+
Trh-GAL4/+
UAS-shi ts1 >Trh-GAL4
f
Serotonin (pmole/fly)
0.6
0.4
0.2
0.0
Fully-fed
Starved
Yeast
BSA
g
mRNA Fold-change
Starved
3h refed
24h refed
1a
1b
2a
2b
7
Serotonin receptor
h
UAS-dTrpA1/+
Trh-GAL4/+
UAS-dTrpA1>Trh-GAL4
Feeding time (sec)/h
Preference Index

Figure 4.2. Serotonin signaling through receptor 2a modulates protein preference.
(A) Summary results from a candidate reverse genetic screen. We found that disruption of serotonin signaling consistently abrogates protein preference (See Supplemental Table 1 for listed candidates). We used BSA as the protein source. Blue bars indicate manipulations of serotonin signaling that strongly disrupted protein preference. (B-C) Time-dependent preference index (PI) plot from 24h-starved Trh mutant and control flies given a choice between sucrose-only or sucrose plus autolyzed yeast or BSA. When the protein source was autolyzed yeast, the cumulative preference index for Canton S files was 0.43 ± 0.1 (Mean +/- SEM) whereas Trh mutant flies was -0.18 ± 0.06 (Student's t-test; P ≤ 0.001). When the protein source was BSA, cumulative PI for Canton S. files was 0 +/- 0.1 whereas Trh mutant flies was -0.43 ± 0.2 (Student's t-test; P ≤ 0.05). (D) Time-dependent PI plot from 24h-starved flies with 5HT2aPL00052 mutant allele and Canton S., control flies given a choice between sucrose-only or sucrose plus BSA. Cumulative PI for the control was 0.2 ± 0.2 and for the mutant was -0.21 ± 0.2 (Student's t-test). (E) Serotonin signaling is required during the choice test to develop protein preference. Flies were placed in either 23°C or 29°C during starvation or choice test as noted in the diagram above each plot. The box plots indicate mean and SEM. Statistical significance for genotype effect within each temperature-shift experiment was determined using one-way ANOVA followed by Fisher's multiple-comparison, N=8-11/genotype (* P ≤0.05). (F) Serotonin abundance in the heads of flies following specified diet treatment. Serotonin significantly increased when animals were allowed to refeed on autolyzed yeast or BSA for three hours after 24hr starvation. Individual symbols represent measures based on 5 fly heads, and lines denote the mean. Statistical significance for diet effect was determined using one-way ANOVA followed by Tukey's multiple-comparison; N=7-10/treatment (** P ≤0.01, **** P≤0.0001). (G) Neuronal mRNA abundance of five serotonin receptors. Abundance of 5HT2a transcript acutely increased during 3hr protein refeeding after starvation. Statistical significance for the treatment effect was determined using one-way ANOVA followed by Tukey's multiple-comparison (** P ≤0.01). (H) Hyper-activation of serotonergic neurons is sufficient to suppress feeding behavior (left) and induce protein preference even in the absence of starvation (right). We observed feeding behaviors in 7 out of 14 flies during the choice test. Individual symbols indicate measures from single flies, and lines denote the mean value among biological replicates. Statistical significance for the genotype effect was determined using one-way ANOVA followed by Tukey's multiple-comparison (* P≤0.05, **** P≤0.0001).

Loss of function in three other serotonin receptors (*5HT1a, 5HT1b*, and *5HT2b;* 5HT7 mutant animals were not viable) had no effect on the preference phenotype (Supplemental Figure 4.2c). Disruption in serotonin signaling seems to affect only protein preference because *Trh* and *5HT2a* mutant flies exhibited normal choice behaviors when presented with sweet vs bitter tastes or sweet vs sweeter food (Supplemental Figure 4.2d)

We next sought to better understand the temporal dynamics through which serotonin regulates protein preference. To determine whether serotonin signaling is required during starvation and/or during food choice, we selectively inactivated serotonergic neurons (*Trh-GAL4>UAS-shi^{ts}*) during specific periods of the experiment. We found that flies retained their preference for dietary protein when serotonergic neurons were inactivated only during the starvation phase. Inactivation only during the choice phase, however, was sufficient to abolish preference and phenocopy the *Trh* mutant, implying that serotonin may be involved specifically in protein

reward after starvation (Figure 4.2e). Inhibition of serotonin signaling did not alter total food intake during the choice test, demonstrating that the lack of protein preference seen in transgenic flies is not an artifact of reduced feeding (Supplementary Figure 4.2e). If serotonin is involved in post-ingestive reward, then protein intake, but not starvation, would be expected to increase serotonin levels in the central nervous system. Indeed, we observed that serotonin levels in the head were unchanged after starvation but increased 100-200% after flies consumed protein compared with the fully-fed condition (Figure 4.2f). Because transcriptional regulation of serotonin receptors can directly affect behavioral output (Albert, 2010), we examined whether mRNA levels of serotonin receptors were altered in the head of the fly following protein ingestion. Much like the temporal profile of serotonin concentration, we observed an acute increase in the abundance of *5HT2a* mRNA, but not transcript from other serotonin receptors, in the heads of flies that were

starved of all nutrients and refed with protein for 3h (Figure 4.2g). *5HT2a* mRNA abundance was not affected by starvation alone, and it returned to the level of fully-fed animals after 24h of protein feeding, which is coincident with their loss of feeding preference (e.g., Figure 4.1a). Together, these results suggest that protein reward and preference are established in a relatively short time (<3h) and regulated by serotonin signaling through 5HT2a.

Serotonin signaling through 5HT2a establishes the value of dietary protein

We envisioned at least two ways that serotonin might influence protein reward. First, it may be involved in transducing sensory perception of protein. Second, it may be important for higher-order processing of the value of ingested protein. To distinguish these hypotheses, we hyper-activated serotonergic neurons in fully-fed flies using targeted expression of a heat-sensitive Trp channel (*Trh-GAL4>UAS-dTrpA1)*. If serotonin acts in protein sensing, both foods would be interpreted equally as containing protein, and thus we would expect flies to lack preference in our choice test regardless of their starvation state. In contrast, if serotonin acts to increase the value of consumed protein, we would predict that hyper-activation of serotonergic signaling would add value to protein meals and reinforce protein feeding in the absence of starvation. Although activation of serotonergic neurons suppressed feeding as we expected (Gasque et al., 2013) (Figure 4.2h left), when fully-fed flies did eat, they showed a near absolute preference for protein-containing food (Figure 4.2h right). These results support the notion that serotonergic signaling increases the value of dietary protein in an energy-state dependent manner. Mammalian studies have revealed a role for serotonin in carbohydrate satiety and have indicated the possibility that it may also influence protein or lipid feeding (Leibowitz et al., 1993; 1989). Our results establish that, in an invertebrate model, serotonin signaling directly modulates the value of dietary protein to adjust the animals' preference to favor a protein-

containing meal, and they suggest a complex role for this monoamine neurotransmitter in macronutrient selection across taxa.

The perceived value of dietary protein modulates lifespan via serotonin signaling

We hypothesized that serotonin's role in ascribing value to ingested protein may be important for lifespan, given the noted importance of this nutrient in aging (Gallinetti et al., 2012; Mair et al., 2005). Laboratory protocols that are standard in the aging field employ fly diets that consist of sugar and brewer's yeast, the sole source of dietary protein, combined in an agar medium in fixed ratios. On a standard laboratory diet of low or intermediate yeast content, *Trh* mutants were long-lived and loss of *5HT2a* did not affect lifespan (Supplementary Figure 4.3a,b). If serotonin influences aging primarily through the physiological changes in response to the amount of protein consumed, then loss of *Trh* or *5HT2a* would be expected to reduce or eliminate diet-dependent changes in lifespan in fixed-diet conditions (Skorupa et al., 2008). We found, however, that *Trh* and *5HT2a* mutant flies responded similarly to control animals when diet was manipulated in this traditional manner (Supplementary Figure 4.3a.b).

While conventional fixed diets have been used effectively to examine physiology in response to total nutrient availability, we reasoned they would obfuscate serotonin's regulatory effects on aging because the animals would not be able to perceive and respond to nutrients individually. We therefore aged flies in more complex dietary environments where they were allowed to freely choose between carbohydrate and protein sources. For these experiments we created food dividers using a 3D-printer that allowed flies to age in the presence of two distinct food sources (Supplementary Figure.4.3c). In three control diets flies were provided with either a 10% sucrose-only, 10% yeast-only, or a fixed 10% sucrose/10% yeast diet on both sides of the divider. For the complex choice environment, flies were provided with 10% sucrose on one side of the divider and 10% yeast on the other, allowing them to freely interact with individual macronutrients.

We observed striking effects of the choice environment on lifespan and physiology. As expected, control flies were shortest-lived in a sucrose-only diet (Good and Tatar, 2001). On the other hand, we were surprised to observe that control animals in the choice environment lived substantially shorter than animals maintained in an isocaloric fixed diet or in one consisting of yeast-only (Figure 4.3a). Consistent with our observations using standard protocols, *Trh* mutant flies lived modestly longer than control flies on all control diets (Figure 4.3a; Sucrose-only, Yeast-only, and Fixed diet), while *5HT2a* mutants did not. However, when the flies were aged in the choice environment we observed a near doubling (90% increase) of mean lifespan of both *Trh* and *5HT2a* mutant animals (Figure 4.3a, Choice diet).

Why is it that dietary choice reduces lifespan in control flies and that *Trh* and *5HT2a* mutant animals are much longer-lived in these conditions? Using an indigestible dye mixed in both food wells we found that all flies consumed much more food in the choice environment compared to an isocaloric fixed diet (Fig 4.3b, left two bar groups). By labeling foods in individual wells we found that most of that increased consumption was due to a boost in sugar feeding (Figure 4.3b, right two bar groups). The increase in total feeding is unexpected, but the compositional intake is consistent with previous reports (Lee et al., 2008) and with our data (Figure 4.1a) showing that fed flies, similar to mammals, choose to consume proportionally more carbohydrate than protein. Fixed diets of this composition are associated with high reproductive output and a reduced lifespan (Skorupa et al., 2008). Indeed, it seems that a choice environment allows for more natural feeding conditions that may better reflect dietary habits in human populations.

One possible explanation for the exceptional longevity of *Trh* and *5HT2a* mutant flies in the complex nutrient environment is behavioral protein restriction or reduction in total food consumption. However, if mutant animals were simply eating less, we would expect to observe a similar degree of life extension in all diets, which we did not (e.g., Figure 4.3a). More importantly, we found no differences among genotypes in total food consumption in any condition, and in the choice environment, where lifespan extension was greatest, mutant flies consumed a sugar:protein ratio that was statistically indistinguishable from control animals (Figure 4.3b right two bar groups). We therefore find no evidence that serotonergic modulation of lifespan is due to self-induced PR.

A second possible explanation is that increased carbohydrate consumption alone causes shorter lifespan and that disruption of serotonin signaling protects animals from this effect. Several lines of evidence make it clear that this is not the case. First, mutant flies show the same extent of

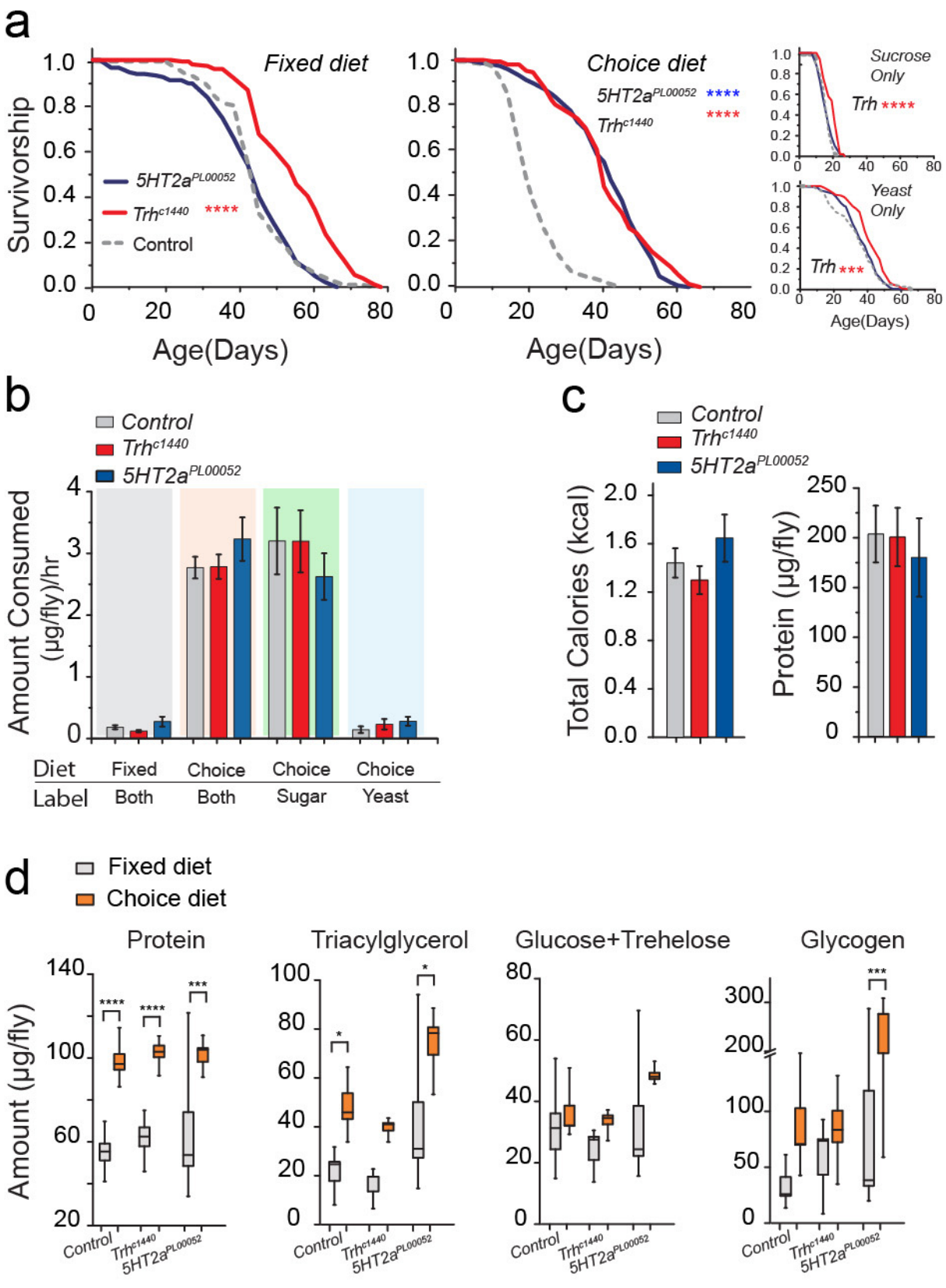

a
Fixed diet
Choice diet
Survivorship
Age(Days)
5HT2a^PL00052
Trh^c1440
Control
****
Sucrose Only
Yeast Only
Trh
***
b
Amount Consumed (µg/fly)/hr
Diet
Label
Fixed
Choice
Both
Sugar
Yeast
c
Total Calories (kcal)
Protein (µg/fly)
d
Fixed diet
Choice diet
Protein
Triacylglycerol
Glucose+Trehelose
Glycogen
Amount (µg/fly)

Figure 4.3. Serotonin modulates lifespan.
(A) Trh mutants live significantly longer than control flies when aged on the fixed diet (log-rank test), as well as on the choice diet (long-rank test). 5HT2a mutants live significantly longer than control flies on the choice diet (log-rank test). Trh mutants also live significantly longer in the sucrose-only and yeast-only diets. (*** P≤0.001, **** P≤0.0001) (B) The amounts of total food (both fixed and choice diets) and individual nutrients (choice diet only) consumed by Trh and 5HT2a mutant flies were statistically indistinguishable from control flies regardless of the diet environment (two-way ANOVA, all P>0.05). (C) Stored energy and protein levels in whole body homogenates of fully-fed Trh and 5HT2a mutant flies were not significantly different from control animals (one-way ANOVA). (D) Major stored metabolites in flies after they were kept in either the fixed or choice diets for 7 days. Box indicates median and 1 SEM with whiskers showing 10-90% quantile. Significance of the diet effect within the genotype per metabolite was determined by Tukey's multiple-comparison after two-way ANOVA (* P≤0.05, *** P≤0.001, P**** P≤0.0001).

increased sugar consumption as control animals in the choice environment (Figure 4.3b, third bar group). Second, the lifespans of *Trh* and *5HT2a* mutant flies are reduced to a similar extent as control animals when the carbohydrate content of a fixed diet is increased (Supplemental Figure 4.3d,e). So their lifespan is equally or more sensitive to it. Third, gross metabolites that are known to be influenced by food intake, including total protein, fat, and carbohydrate abundances (Skorupa et al., 2008), are strongly increased in the choice environment, and mutant and control animals are similarly affected (Figure 4.3d). It appears, therefore, that *Trh* and *5HT2a* mutant flies are eating the same amount of the same food, and they are processing nutrients similarly to control animals regardless of how foods are presented to them. Yet lifespan of the mutant flies is dramatically longer in the complex dietary environment. Based on our results showing a key role for serotonin signaling in valuation of dietary protein, we propose that the macronutrient valuation process itself is a potent factor that modulates organismal aging independent of food consumption.

<u>An amino acid transporter, JhI-21, is required for serotonergic valuation of protein</u>

The cellular and metabolic processes upstream of serotonin that are required for protein valuation remain to be determined. Sensory perception is likely to be important, but at present, specific amino acid taste receptors have not been identified in *Drosophila*, although this is an active area of research. Our examination of chemosensory receptors known to affect lifespan failed to reveal effects on protein sensing (Supplementary Table 1). Furthermore, manipulation of canonical intracellular amino acid sensing, such as RNAi-mediated knocked-down of GCN2 in starved animals (Figure.4.2a, Supplementary Table 4.1 candidate #23) or suppression of TOR signaling through rapamycin or overexpression of dominant negative RagA in fully-fed animals, had no effect on protein preference (Supplementary Figure 4.4a,b). In light of studies suggesting that amino acid transporters can act as transreceptors (Hyde et al., 2003; Nicklin et al., 2009)

and that particular solute carrier 7 protein family members (e.g., SLC7A5) interact with a cellular amino acid sensing systems such as TOR signaling (Taylor, 2013; Verrey et al., 2004), we examined protein preference of flies with mutation in *juvenile hormone inducible 21* (*JhI-21),* a *Drosophila* SLC7A5 protein (Piyankarage et al., 2010; Ziegler et al., 2013).

Interestingly, we found that mutation in *JhI-21* abolished protein preference in starved flies (Figure 4.4a). We also discovered evidence that JhI-21 acts upstream of serotonin signaling; *JhI-21* mutant animals failed to increase neuronal serotonin after 3h of yeast feeding, establishing that it is required for protein-dependent serotonin production (Figure 4.4b). *JhI-21* mutant females exhibited a normal increase in egg production with dietary protein concentration (Fig. 4c), and they showed normal feeding in both fixed and choice environments (Figure 4.4d). Nevertheless, mutation in *JhI-21* recapitulated the extended lifespan pattern observed in *Trh* and *5HT2a* mutant flies with a modest (13%) increase in mean lifespan in a fixed-diet condition (Supplementary Figure 4.4c) and a greater (32%) mean lifespan extension in a choice-diet environment (Figure 4.4e). These data suggest that the *JhI-21* amino acid transporter, and possibly SLC7 protein family members in general, are conserved regulators of protein-dependent behavior and physiology and that they may function together with serotonin signaling to modulate aging independently of mechanisms that regulate reproduction and total food intake.

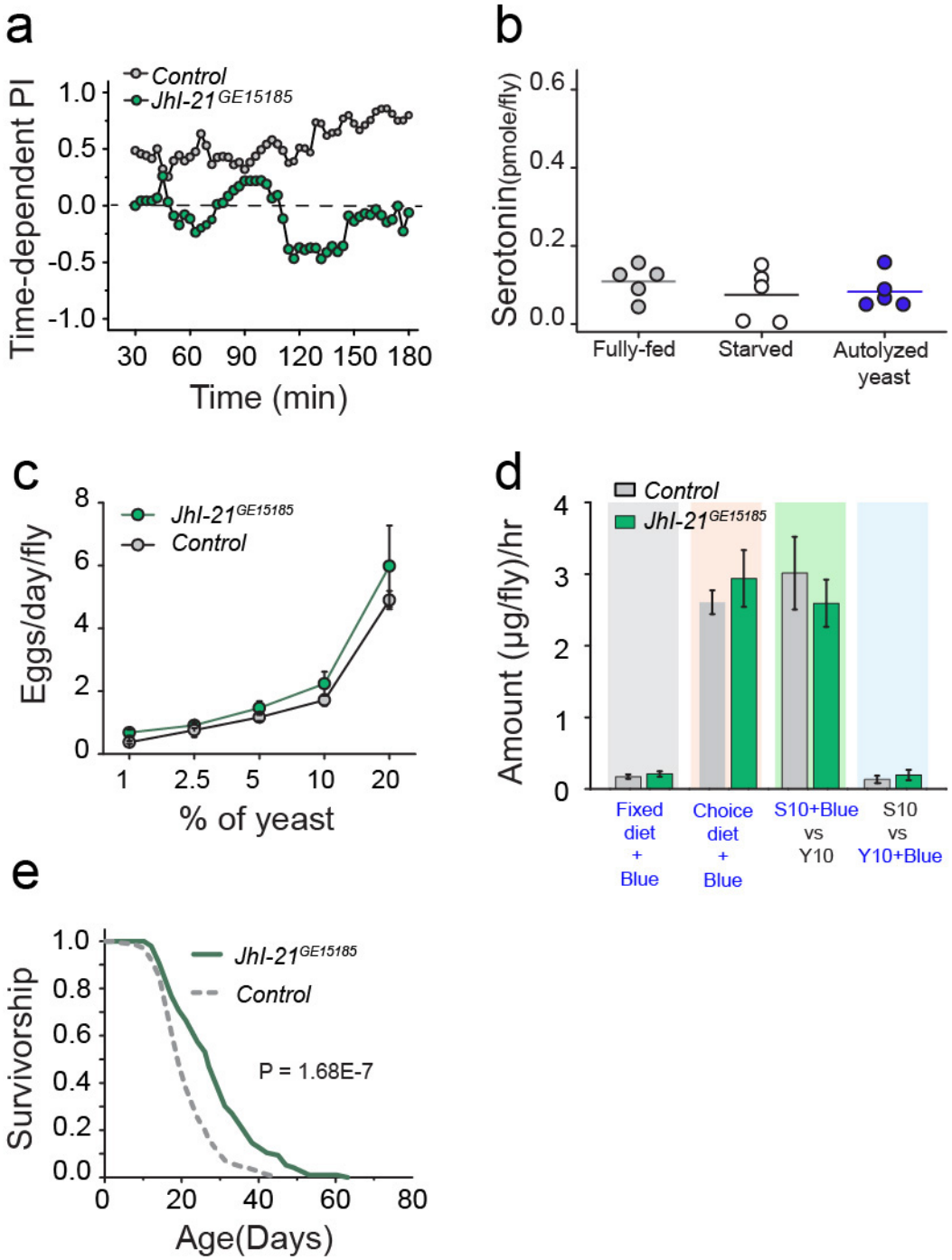
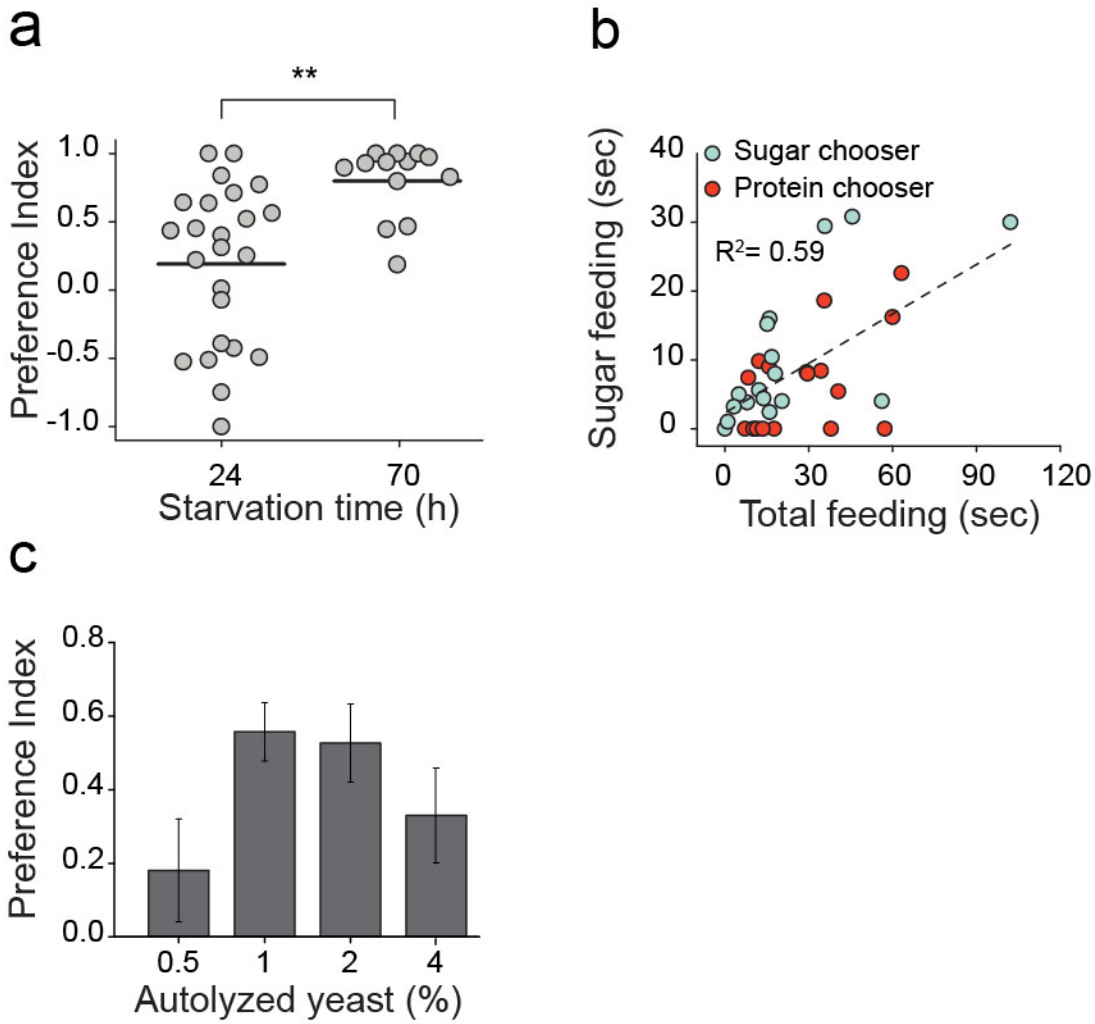
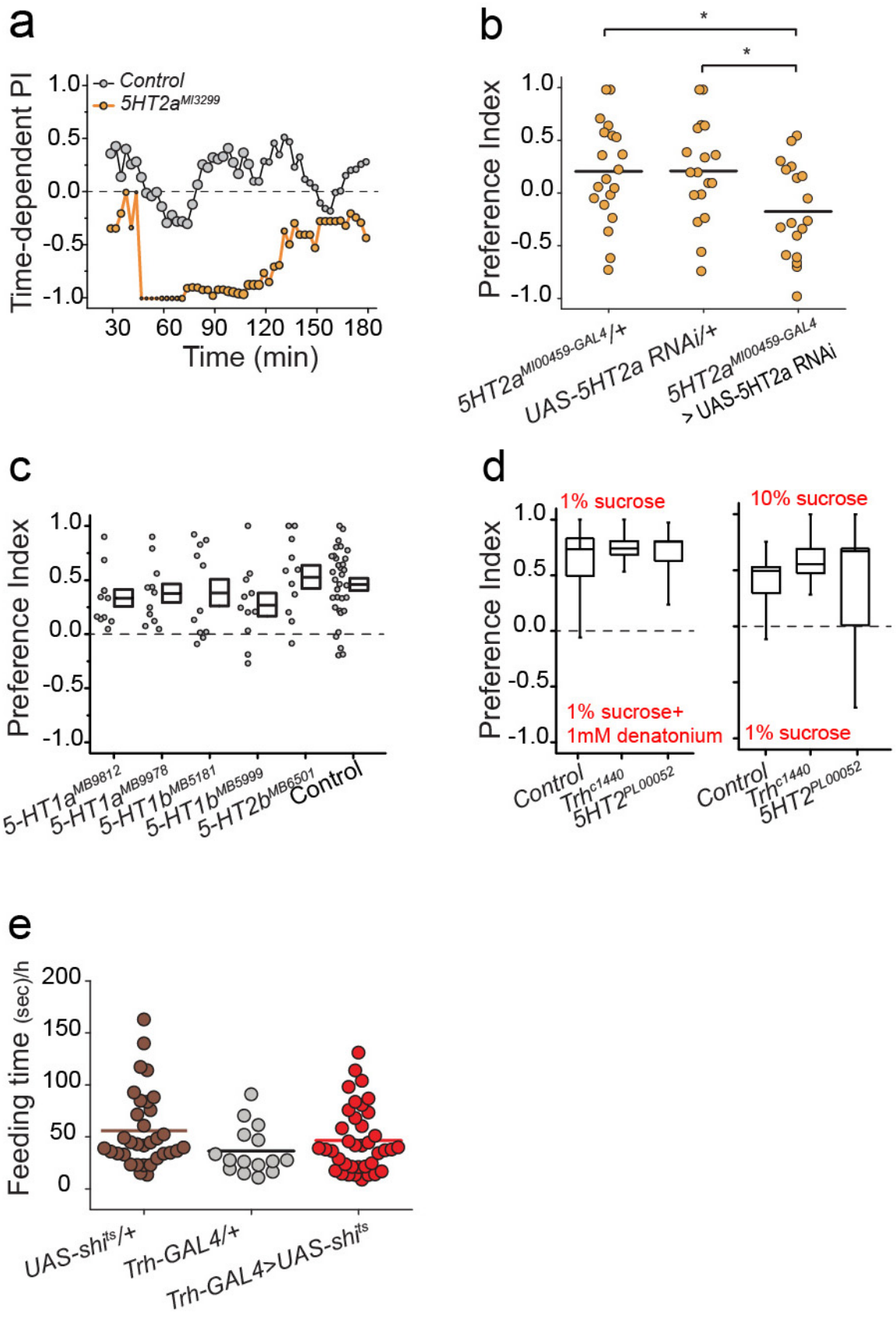

a
Control
Jhl-21GE15185
Time-dependent PI
1.0
0.5
0.0
-0.5
-1.0
30 60 90 120 150 180
Time (min)
b
Serotonin(pmole/fly)
0.6
0.4
0.2
0.0
Fully-fed
Starved
Autolyzed yeast
c
Jhl-21GE15185
Control
Eggs/day/fly
8
6
4
2
0
1 2.5 5 10 20
% of yeast
d
Control
Jhl-21GE15185
Amount (µg/fly)/hr
4
3
2
1
0
Fixed diet + Blue
Choice diet + Blue
S10+Blue vs Y10
S10 vs Y10+Blue
e
Jhl-21GE15185
Control
P = 1.68E-7
Survivorship
1.0
0.8
0.6
0.4
0.2
0.0
0 20 40 60 80
Age(Days)

Figure 4.4. JhI-21 functions upstream of serotonin to modulate protein preference.
(A)Time-dependent protein preference of flies with mutation in one of the Drosophila SLC7A5 proteins, JhI-21. Mutation in JhI-21, abolished protein preference. The choice was given as 1% sucrose vs 1% sucrose+ 2% autolyzed yeast. Cumulative preference index for Canton S files was 0.6 +/- 0.08 whereas JhI-21 mutant flies was -0.02 +/- 0.1 (Student's t-test; $P \leq 0.001$). (B) Serotonin abundance in the heads of JhI-21 mutant flies after specified diet treatments. There was no change in serotonin abundance following 24h starvation or 3h of autolyzed yeast refeeding after starvation. Individual symbols represent measures based on 10 fly heads, and lines denote the mean (one-way ANOVA; N=5 biological replicates/treatment). (C) JhI-21 mutant flies increase reproductive output normally as concentration of dietary protein increases. (D) JhI-21 mutant flies consume the same amount of sucrose and yeast as control flies regardless of the diet environment (two-way ANOVA). (e) Survivorship of JhI-21 mutants aged on the choice diet. Mutants live significantly longer in these conditions compared with the control flies (log-rank test).

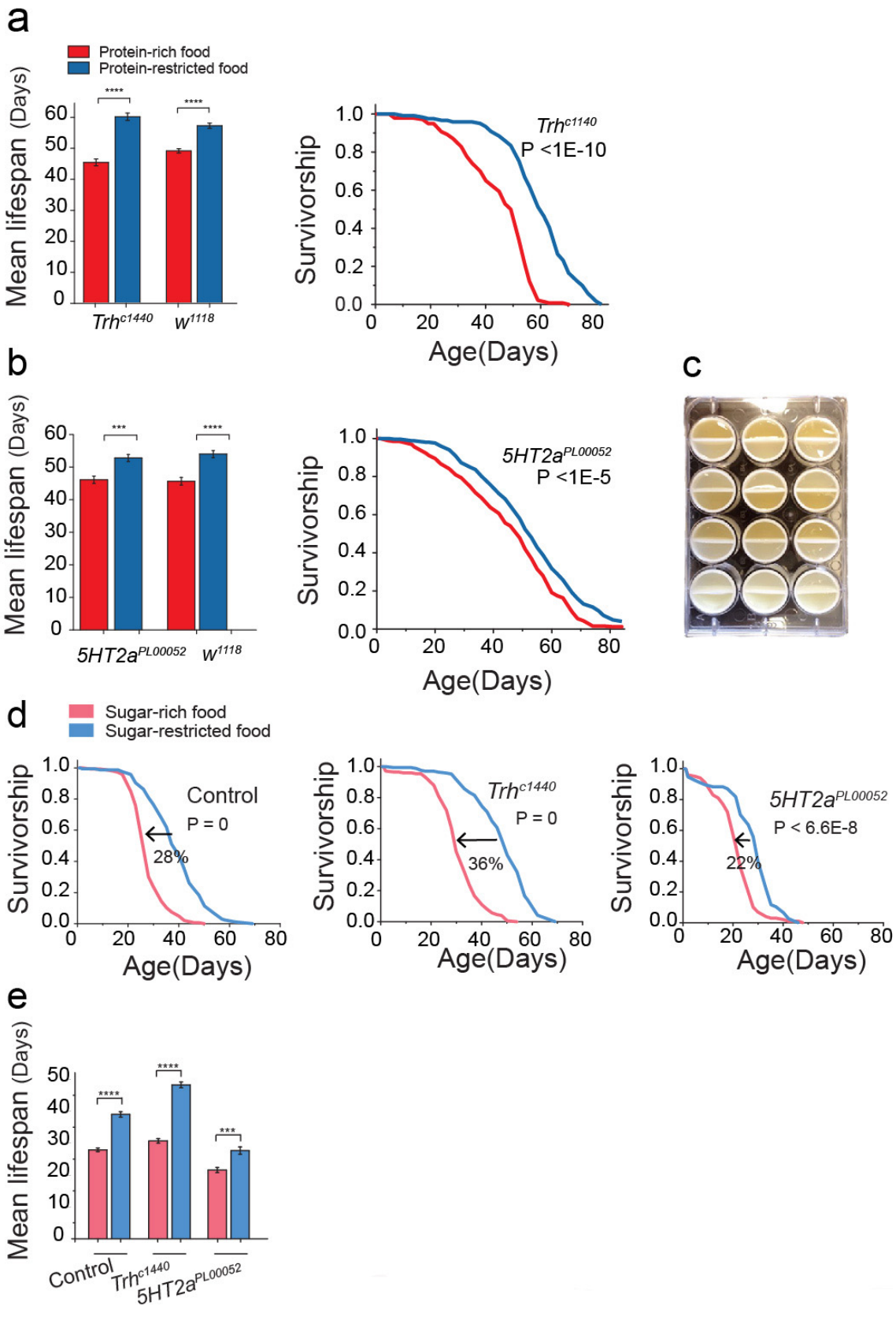
Supplemental Information



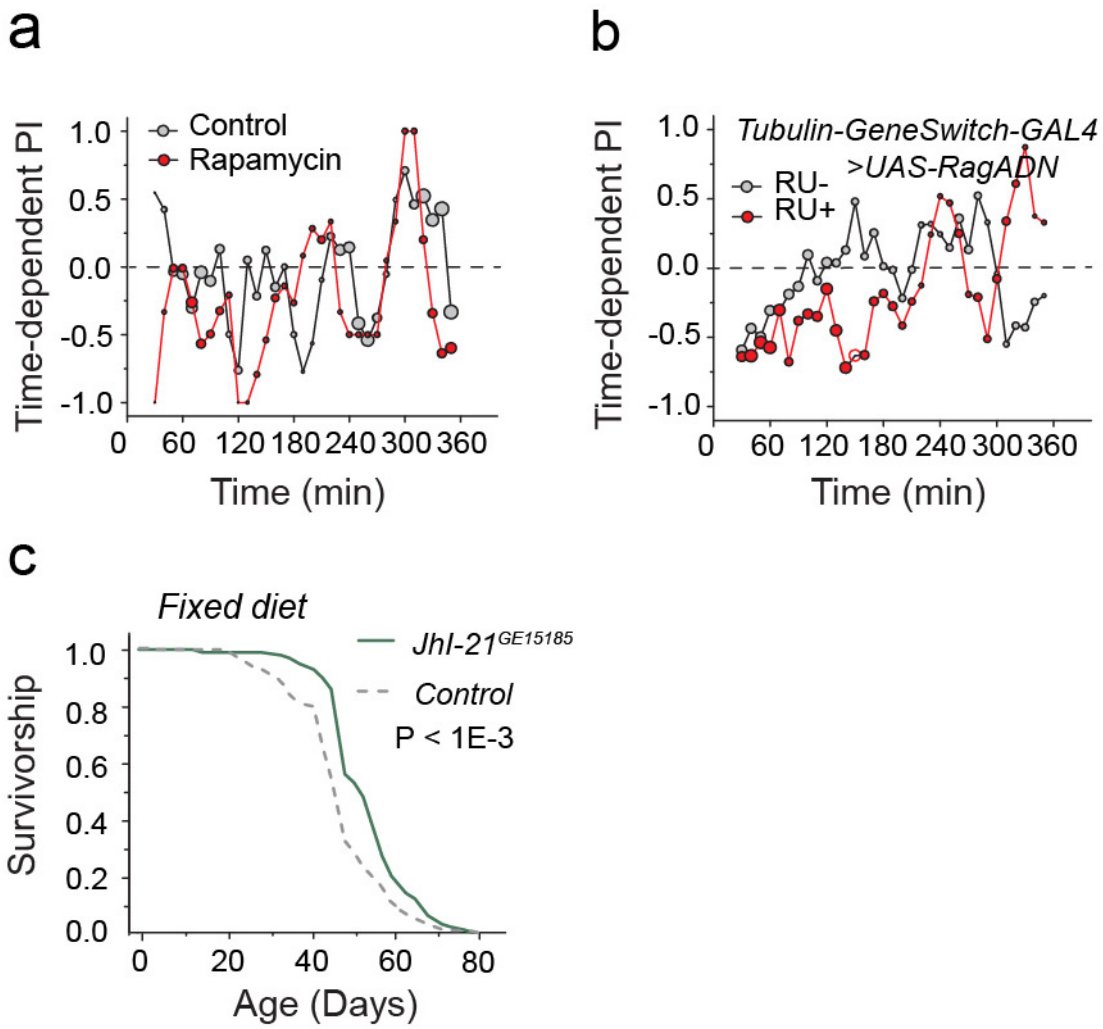
Supplemental Figure 4.1. Characteristics of protein feeding behavior in flies.
(A) Protein choice is energy state-dependent. Canton S. females starved for longer periods of time (e.g., 24h vs. 70h) had stronger protein preference (Student's t-test, ** P≤0.01). (B) When given a choice between isocaloric 1% autolyzed yeast vs 1% sucrose choice, 24h-starved Canton S. female flies show modest positive correlation between total feeding time during the choice test and time spent consuming the sugar meal ($F(1,32)=17.2$, $P < 2.4E-4$). Individual flies' first meal choice did not affect relationship between these correlates. (C) Starved female flies preferred wide concentrations of autolyzed yeast. The choice was given to 24h starved Canton S. female as 1% sucrose vs. 1% sucrose with different concentrations of autolyzed yeast. Bars indicate mean and standard error of mean (SEM). (N= 6 per each concentration treatment; one-way ANOVA).


a
Time-dependent PI
1.0
0.5
0.0
-0.5
-1.0
Control
5HT2a MI3299
30 60 90 120 150 180
Time (min)
b
Preference Index
1.0
0.5
0.0
-0.5
-1.0
*
*
5HT2a MI00459-GAL4 /+
UAS-5HT2a RNAi/+
5HT2a MI00459-GAL4 > UAS-5HT2a RNAi
c
Preference Index
1.0
0.5
0.0
-0.5
-1.0
5-HT1a MB9812
5-HT1a MB9978
5-HT1b MB5181
5-HT1b MB5999
5-HT2b MB6501
Control
d
Preference Index
1.0
0.5
0.0
-0.5
-1.0
1% sucrose
1% sucrose+ 1mM denatonium
10% sucrose
1% sucrose
Control
Trh c1440
5HT2 PL00052
Control
Trh c1440
5HT2 PL00052
e
Feeding time (sec)/h
200
150
100
50
0
UAS-shi ts /+
Trh-GAL4/+
Trh-GAL4>UAS-shi ts

Supplemental Figure 4.2. Serotonin receptor 2a is required for protein preference and mutation in Trh or 5HT2a does not affect other taste modality-dependent choice behavior.
(A) We genetically knocked-down 5HT2a transcript level in the 5HT2a-positive neurons using $5HT2a^{Mi00459\text{-}GAL4}$> UAS-5HT2a RNAi and tested protein preference of female flies. The choice was given as 2% autolyzed yeast +1% sucrose vs. 1% sucrose. Genotype effect was tested with one-way ANOVA followed by Tukey's multiple comparison (* $P \leq 0.05$). (B) Time-dependent preference plot of flies carrying another mutant allele of 5HT2a ($5HT2a^{MI3299}$). The preference was abrogated as seen in other 5HT2a mutant. The choice was given as 2% autolyzed yeast +1% sucrose vs. 1% sucrose. (C) Protein preference of three serotonin receptor mutants. We tested 2 mutant alleles for 5HT1a and 5HT1b mutation. We used BSA as a protein source. Individual symbols indicate average PI from three female flies. Boxes denote mean and SEM among biological replicates. Statistical significance for the genotype effect was determined using one-way ANOVA. (D) When given a choice between sweet vs. bitter food, Trh and 5HT2a mutant flies correctly distinguish foods and choose the sweet food like Canton S., control animals. When given a choice between sweet vs. sweeter food, Trh and 5HT2a mutant flies correctly distinguish foods and choose the sweeter food like Canton S, control animals. (E) To determine whether silencing serotonergic neurons disrupts normal feeding activity, we calculated the total time that flies spent interacting with the food during putative feeding bouts during the choice test and found no difference between control and test genotypes. Symbols indicate individual flies, and bars indicate mean among biological replicates (one-way ANOVA).


a
Protein-rich food
Protein-restricted food
Mean lifespan (Days)
Trhc1440
w1118
Survivorship
Trhc1140
P <1E-10
Age(Days)
b
5HT2aPL00052
w1118
5HT2aPL00052
P <1E-5
c
d
Sugar-rich food
Sugar-restricted food
Control
P = 0
28%
Trhc1440
P = 0
36%
5HT2aPL00052
P < 6.6E-8
22%
e
Control
Trhc1440
5HT2aPL00052

Supplemental Figure 4.3. Lifespan analysis of Trh and 5HT2a mutants in various diet conditions.
Trh (A) and 5HT2a (B) mutants showed a modest, but statistically significant, life extension under protein restriction. For mean lifespan, statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison. For diet-dependent survival, significance was determined using log-rank test. Based on cox-regression analysis, we found significant interaction between genotype and diet effect for Trh mutant ($P< 0.01$) and 5HT2a mutant ($P < 0.05$). (C) We printed plastic vial dividers using a Form 1+ 3-D printer to retrofit standard 12-well cell culture plates. These were then placed underneath acrylic chambers to create conditions in which aging flies were able to choose from two different feeding wells throughout their lifespan. These wells were then filled with identical (control) or nutrient-specific diets. (D) The high sucrose diet significantly reduces lifespan of control (w^{1118}) flies and flies with mutation in Trh and 5HT2a (log-rank test). (E) Mean lifespan of flies exposed to either sugar-rich or -restricted diets. For mean lifespan, statistical significance was determined by one-way ANOVA followed by Tukey's multiple-comparison (*** $P \leq 0.001$. **** $P \leq 0.0001$). Based on cox-regression analysis, we found significant interaction between genotype and diet effect for Trh mutant (P=1.1E-1) and 5HT2a mutant (P=3.1E-2).



Supplemental Figure 4.4. TOR signaling does not influence protein choice, and Jhl-21 mutant flies are long-lived when fed a conventional fixed diet.

We tested the effect of down-regulation of TOR signaling on protein preference in fully-fed flies in the FLIC assay by (A) treating flies with Rapamycin, or (B) overexpressing a dominant negative form of RagA. We used autolyzed yeast as the protein source in these experiments. Down-regulation of TOR signaling using either manipulation was not sufficient to mimic starvation and induce protein preference in fully-fed animals. (C) Jhl-21 mutants live significantly (13 %) longer than control flies when aged on a fixed 10% sugar and yeast (w/v) diet (log-rank test).

Supplemental Table 4.1. A list of candidates used in the reverse genetic screen of protein preference.

The list is showing the genotype or treatment of candidates in descending orders of average preference index (PI). The summary of PI and candidate is graphically depicted in Figure 4.2a.

Cadidate ID	*Candidates*
1	*NpfR1^{c01896}*
2	*Rut^{1}*
3	*5HT2b^{MB06501}*
4	*UAS-DopECR RNAi;Elav-GeneSwitch-GAL4*
5	*Gr68a$^{\Delta 2}$*
6	*AstC$^{\Delta}$ #2*
7	*AstC$^{\Delta}$ #30*
8	*Orco^{2}*
9	*ppk28$^{\Delta}$*
10	*Tub-GeneSwitch-GAL4>UAS-obp99a*
11	*chico^{1}*
12	*Dnc^{1}*
13	*Poxn$^{\Delta M22\text{-}B5\text{-}\Delta XB}$*
14	*GH146-GAL4 >UAS-shi^{ts}*
15	*CS* treated Haloperidol 4 days
16	*D2R^{f06521}*
17	*Foxo$^{\Delta 94}$*
18	*Tubulin-GeneSwitch-GAL4>UAS-Megalin RNAi*
19	*Gr93a^{3}*
20	*5HT1b^{MB5181}*
21	*CS* treated 3IY 6 days
22	*Gr64f^{MB12243}*
23	*Tubulin-GeneSwitch-GAL4>UAS-GCN2 RNAi*
24	*CS* treated 3IY 5 days
25	*Dilp2-GAL4>UAS-dsk RNAi*
26	*Poxn$^{\Delta M22\text{-}B5\text{-}Full1}$*
27	*Gr32a^{1}*
28	*Gr33a^{1}*
29	*Trh-GAL4 >UAS-Kir 2.1*
30	*Tdc2-GAL4>UAS-shi^{ts}*
31	*w^{1118}*
32	*Tubulin-GeneSwitch-GAL4>UAS-Cublin RNAi*
33	*Gr66a-GAL4 >UAS-rpr*
34	*DopR1^{f02676}*
35	*5HT1a^{MB9978}*
36	*5HT1b^{MB5999}*
37	*Thor1^{null}*
38	*ign $^{\Delta 58\text{-}1}$*
39	*Elav-GeneSwitch-GAL4 >UAS-DopECR*
40	*5HT1a^{MB9812}*
41	*CS* treated 100uM Mianserin 8days
42	*Gr5a$^{\Delta 5}$*
43	*Gr66a$^{Df(3L)ex83}$*
44	*Gr5a-GAL4>UAS-rpr*
45	*CS* treated 1mM Ketanserin
46	*Dnc^{1395}*
47	*Dilp2-GAL4>UAS-dsk;*
48	*CS* treated Haloperidol 5 days
49	*Ir64a^{MB05283}*
50	*CCKLR 17D1^{MB02688}*
51	*AstC $^{\Delta}$ #16*
52	*S6KII^{g1846}*
53	*AstC$^{\Delta}$ #23*
54	*CS* treated 50uM Mianserin_8days
55	*yw*
56	*w[CS]*
57	*ppk28-GAL4>UAS-shi^{ts}*
58	*Poxn$^{\Delta M22\text{-}B5\text{-}SuperA}$*
59	*Elav-GeneSwitch-GAL4>UAS-dsk*
60	*Th-GAL4>UAS-shi^{ts}*
61	*c772-GAL4>UAS-shi^{ts}*
62	*ppk28-GAL4>UAS-rpr*
63	*5HT2a^{PL00052}*
64	*Gr33a-GAL4>UAS-rpr*
65	*CS* treated 100uM Mianserin_8days
66	*CS* treated 3IY 4 days
67	*Trh-GAL4>UAS-shi^{ts}*
68	*CS* treated Ketanserin 3days

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Chapter 5

Gustatory and Metabolic Perception of Nutrient Stress in *Drosophila*[1]

Abstract

Sleep loss is an adaptive response to nutrient deprivation that alters behavior to maximize the chances of feeding prior to imminent death. Organisms must maintain systems for detecting the quality of the food source to resume healthy levels of sleep when the stress is alleviated. We have determined that gustatory perception of sweetness is both necessary and sufficient to suppress starvation-induced sleep loss when animals encounter nutrient-poor food sources. We further find that blocking specific dopaminergic neurons phenocopies the absence of gustatory stimulation, suggesting a specific role for these neurons in transducing taste information to sleep centers in the brain. Finally, we show that gustatory perception is required for survival, specifically in a low nutrient environment. Overall, these results demonstrate an important role for gustatory perception when environmental food availability approaches zero and illustrate the interplay between sensory and metabolic perception of nutrient availability in regulating behavioral state.

Introduction

Starvation is a condition of extreme nutrient stress that leads to rapid death. Upon detecting the absence of environmental nutrient sources, organisms use multiple strategies to adjust resource allocation in order to maximize the chances of finding a food source, including inducing longer foraging searches (Wiersma et al., 2005) and limiting sleep behavior (Keene et al., 2010; MacFadyen et al., 1973) Sleep loss in *Drosophila melanogaster* is a characteristic response to nutrient deprivation that appears approximately 12 hours after the removal of a food source, in males it is followed by death in another 12 hours (Keene et al., 2010) . Sleep loss is thought to represent a cost to the organism (Bergmann et al., 1996; Rechtschaffen et al., 1983; Shaw et al., 2002), and mechanisms for evaluating the environment and terminating

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this behavioral response when food is available would likely confer an adaptive benefit. A deeper understanding of how organisms perceive and respond to environmental stress could offer substantial benefit to humans attempting to maintain maximal health in the face of food shortages and unstable environmental conditions. The strategies used by organisms to evaluate the sufficiency of a food source and to initiate or suppress sleep loss under very low nutrient conditions remain largely unknown and represent one path toward understanding global stress response.

Materials and Methods

Husbandry

All fly stocks were maintained on a standard cornmeal-based larval growth medium and maintained in a temperature and humidity-controlled environment (25°C, 60% humidity) with a 12:12 hour light:dark cycle. Prior to experimentation, the F0 generation was mated in egg-collection chambers and eggs were collected on grape agar medium and distributed at 10ul per vial to provide consistent larval density. Following eclosion, flies were mated for 2 days and then males were separated into SY10 medium at a constant density of 25 flies/vial for 2-4 days (Linford et al., 2013) prior to experimentation.

Fly Stocks

The genotype of the *Δ*Gr64 line was *R1;R2/+;ΔGr64/ΔGr64* and the rescue was *R1;R2/Gr5a-GAL4; ΔGr64,UAS-Gr64abcdGFPf/ΔGr64.* Heterozygous controls used for comparison to the ΔGr64 mutant were *R1;R2/+; ΔGr64/+.* These stocks were a kind gift from the Amrein laboratory (Slone et al., 2007). The *Gr64* genomic rescue flies were made by inserting the CH322-176A20 genomic fragment (Venken et al., 2009) into the 59D3 chromosome location. Transgenesis was performed by BestGene, Inc. (Irvine, CA). *Gr64a*^{2}, *Gr5a-Gal4*, *Gr66a-Gal4*, and *UAS-Kir*$^{2.1}$ flies were kind gifts from A. Dahanukar (Dahanukar et al., 2007) K. Scott (Marella et al., 2006), J. Carlson (Weiss et al., 2011), and R. Baines (Baines et al., 2001), respectively. The *TH-Gal4* subset lines (C1-G1) were a kind gift from M. Wu(Liu et al., 2012). *tubGAL80*^{ts} (7017), *UAS-tntG* (28838), *and TH-Gal4* (8848) were from the Bloomington Stock Center and the *UAS* and *Gal4* lines were backcrossed 6 generations into the *w*^{1118} background. All other experiments used the Canton-S control strain.

Sleep behavior

Flies were transferred to 5mm polycarbonate tubes fitted with a food cup (trimmed pipet

tip with glued end) containing 50µl of SY10 medium and allowed to equilibrate within a Trikinetics activity monitoring apparatus (Trikinetics, Watham MA) for at least 1 day (Day 0). For all temperature manipulations, the flies were placed at the test temperature at the start of day 0. Baseline sleep behavior was subsequently monitored for 24 hours (day 1 ZT0-24). At ZT0 (lights-on) of day 2, the food cup was replaced with the specified test medium (1% Bacto-agar with or without additions) with minimal disturbance to the fly. Flies were not starved prior to placement on the test medium. Sleep behavior was monitored for at least 24h on the test medium. Sleep was calculated as the total time spent in periods of 5 or more minutes of continuous inactivity (34) and grouped into 30 minute bins. The percent sleep change was calculated for each fly during hours 12-24 of each day using the following equation: (Day 1 – Day 2) / Day 1 X 100. If a fly died within the Test day (no further activity counts), the sleep calculation was adjusted to include only the window of time over which the fly survived on both day 1 and day 2. Within each experimental cohort the values were normalized to a group of flies experiencing SY10 on both Day 1 and Day 2 in order to correct for time-dependent effects (typically less that 10%). All analysis was conducted using custom scripts in the R statistical software package that are available from the authors upon request. The observed total sleep levels on Day 1 for the genotypes used in this manuscript are listed in Supplemental Table 5.3.

Feeding Behavior

Abdominal Blue: Food. Flies were transferred from SY10 medium to vials containing the test medium for 24 hours and then transferred to test medium containing 0.5% FD&C blue #1 for 2 hours (Tanimura et al., 1982). Individual flies were frozen and homogenized in 40µl of PBS + 0.01% Triton X-100 using a Qiagen TissueLyser. The lysate was centrifuged at 2250 x g for 20 minutes. 20 µl of the resulting supernatant was analyzed at 630nm using a half-diameter 96-well plate using a standard curve with the blue dye. A control group fed without blue dye was run simultaneously to determine the nonspecific 630nm absorbance and this value was subtracted from all measurements.

Blue Frass. Groups of 15 flies were placed on SY10 medium containing 0.5% FD&C blue #1 for 24 hours (day 1, baseline day) and then transferred to test medium containing 0.5% FD&C blue #1 in 28.5x95 mm (standard wide) vials fitted with a layer of transparency film on the inner surface of the vial. After 24h, the transparency film was removed and imaged to determine the total number of spots and the area of each spot.

Food Interactions. Individual interactions with the food were counted using the FLIC, a novel apparatus that continuously (roughly 500 times/second) monitors feeding behavior by recording an electrical signal for every interaction a fly makes with a liquid food source (for details see ref.

(Ro et al., 2014)). Flies were placed individually into FLIC measurement arenas for 6 hours with the indicated food type, and the total number of seconds spent interacting with the food was recorded.

Video analysis. We observed and recorded the position of flies in 5mm activity monitoring tubes as described previously (Linford et al., 2012). Briefly, we recorded movies at 1 frame/second and used an in-house software system (DTrack) to calculate the centroid position for each fly and plotted the position along the axis of the tube over time. This software is available from the authors upon request.

Survival. Flies were prepared for survival experiments as described (Linford et al., 2013) with a slight modification. Male flies were transferred to the test medium (1% agar with or without the indicated carbohydrate) between day 3 and 10 post-eclosion and the time of transfer is indicated as time 0. Flies were transferred to new food 3x per week and survival was recorded every 1-2 days.

Results

We have observed, as previously reported, that adult *Drosophila* spontaneously adjust their behavioral patterns to reduce sleep when starved (Figure 5.1a) (Erion et al., 2012; Keene et al., 2010). To more completely understand how organisms modulate sleep in response to food availability, we measured the extent of nutrient deprivation required to induce sleep loss. These assays consist of monitoring the activity of male Canton-S flies on a complete medium (10% sugar:Brewer's yeast medium, see methods) for one day (Day 1) to estimate baseline sleep in the fully-fed condition, followed by data collection from one or more days on a 1% agar-only starvation medium (Day 2+), which provides water and humidity but not nutrients. We modified this procedure by augmenting the agar medium with either 50mM D-glucose or 550mM D-glucose. Interestingly, 50mM D-glucose was sufficient to promote normal sleep patterns (Figure 5.1b), despite evidence that the animals remained in a state of severe malnourishment (Figure 5,1c). Indeed, median survival on 50mM D-glucose medium was only 5.1 days, which was only slightly longer than complete starvation (1.1 days) and substantially less than 550mM D-glucose (21.2 days; Figure 5.1c). The discordance between the amount of nutrients apparently required to support life and the amount sufficient to eliminate starvation–induced sleep loss suggested that the animals may be capable of regulating sleep through evaluations of the food source that are independent of its energetic value.

Previous work has established that sensory perception of the food source influences sleep

architecture but not total sleep in fully fed *Drosophila*(Linford et al., 2012), and we wondered whether it also regulates sleep loss in response to nutrient stress. We tested modulatory roles for gustatory perception using a mutant line containing a deletion of the *Gr64a-f* sweet-sensing receptor gene cluster (ΔGr64)(Slone et al., 2007), which has been previously reported to suppress proboscis extension in response to glucose as well as to several other sweet tastants (trehalose, arabinose, maltose, sucrose, and glycerol) but not to fructose. Concentrations of D-glucose that promoted normal sleep behavior in control (ΔGr64/+) heterozygous flies were ineffective in flies homozygous for the ΔGr64 mutation (Figure 5.2a), with the defect particularly apparent at low nutrient concentrations. This defect was fully rescued by *Gal4-UAS*-based rescue of the ΔGr64 deletion (Figure 5.2a) or by transgenic expression of an ectopic copy of the *Gr64a-f* genomic region (Supplemental Figure 5.1a). We note that high concentrations of D-glucose still promoted sleep in ΔGr64 mutant flies, albeit not to the extent observed in control animals, suggesting that taste perception may be particularly important at low nutrient concentrations. There are multiple reports of a gustatory-independent nutrient sensor that regulates behavior and food preference under starvation conditions(Burke and Waddell, 2011; Dus et al., 2011; Fujita and Tanimura, 2011; Linford et al., 2012; Stafford et al., 2012), and it is likely that this sensor compensates for lack of gustatory perception to promote normal sleep behavior when nutrients are replete.

To ensure that the sleep loss phenotype observed in ΔGr64 mutant flies is due to their failure to taste glucose rather than a deficiency in their metabolic response to nutrients in general, we repeated the experiments, replacing D-glucose with fructose, which has roughly the same caloric value but can be perceived by ΔGr64 mutant flies (Jiao et al., 2007; 2008; Linford et al., 2012; Slone et al., 2007). Consistent with taste as a causal factor, ΔGr64 mutants exhibited a normal pattern of sleep behavior that was statistically indistinguishable from the control and genetic rescue lines (Figure 5.2a, Supplemental Figure 5.1b). Together, these data suggest that behavioral decisions affecting sleep regulation rely heavily on gustatory perception when nutrients are scarce.

One alternative interpretation of the data presented thus far is that sleep loss is not regulated *per se* but instead occurs as flies approach death—at any given point in time, a greater proportion of flies in low nutrient environments will be near death than their better-fed siblings. Data from flies carrying the ΔGr64 mutation effectively refute this hypothesis. On their first day of 50mM D-glucose feeding, ΔGr64 flies exhibit nearly identical sleep loss to levels observed using starved flies (either ΔGr64 or control, Figure 5.2b*).* However, the fraction of each population that is predicted to be near death is very different; 100% of the starved population will die in the subsequent 24 hours while less than 5% of 50mM-fed mutants will do so (see

also Figure 5.5). Furthermore, if sleep loss was strongly coupled to death, we would expect to observe its onset much later in the ΔGr64 mutant animals on 50mM D-glucose compared to starved controls, which does not happen (Figure5.2b). The sustained sleep loss in the ΔGr64 mutant flies, therefore, is consistent with a model where the animals regulate sleep based on the perception of food.

A second alternative explanation is that ΔGr64 mutant flies respond normally to a given amount of nutrient intake but are effectively "self-starving" through an undocumented effect of the mutation on feeding behavior. To test this model, we estimated food intake in three ways. First, we replaced the test medium with a blue dye-labeled medium for 2 hours near the end of the assay, which corresponds to the period of the circadian day when food intake is thought to be maximal(Xu et al., 2008). There was no difference in the amounts of blue dye between mutant and control flies (Figure 5.2c). Second, we measured 24-hour fecal deposition by pre-loading the flies on blue dye during the pre-treatment period on complete medium and measuring blue frass (excrement) deposits on the side of the vial during a 24-hour exposure to the 50mM D-glucose test medium, on which the mutant phenotype was most prominent(Cognigni et al., 2011). There was no difference in the total deposition per fly (Figure 5.2d). Third, we used a novel assay (the Fly Liquid food Interaction Counter, or FLIC) that quantifies food interactions by allowing the fly to complete a low-voltage electrical circuit by touching or consuming a liquid food while standing on a metal base (Ro et al., 2014). We found that there was no difference between the ΔGr64 and control flies in the amount of time spent interacting with the food (P=0.56; Figure 5.2e).

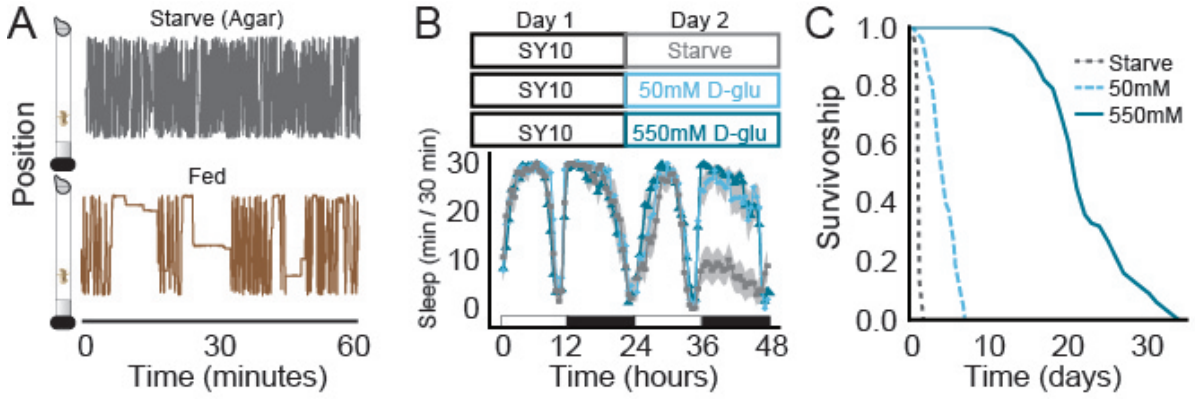


Figure 5.1. Sleep behavior is regulated by nutrient availability.
(A) Sample video trace of the fly (Canton S male) position over time on a complete food (10% sugar:yeast, SY10) or after 20h starvation. The y-axis represents the fly position in a tube resting horizontally with a video camera above. (B) Sleep behavior (30min bins) during day 1 on SY10 food followed by day 2 on the indicated test medium. (C) Survival on starvation medium or the indicated amount of D-glucose. All points with error bars represent the mean ± SEM from 30 to 100 flies

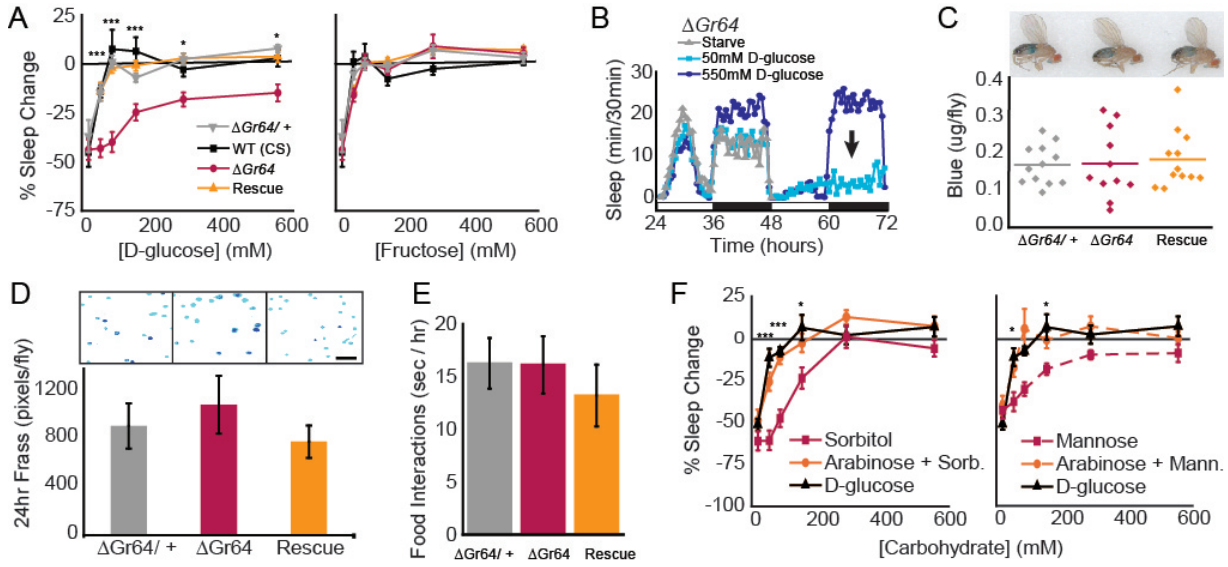


Figure 5.2. Appetitive gustatory perception is required for low environmental nutrient density to promote sleep.

(A) Deletion of the Gr64a-f gene cluster significantly impaired normal sleep behavior on a D-glucose but not fructose medium. Where noted, the ΔGr64 mutant was significantly different from all other groups with no differences between the other groups. For A and F, all points with error bars represent the mean ± SEM from 30 to 100 flies. Statistical significance was determined by one-way ANOVA with a Tukey posttest. (B) Sleep behavior (30min bins) for ΔGr64 on the test day (24–48 h) and the following day. The arrow indicates the persistence of sleep loss on 50 mM D-glucose. (C) Abdominal food content after 2 h on 50 mM D-glucose containing 0.5% FD&C Blue #1. P = 0.89, one-way ANOVA. (D) Frass production during the test day on 50 mM D-glucose containing 0.5% FD&C Blue #1. n = 3 vials of 15 flies. (Scale bar, 1 mm.) P = 0.56, one-way ANOVA. (E) Food interactions during day 2 for flies exposed to 50 mM D-glucose in liquid. n = 4–6 flies per group. P = 0.43, one-way ANOVA. (F) Sleep change when flies (Canton-S) Ire fed the indicated concentrations of carbohydrates that were nutritious but nonsweet (sorbitol or mannose) relative to D-glucose and a dietary rescue with the addition of arabinose (Table S1). Where noted, sorbitol or mannose was significantly different from the other groups with no differences between groups. *P < 0.05; **P < 0.01; ***P < 0.001. See also Supplemental Figure 5.1

The abnormal sleep response profile observed in ΔGr64 mutants can be recapitulated in "wild-type" (Canton S) animals using alternative nutrient sources that offer nutrition without stimulating gustatory sensilla (sorbitol and mannose) (Burke and Waddell, 2011; Dahanukar et al., 2007; Fujita and Tanimura, 2011; Stafford et al., 2012). We found that providing carbohydrate nutrition without sweetness in the feeding medium led to a response profile that largely phenocopied the concentration-dependent sleep response of the gustatory mutant, with "starvation-like" sleep loss at low environmental nutrient concentrations relative to control groups exposed to D-glucose (Figure 5.2f, Supplemental Table 5.1). This defect was fully rescued by the addition of a non-nutritive sweetener (arabinose or L-glucose) (Burke and Waddell, 2011; Fujita and Tanimura, 2011; Stafford et al., 2012). to the feeding medium in combination with

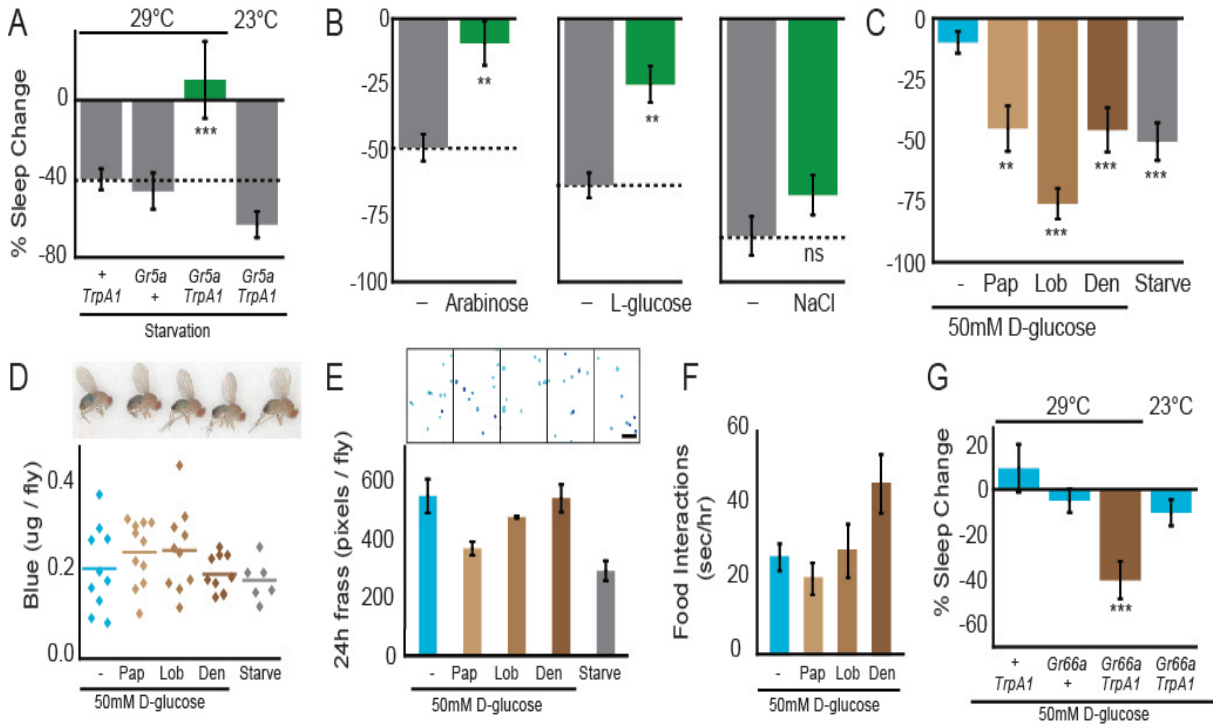


Figure 5.3. Appetitive gustatory perception is sufficient to prevent sleep loss.
(A) Activation of Gr5a-containing sweet-sensing neurons with Gr5a-Gal4/UASTrpA1 (29 °C) on 1% agar starvation medium was sufficient to promote an increase in sleep relative to each of the control groups. One-way ANOVA with Tukey's post test, n = 30–100. (B) Feeding with sweet non-nutritious compounds (250 mM arabinose or L-glucose) but not a salty compound (100 mM NaCl) was sufficient to promote an increase in sleep relative to starvation. Student t-test. Each compound is compared with its contemporaneous control, n = 30–100. (C) Addition of 1 mM of the bitter tastants papaverine (Pap), lobeline (Lob), and denatonium (Den) to 50 mM D-glucose caused sleep loss relative to glucose alone. One-way ANOVA with Tukey's posttest, n = 30–100. (D) Abdominal food content following 2 h on the indicated test food containing 0.5% FD&C Blue #1. P = 0.27, one-way ANOVA. (E) Frass production on the test day for the indicated foods containing 0.5% FD&C Blue #1 relative to glucose alone. n = 3 vials of 15 flies. (Scale bar, 1 mm.) One-way ANOVA with Tukey's post test. (F) Total time of interactions between individual flies and the indicated foods. Adding a bitter tastant to 50 mM D-glucose did not change flies' tendency to interact with a food. One-way ANOVA with Tukey's posttest, n = 9–16 flies per group. Flies' interaction with a food containing Pap was significantly lower than a food containing Den (P = 0.034). (G) Activation of Gr66a-containing bitter-sensing neurons with Gr66a-Gal4/UAS-TrpA1 (29 °C) on 50 mM D-glucose was sufficient to promote a decrease in sleep relative to all of the control groups. One-way ANOVA with Tukey's posttest, n = 30–100. Summary data are expressed as the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. See also Supplemental Figure 5.2.

either mannose or sorbitol (Figure 5.2f, Supplemental Figure 5.1c, Supplemental Table 5.1). As before, these results were not driven by differences in food uptake; we confirmed the presence of blue dye in the abdomens of flies exposed to all concentrations of mannose and sorbitol (Supplemental Figure 5.1d). These results further support the notion that appetitive gustatory

perception is required to promote normal sleep behavior, particularly when environmental nutrient availability is low.

Having established that gustatory perception is required to promote normal sleep behavior in the presence of nutrients, we next asked whether appetitive signals alone are sufficient to prevent starvation-induced sleep loss. To simulate sweet taste in the absence of nutrients, we expressed the temperature-sensitive activating ion channel TRPA1 (Kang et al., 2010) under control of the *Gr5a-GAL4* driver, which is broadly expressed in sweet-sensing neurons(Marella et al., 2006). We found that this manipulation eliminated starvation-induced sleep loss (Figure 5.3a) when the neurons are activated (29°C) but not in control, non-activating conditions (23°C). Neuronal activation only during the daytime period when flies are most actively feeding recapitulated the reversal of sleep loss observed when those same neurons were activated continuously during the 48hours of starvation (Supplemental Figure 5.2). We also tested two sweet but non-nutritional sugars, arabinose and L-glucose, and both significantly suppressed sleep loss (81% and 60%, respectively, Figure 5.3b, Supplemental Figure 5.3a-d, Supplemental Table 5.1). On the other hand, salt (NaCl, 100mM) had no significant effect (19%, Figure 5.3b, Supplemental Figure 5.3a-b). We conclude that gustatory perception of sweetness is sufficient to promote normal sleep in the absence of available nutrients, even when death is imminent (Supplemental Figure 5.3d).If gustatory perception of sweetness regulates sleep behavior, we would anticipate that the gustatory perception of bitterness (Masek and Scott, 2010; Zhang et al., 2013), which has been shown to counteract sweet-responsive phenotypes, would block the ability of sweet taste inputs to promote sleep. We found that addition of either 1mM denatonium, lobeline, or papaverine (Weiss et al., 2011) to 50mM D-glucose substantially blocked the capacity for this sugar to suppress starvation-induced sleep loss (Figure 5.3c). As before, we ruled out feeding differences as the cause for these observations. Abdominal blue dye levels from the 2 hour dye feeding assay (Figure 5.3d), frass deposition (Figure 5.3e, Supplemental Figure 5.3e), and total food interactions (Figure 5.3f, Supplemental Figure 5.3f) were not decreased by the addition of bitter substances, which is consistent with idea that flies rapidly habituate to noxious but non-toxic tastants when there is no better choice available(Zhang et al., 2013). Furthermore, expression of TRPA1 under control of the *Gr66a-GAL4* driver, which is broadly expressed in bitter-sensing neurons (Marella et al., 2006), led to sleep loss in the presence of 50mM D-glucose (Figure 5.3g) when the neurons were activated (29°C) but not at a lower temperature, where the neurons fired normally (23°C). Together, these results indicate that multiple sensory modalities coordinate information about the potential quality of the food source to regulate sleep behavior, as predicted by our model.

We note that our findings, which indicate a strong relationship between gustatory perception

and starvation-induced sleep loss, differ from a prior report demonstrating that a non-nutritive sweetener, sucralose, failed to suppress sleep loss (Keene et al., 2010). We confirmed this prior observation. However, while we observed that sucralose was appetitive to control flies, it was also aversive to ΔGr64 mutant animals (Supplemental Figure 5.3g). The receptor-mediated signaling pathway for sucralose is not fully described in *Drosophila,* and these data suggest that sucralose may activate both sweet- and bitter-sensing neurons, making it a more complicated stimulus than is currently appreciated. Based on our model, a compound with both bitter and sweet properties would be unable to attenuate starvation-induced sleep loss.

Dopamine-containing neurons have been implicated as downstream mediators of sweet sensory input for other behavioral outputs including proboscis extension respons (Inagaki et al., 2012; Marella et al., 2012). We therefore tested whether dopaminergic neurons play a role in transducing sensory information to sleep regulatory centers. We selectively suppressed the activity of dopaminergic (tyrosine hydroxylase (TH)-containing) neurons by expressing the inward-rectifying potassium channel $KIR^{2.1}$ (Nitabach et al., 2002) in combination with *tub-Gal80^{ts}* (*TARGET*) (McGuire et al., 2004)under control of the *TH-Gal4* driver. The *TH-Gal4* construct has been shown to express in all of the major subsets of dopaminergic neurons(Liu et al., 2012). This approach allowed us to bypass developmental effects and only adjust neuronal activity during the experimental period. If gustatory stimulation leads to activation of dopaminergic neurotransmission, we would predict that blockade of TH-containing neurons (*TH-Gal4 / UAS-Kir$^{2.1}$;TARGET*) would phenocopy the ΔGr64 defect and lead to sleep loss at low nutrient concentrations. We found exactly this (Figure 5.4a,b). When the flies were

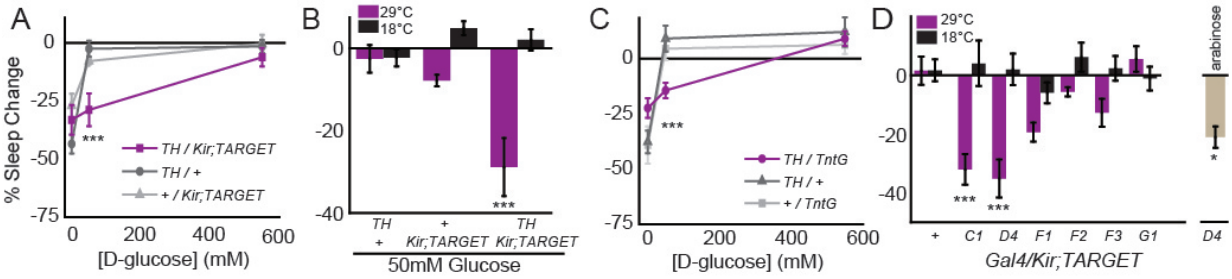


Figure 5.4. Blockade of dopaminergic neurotransmission phenocopies loss of appetitive gustatory perception.

(A and B) Blockade of TH-expressing neurons with TH-Gal4/UAS-Kir2.1;tub-Gal80ts (TARGET) at 29°C or (C) constitutive blockade using TH-GAL4/UAS-TntG at 25 °C suppressed sleep at 50 mM D-glucose relative to genetic controls at that glucose concentration. (D) Sleep loss on 50 mM D-glucose or 200 mM arabinose (where indicated) in the presence of dopaminergic subset Gal4 drivers with UAS-Kir2.1; TARGET relative to the UAS-Kir2.1; TARGET alone control. *P < 0.05; **P < 0.01; ***P < 0.001, one-way ANOVA with Tukey's post test. All data are expressed as the mean ± SEM from 30 to 100 flies. See also Supplemental Figure 5.3.

exposed to a high temperature (29°C, neurons blocked) only during the experiment, we saw a specific impairment of the response to low glucose (Figure 5.4a) that was not present at the low temperature (18°C, neurons firing normally, Figure 5.4b). We observed a similar result with constitutive expression of the tetanus toxin light chain (*UAS-tntG*), which blocks neurotransmission through an independent mechanism and does not require temperature manipulations (Figure 5.4c).

To begin narrowing down the specific neurons involved, we tested other *Gal4* drivers (labeled with the letters C-G) that were created in the laboratory of Mark Wu from fragments of the *TH* enhancer region. These drivers have been shown in a prior report to express in more confined, dopamine-producing neuron subsets based on TH antibody co-labeling (Liu et al., 2012). We found that expression of *UAS-Kir$^{2.1}$;TARGET* in both the *D4* and *C1* subsets eliminated the ability of 50mM D-glucose to suppress starvation-induced sleep loss (Figure 5.4D, Supplemental Figure 5.4a). Expression by *G1* and *F2* had no effect, while *F1* and *F3* partially suppressed sleep loss but the effect was not statistically significant. The *D1* driver combined with *UAS-Kir$^{2.1}$;TARGET* caused death within 24 hours when placed at 29°C. Based on the expression profile of the *D4* driver, we can effectively say that the PAM, PAL, PPM1, and PPL2 dopaminergic clusters are unlikely to be involved. Notably, the PPM1 neuron subset contains *TH-Gal4*-positive non-dopaminergic cells that we can effectively rule out with this analysis. To confirm that D4 dopaminergic neurons are in the gustatory sensing pathway, we also tested flies expressing *UAS-Kir$^{2.1}$;TARGET* under control of the *D4* driver using the sweet but non-nutritive sugar arabinose and also observed sleep suppression, consistent with a role for these dopaminergic neurons in the regulation of sleep by gustatory cues (Figure 5.4D). Further work will be required to determine the specific neuron(s) that relay gustatory signals to the sleep centers of the brain and whether dopamine alone, or a combination of neurotransmitters and neuropeptides, are involved.

Finally, we asked whether the lack of appetitive gustatory perception and its associated sleep dysregulation have broader consequences on organismal health. ΔGr64 mutants are not impaired in their survival under starvation (Figure 5.5a, Supplemental Table 5.2), and their lifespan is not adversely affected by sleep deprivation using the guest-host paradigm, which is a model for sleep stress where the pairing of a male and female in the same activity tube significantly reduces sleep in both animals (Gilestro et al., 2009) and eventually leads to death (Figure 5.5b, Supplemental Table 5.2). Thus, we conclude that the ΔGr64 animals are not 'sick' or broadly stress-sensitive.

Our model predicts that the negative consequences of losing sweet taste sensitivity would be most substantial under conditions of low nutrient availability. Consistent with this, we see that

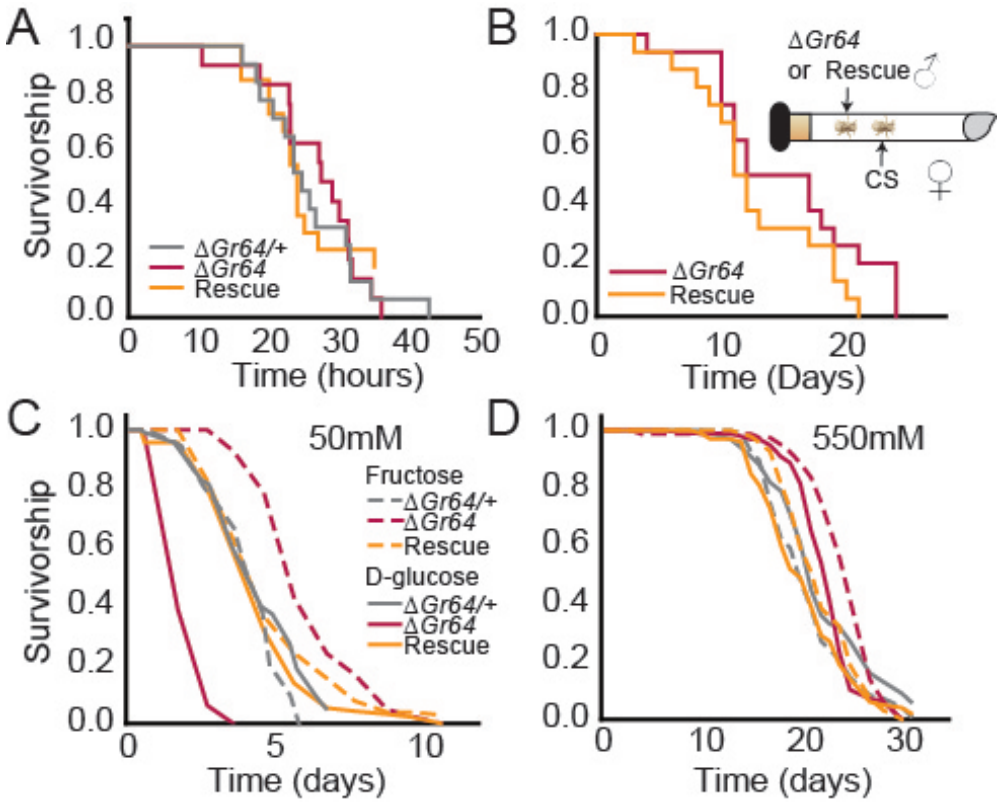
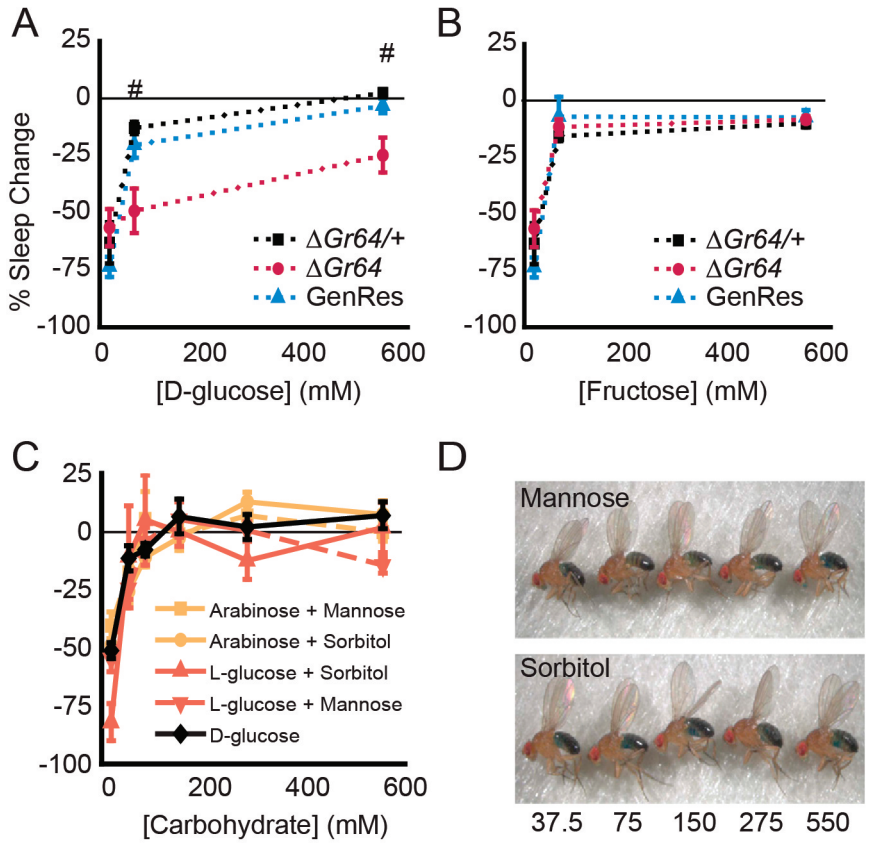


Figure 5.5. Loss of appetitive gustatory perception impairs survival in low nutrient environments. Survival in response to starvation (A) or sleep deprivation stress from the presence of a female "guest" in an activity monitor tube on a complete SY10 food (B) is not impaired by the ΔGr64 deletion. Survival on 50 mM (C) but not 550 mM (D) D-glucose is impaired by the ΔGr64 deletion relative to the fructose control. Number of flies, median survival, and P values are in Supplemental Table 5.2.

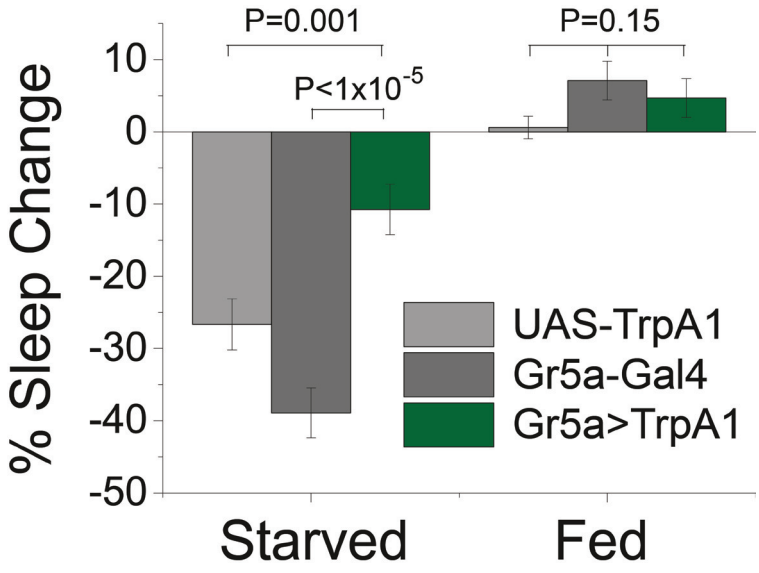
the ΔGr64 mutants are short-lived on the diet to which they are taste-blind (D-glucose) relative to fructose when the nutrient concentration is 50mM (69% difference in mean lifespan, Figure 5.5c, Supplemental Table 5.2). There is no significant difference between fructose and D-glucose for either the ΔGr64/+ control (1% lifespan difference, Figure 5.5c, Supplemental Table 5.2) or the genomic rescue lines (<1% lifespan difference, Figure 5.5c, Supplemental Table 5.2). We observed a similar reduced survival on low nutrients when the ΔGr64 deletion mutant was crossed to a different *Gr64* mutant that contains a deletion only in *Gr64a-c* (Dahanukar et al., 2007), indicating that the a-c region of the gene cluster may be important for survival under nutrient stress (Supplemental Table 5.2). However, under conditions of high nutrient density, the effects of the ΔGr64 mutation on survival are largely absent (Figure 5.5d, Supplemental Table 5.2). Thus, the negative effects of losing sweet-sensory perception are dependent on the environmental nutrient concentration. Interestingly, ΔGr64 mutant survival on both low and high fructose (which the animals can taste) is significantly enhanced relative to the two control backgrounds (Figure 5.5c,d, Supplemental Table 5.2). Together our results suggest an essential

role for gustatory perception in defining normal behavioral and physiological responses to nutrient variability, particularly under conditions of nutrient stress.

Supplemental Information

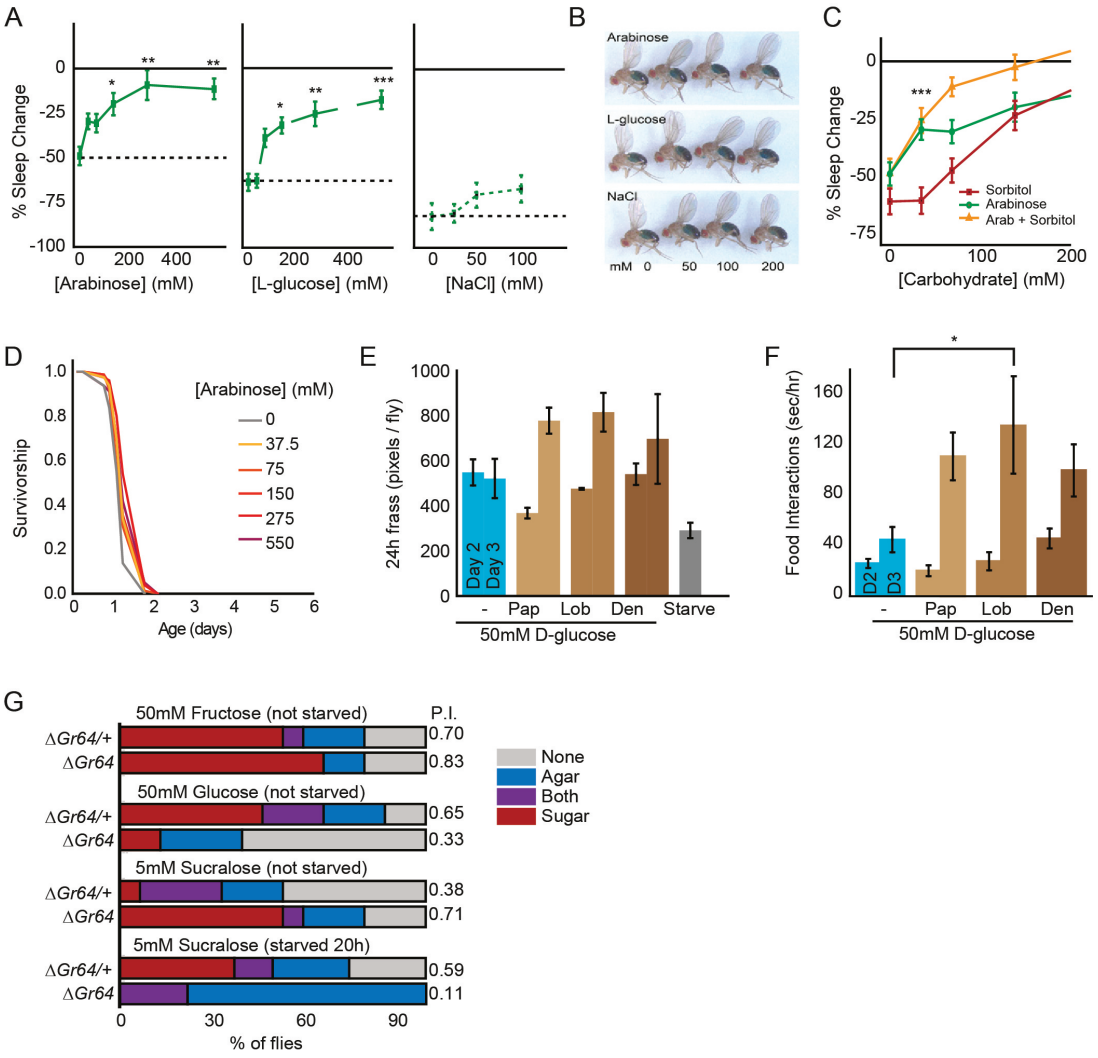


Supplemental figure 5.1 An ectopic copy of the Gr64a-f genomic region or yeast feeding can rescue the effects of Gr64 deletion (related to Figure 5.2).
Genomic rescue of ΔGr64a-f was sufficient to block the effects of ΔGr64 mutation on (A) D-glucose test food and had no effect on (B) fructose control food. Significance at each D-glucose concentration was determined by one-way ANOVA followed by Tukey's posttest. ΔGr64 mutant was different from all other groups with no differences between the other groups. (C) The combination of sweet-only (L-glucose or arabinose) and nutritional-only (sorbitol or mannose) produced a sleep response that was not distinguishable from D-glucose alone (one-way ANOVA at each concentration). (D) Abdominal color following 2 h of feeding at the indicated concentrations of mannose or sorbitol (mM) supplemented with 0.5% FD&C Blue #1.

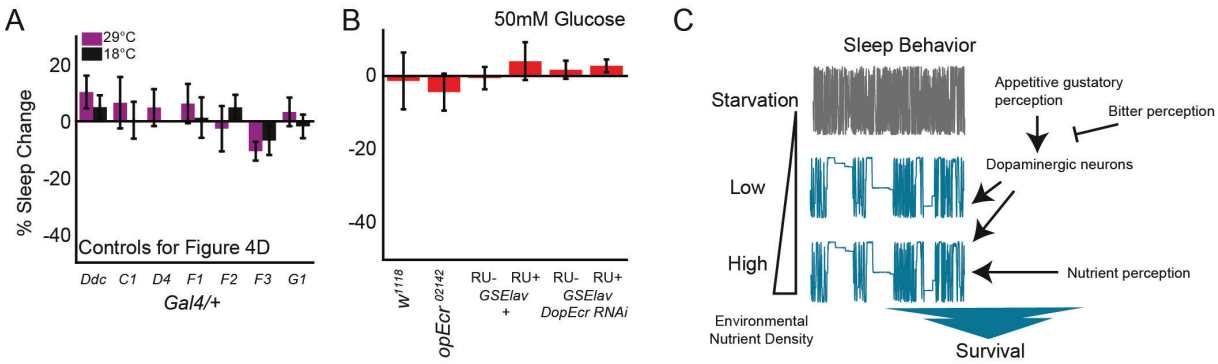


Supplemental figure 5.2 Acute activation of Gr5a neurons significant reverses starvation-induced sleep loss.

Activation of Gr5a-containing sweet-sensing neurons with Gr5a-Gal4/UAS-TrpA1 (29 °C) only during the daytime period, when flies are expected to be actively feeding significantly reduced the amount of sleep loss normally occurring on 1% agar starvation medium. One-way ANOVA among genotypes in the starved condition resulted in $P = 1.0 \times 10^{-6}$. Post hoc t tests indicate significant differences between the treatment and control animals with P values as indicated. n = 35–36 flies per treatment. One-way ANOVA among genotypes in the fed condition resulted in P = 0.15. Shorter periods of activation were less effective in reducing sleep loss.



Supplemental figure 5.3 Responses to nonnutritional sweeteners (related to Fig. 3).
(A) Concentration dependence of responses to arabinose, l-glucose, and NaCl. Statistical comparisons to determine sufficiency are relative to starvation (dashed lines). (B) Abdominal color following 2 h of feeding at the indicated concentrations of tastant supplemented with 0.5% FD&C Blue #1. (C) Comparison of sorbitol, arabinose, and both compounds reveals a sorbitol-specific sleep deficit when dietary availability is low. (D) Arabinose in the agar medium does not confer a survival advantage ($P > 0.05$ relative to starvation, log rank test, n = 100–200 flies per group). (E) Frass production during days 2 and 3 on the indicated foods containing 0.5% FD&C Blue #1. n = 3 vials of 15 flies. (Scale bar, 1 mm.) $P = 0.33$ for the effect of food, $P = 0.0035$ for the effect of time, two-way ANOVA. (F) Food interactions during day 2 or 3 for the indicated test food in liquid. n = 9–16 flies per group. $P = 0.029$ for the effect of food, $P = 2.5 \times 10^{-7}$ for the effect of time, two-way ANOVA. (G) Feeding preference for the indicated compound relative to agar for the indicated genotype. PI is the preference index for the sweet substance (number eating sugar + 0.5 × number eating both)/(total number that ate). Statistical significance was determined by one-way ANOVA with Tukey's posttest. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.



Supplemental figure 5.4 TH subset Gal4 controls do not have a deficit in sleep behavior (related to Fig. 4).

(A) The Gal4 driver lines from Fig. 4 were crossed to the w^{1118} control line and assayed with 50 mM D-glucose in the test medium at the indicated temperature. P = 0.23, one-way ANOVA for each temperature. (B) DopEcR is not required for normal sleep behavior in the presence of 50 mM D-glucose. Flies of the indicated genotypes were tested on food containing 50 mM D-glucose. Flies containing the GSElav neuron-specific conditional GeneSwitch driver construct were tested in food containing 0.1% ethanol with and without 200 μM RU486. Flies were pretreated for 3 d with RU486 where indicated. P = 0.30, one-way ANOVA. Each column is the mean ± SEM from 16 to 32 flies. (C) Working model for the regulation of sleep behavior by environmental nutrient density. We propose that gustatory perception is alerts the organism, through dopamine signaling, to the present of nutrients when the nutrient density is low and blocks starvation-induced sleep loss. When nutrient density is high, we propose that there are redundant mechanisms involving both gustatory perception and metabolic nutrient perception that maintain consistent patterns of sleep behavior.

Supplemental Table 5.1. Description of the sensory and metabolic characteristics of the carbohydrates used in this study.

Carbohydrate	Sweet	Nutritious
D-Glucose	+	+
Sorbitol		+
Mannose		+
Arabinose	+	
L-Glucose	+	

Supplemental Table 5.2. Survival Parameters for young male flies on the indicated sugars in a 1% agar base. P values are derived from the log rank test.

Genotype	Treatment	N	Mean	SEM	Median	P value	Percent change
R1;R2/+;ΔGr64/ΔGr64	550 mM Fru	80	24.6	0.4	25.1	1.0×10^{-4}	8
	550 mM D-glu	98	22.7	0.3	23.0		
R1;R2/R2; ΔGr64/ΔGr64,UAS-Gr64abcdGFPf	550 mM Fru	57	20.4	0.3	21.1	8.0×10^{-1}	5
	550 mM D-glu	53	20.4	0.3	20.1		
R1;R2/R2; ΔGr64/ΔGr64,Gr5a-Gal4/+	550 mM Fru	98	21.7	0.4	22.2	7.0×10^{-13}	14
	550 mM D-glu	97	17.8	0.4	19.0		
R1;R2/+;ΔGr64/+	550 mM Fru	96	21.0	0.4	20.1	4.4×10^{-2}	−5
	550 mM D-glu	100	22.2	0.5	21.1		
R1;R2/Gr64rescue; ΔGr64/ΔGr64	550 mM Fru	96	22.2	0.4	22.2	1.1×10^{-1}	9
	550 mM D-glu	100	20.6	0.5	20.1		
R1;R2/+;ΔGr64/Gr64a[2]	550 mM Fru	105	21.1	0.4	21.1	2.3×10^{-1}	0
	550 mM D-glu	88	20.7	0.3	21.1		
R1;R2/+;ΔGr64/ΔGr64	50 mM Fru	42	6.4	0.3	5.7	$<10^{-15}$	69
	50 mM D-glu	49	2.2	0.1	1.8		
R1;R2/R2; ΔGr64/ΔGr64,UAS-Gr64abcdGFPf	50 mM Fru	94	6.2	0.2	5.7	7.0×10^{-11}	36
	50 mM D-glu	89	4.4	0.1	3.7		
R1;R2/R2; ΔGr64/ΔGr64,Gr5a-Gal4/+	50 mM Fru	99	5.8	0.2	5.7	$<10^{-15}$	52
	50 mM D-glu	99	2.7	0.1	2.8		
R1;R2/+;ΔGr64/+	50 mM Fru	145	4.6	0.1	4.6	1.5×10^{-1}	1
	50 mM D-glu	130	4.9	0.2	4.6		
R1;R2/Gr64rescue; ΔGr64/ΔGr64	50 mM Fru	104	5.1	0.2	4.7	1.1×10^{-1}	0
	50 mM D-glu	95	4.6	0.2	4.7		
R1;R2/+;ΔGr64/Gr64a[2]	50 mM Fru	97	7.0	0.2	6.8	3.0×10^{-6}	31
	50 mM D-glu	93	5.1	0.2	4.7		
R1;R2/+;ΔGr64	CS female, SY10	16	15.0	1.5	14.3	0.138	
R1;R2/Gr64rescue; ΔGr64	CS female, SY10	16	12.4	1.3	11.3		
R1;R2/+;ΔGr64/+	Starve (h)	16	22.6	1.2	24.6	0.146	
R1;R2/+;ΔGr64	Starve (h)	16	26.9	1.7	28.2		
R1;R2/Gr64rescue; ΔGr64	Starve (h)	16	26.1	1.8	23.1		

P values are derived from the log rank test.

Supplemental Table 5.3. Average total sleep on SY10 (complete) food for adult males maintained under 12h:12h light:dark conditions at 25°C (except where noted).

Experimental group	Genotype	Total sleep (min/h)	SEM	N
Typical laboratory control	Canton S	36.8	0.4	769
ΔGr64 /+	R1;R2/ +;ΔGr64/ +	47.8	0.3	155
ΔGr64 mutant	R1;R2/ +;ΔGr64 4 4/ ΔGr64	51.8	0.3	298
ΔGr64 rescue (Gal4 -UAS)	R1;R2/R2; ΔGr64/ ΔGr64,UAS -Gr64abcdGFPf	48.8	0.3	309
ΔGr64 rescue (GenRes)	R1;R2/Gr64r escue; ΔGr64	50.8	0.7	79
Gr5a-Gal4/ + (29 °C)	w ^{1118};Gr5a-Gal4/ +	35.5	1.2	30
+/UAS-TrpA1(29 °C)	w ^{1118};UAS-TrpA1/ +	37.4	1.0	32
Gr5a-Gal4/ UAS-TrpA 1 (29 °C)	w ^{1118};Gr5a-Gal4 /UAS-TrpA1	31.1	1.2	30
Gr66a-Gal4/ + (29 °C)	w ^{1118};Gr66a-Gal4/ +	37.9	1.0	30
Gr66a-Gal4/UAS -TrpA 1 (29 °C)	w ^{1118};Gr66a-Gal4 /UAS-TrpA1	27.8	1.2	32
TH-Gal4 /+	w ^{1118};;TH-Gal4/ +	46.4	0.8	62
+/UA S-TntG	w ^{1118}; UAS - tntG/ +	45.5	0.9	63
TH-Gal4 /UAS - tntG	w ^{1118};UAS - tntG/ +;TH -Gal4/ +	48.3	0.8	90
TH-Gal4 /+ (29 °C)	w ^{1118};;TH-Gal4/ +	36.9	1.1	61
+/UAS-Kir $^{2.1}$;TARGET (29 °C)	w ^{1118};UAS - Kir $^{2.1}$/+;tubGa l80 ^{ts} /+	42.9	0.6	62
TH-Gal4/UAS-Kir $^{2.1}$;TARGET (29 °C)	w ^{1118};UAS - Kir $^{2.1}$/+;TH -Gal4 /tubGal80 ^{ts}	55.6	4.4	53

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Chapter 6

Conclusions and Future Directions

This dissertation was motivated by my quest to understand how food affects animals' health and longevity. Casual relationships between food and long-life have been recognized throughout human history, and they have long motivated our ancestors to search for elixirs of immortality. An ancient Chinese tale about the emperor Qin Shi, who believed consuming certain substances could make him immortal, sending his Taoist alchemist, Xu Fu, on an expedition to find the "Mount of the immortals" highlights how primal and strong humans' desire has been to break the secret relationship between food and longevity (Cave, 2012). Over two thousand and two hundred years have passed since the Qin Shi dynasty, and we no longer send alchemists to search for the elixir of immortality. However, our desire to understand how food affects our lifespan is still alive. The root of my aspiration to identify which neurosensory systems interact with key-longevity modulating nutrients and to understand how they regulate physiology perhaps is in line with that of Qin Shi Huang, yet in my case I used a model organism, Drosophila melanogaster and applied modern behavioral, genetic, and biochemical techniques to understand how food impacts aging.

By harnessing facile genetic tools already available in Drosophila melanogaster and developing a novel behavioral assay, I was able to interrogate how sensory processing of dietary protein and carbohydrate affects lifespan and health. Demographic measures of aging are extraordinarily susceptible to even minor variations in experimental design and environment. In Chapter 2, I described a standardized protocol for conducting lifespan experiments. Moreover, I included the use of the dLife software, which was developed by the Pletcher lab to accelerate throughput and promote optimal experimental design in aging studies. Using the standardized lifespan protocol, I was able to control larval developmental environment, adult stress, and bias across experimental groups and controls. In addition, increased throughput by using dLife software allowed me to manage multiple lifespan experiments at once while maintaining adequate sample size in multiple diet conditions and controls. This was important not only from an operational point of view but also for gaining sufficient statistical power to make proper inferences from my study results. In Chapter 3, I developed a novel device called FLIC, which is

a general-purpose system for accurately and continuously measuring feeding-related behaviors in Drosophila. In the FLIC, data from each food source are collected independently, allowing for simultaneous, automated analysis of thousands of flies. Therefore, I was able to obtain continuous trajectories of feeding behavior from individual flies that quantified what they eat, when they eat it, and how much they consume. The FLIC system played a critical role in advancing my thesis research by allowing me to characterize temporal dynamics of food choice as well as to capture subtle feeding and tasting interactions that would have otherwise been impossible to resolve using conventional feeding assays. I believe that the tools I developed during my thesis work facilitated my own research and will function as the basis for future discoveries in the disciplines of physiology, behavior, and neurobiology.

Serotonergic Signaling Modulation of Dietary Protein Reward

The first important discovery emanated from Chapter 4 of my work was the identification of a neural mechanism underlying protein-feeding reward. By using the FLIC continuous feeding monitor, I demonstrated that fruit flies exhibit transient feeding preference for protein-containing food after modest starvation. Through a reverse genetic screen, I identified Trh and 5HT2a as key molecular players in the brain that are important for modulating protein preference. Several lines of evidence from my study suggested that serotonin signaling is a central component of a reward circuit that is important during meal choice. Furthermore, I found that during meal choice, serotonin is involved in the assessment of the value of ingested protein in the context of homeostatic demand.

Serotonin has been implicated in diet balancing either through determining meal size or altering carbohydrate satiety. Pharmacological studies in rodents and cockroaches revealed that increased brain serotonin results in reduced carbohydrate intake or proportionally increased protein consumption (Cohen, 2001; LeBlanc and Thibault, 2003; Leibowitz et al., 1993; Shor-Posner et al., 1991; Thibault and Booth, 1999).

Conversely, similar studies using rainbow trout found that both increasing and decreasing brain serotonin turn over can reduce carbohydrate intake, hinting at a mechanism in which serotonin changes macronutrient selection and suggesting that it may be context-dependent or more complex than researchers originally envisioned (Johnston, 1992).

Unfortunately, previous studies that assessed the role of serotonin in feeding regulation by adding serotonin or serotonin antagonists directly into food confounded the interpretation of a role of central serotonin signaling in macronutrient selection. Most of the serotonin is found

in the gastrointestinal track (Gershon et al., 1965) and the amount of serotonin released in the brain is believed to be less than 5% of the total body serotonin (Gershon and Tack, 2007). Therefore, manipulating serotonin through drug administration in the food can change gut motility independent of type of food consumption and thus it is difficult to judge what effect serotonin had on any central processing such as satiety, reward, and nutrient perception. Some have attempted to address this point by directly injecting serotonin into specific regions in the brain (Leibowitz and Alexander, 1998; Leibowitz et al., 1989). Indeed, these studies were able to more definitively link central serotonin to feeding regulation and nutrient balancing, however, any form of pharmacological manipulation still are limited in temporal dynamics or spatial specificity to dissect specific mechanisms about how serotonin governs meal size and macronutrient selection.

With the powerful genetic tools available in Drosophila, one can manipulate specific neuronal cell populations at will (i.e., with spatial and temporal precision), which make this model system particularly suitable for dissecting neural mechanisms of behaviors. In mammals, there are two distinct gene products regulating serotonin synthesis that is localized either in periphery or in the CNS (Cote et al., 2011). This is also the case in Drosophila (Coleman and Neckameyer, 2005), and using the available genetic tools, I was able to specifically manipulate serotonin signaling in the CNS in my studies. Moreover, using temperature sensitive transgenes under control of a specific upstream activating sequence (Kang et al., 2012; Kitamoto, 2001), I was able to restrict serotonin signaling in specific time points in my experiments. This enabled me to test whether serotonin was involved in recognition or creation of protein demand, or in reward associated with protein feeding. By temporally activating serotonergic neurons, I was also able to instill protein preference in fully fed flies that otherwise would have shown the opposite behavior in non-manipulated state. In doing so, I found that serotonin communicates value of a protein meal to reinforce protein feeding rather than increasing carbohydrate satiety as it was suggested by pharmacological studies (Leibowitz et al., 1993; Shor-Posner et al., 1991; Thibault and Booth, 1999). If central serotonin was exclusively involved in carbohydrate satiety, I expected flies to ceased feeding altogether in my experimental condition because both sides of the choice foods contained the same amount of sugar. Instead, flies ate and demonstrated non-random choice toward the protein containing food. Because I did find that flies with hyperactive serotonergic neurons to significantly reduce their total feeding, it is possible that increased serotonin regulate both carbohydrate meal size as well as protein valuation. This can be tested in future by assessing meal choice when the foods are given as carbohydrate versus protein. If serotonin modulates both carbohydrate meal size as well as protein valuation, I predict flies will still show protein preference but the total consumption of the food during the test period would not be different compared with control flies.

Although it is an active area of research, little is known about how animals sense protein in their food. At least in mammalian species and humans, a heterodimer taste receptor, T1R1/T1R3, is partially responsible for savory taste of proteins. On the other hand, knockout of t1r1 or t1r3 was not sufficient to abrogate mice's ability to discriminate a protein food, suggesting there is more than one taste receptor responsible for dietary protein perception (Delay et al., 2006; Nelson et al., 2002). In addition, T1R1/T1R3 is not evolutionarily conserved, despite a common subset of amino acids that are essential nutrient components in most all animals. Therefore, an evolutionarily conserved dietary protein sensor is yet to be identified.

In last two decades, amino acid transporters have been suggested to play roles in amino acid sensing in animal cells or even in taste perception (Attardo et al., 2006; Chaudhari et al., 2000; Colombani et al., 2003; Hyde et al., 2003). Among the different types of transporters, solute carrier family 6 and 7 (SLC6 and SLC7) proteins have been the center of focus as candidates for evolutionarily conserved amino acid sensors. This is because they are found in apical and basolateral membranes, their biochemical properties promote high-affinity interactions with essential amino acids, and some members have been shown to interact with cellular amino acid sensing pathways, including the activation of target of rapamycin (TOR) (Gallinetti et al., 2012; Metzler et al., 2013; Taylor, 2013). In Drosophila, JhI-21 act as an excitatory amino acid transporter that belongs to the SLC7 (Romero-Calderón et al., 2007). It has also been shown to be involved in antifungal defense and oogenesis (Dubrovsky et al., 2002; Jin et al., 2008).

My work established a novel function of JhI-21 as a potential sensor for dietary protein. According to an in vitro study, JhI-21 functions as 'light chains' in a heterodimeric complex with Drosophila CD98hc and imports neutral amino acids including leucine to activate TOR (Reynolds et al., 2009). Because of its connection to cellular amino acid sensing, I tested whether JhI-21 was involved in perception of dietary protein, and I found that mutation of JhI-21 abolished protein preference in a manner that indicated it acts as an upstream activator of serotonin signaling for protein valuation. This result opens up exciting new avenues of research to study mechanisms of JhI-21 in protein perception. To further elucidate a role of JhI-21 in protein sensing, identifying its tissue-specific expression pattern and biochemically characterizing its amino acid binding capability are critical.

The final important discovery from Chapter 4 of my work is that continuous valuation of nutrients can accelerate or slow aging. My findings suggest that even in simple organisms, the brain continuously evaluates key biological states, including nutrient demand and reward, and actively employs simple decision-making processes to affect behavior and physiology, which in turn influence survival. This is consistent with reports showing that specific genetic and environmental manipulations, such as dietary restriction (Good and Tatar, 2001; Mair et

al., 2003; Smith et al., 2008), insulin signaling (Giannakou et al., 2007) and mate availability (Gendron et al., 2014; Maures et al., 2014) rapidly and reversibly affect mortality rates, often within hours or days. It is also notable that the same neural circuits that evaluate internal and external nutritional status to determine what and when to eat also interact with major hormone axes known to influence aging, such as insulin-like and TGFβ signaling (Domingos et al., 2011; You et al., 2008). Aging, may, therefore have characteristics that resemble a complex behavior that is acutely malleable, susceptible to sensory influences, and strictly controlled by coordinated sets of neurons.

Dopaminergic Circuits Modulating Feeding and Sleep Behavior

In Chapter 5, I have shown that gustatory perception of sweetness is both necessary and sufficient to suppress starvation-induced sleep loss when animals encounter nutrient-poor food sources. Notably, gustatory perception is also required for a normal lifespan, specifically when animals are maintained in a low nutrient environment, establishing that gustatory perception is a critical mechanism for transmitting information about environmental quality to central regulatory centers that control sleep behavior and survival. While there are apparently multiple types of sensors that relay the complexity of dietary information, the impact of gustatory perception on both sleep and survival is most profound when environmental nutrient density is low. Overall, these results demonstrate an important role for gustatory perception when environmental food availability approaches zero, and they illustrate the interplay between sensory and metabolic perception of nutrient availability in regulating physiology and other complex behaviors.

I found that dopaminergic neuron activity is required for appropriate regulation of sleep behavior specifically under conditions of very low nutrient availability. These data support a model where gustatory perception signals through dopaminergic neurons to transmit information about food availability to sleep centers. It is also possible that dopaminergic neurons act to relay the gustatory information back to sensory neurons, as has been reported for starvation (Inagaki et al., 2012). I believe this second model is less likely due to the fact that known dopamine-gustatory feedback is dependent on the DOPECR receptor and that we find no effect of DopEcr mutation or RNAi on gustatory-dependent sleep behavior (Supplemental Figure 5.4b). The precise neurons involved in this interaction remain unknown, but neurons in the PPM2 cluster are strong candidates due to overlapping expression in the C1 and D4 dopamine subset Gal4 lines (Liu et al., 2012). These data and others are beginning to reveal the importance of specific subsets of neurons for regulating distinct behavioral phenotypes.

Why might animals rely on gustatory perception, rather than an assessment of nutritional content per se, to drive key food-related behaviors? One possibility is that in the wild gustatory and nutritional cues are nearly always paired, and therefore taste may be a very reliable predictor of nutrient quality. Metabolic assessment of quality may have lower sensitivity, and it is almost surely slower. As we understand more about the nature of metabolic nutrient sensor(s) in flies and other organisms, the nature of the relationship between the gustatory and metabolic information will clarify. For now, I propose that the gustatory and metabolic processing pathways are mostly distinct. However, the ΔGr64 mutant is partially defective in suppressing starvation-induced sleep loss, even at very high dietary glucose concentrations (Figure 5.2a, Supplemental Figure 5.1a), which leaves open the possibility of either a compensatory change in metabolic processing in response to sensory deficit or more direct cross-talk between gustatory and metabolic signaling.

I speculate that the ability for gustatory stimulation to attenuate starvation-induced sleep loss may be evolutionarily conserved and that this may provide an interesting therapeutic angle for limiting the negative effects of fasting or periods of anticipated food deprivation. Under conditions where sensory perception is impaired, including aging and neurodegenerative disease, nutritional intervention may provide an important approach to limit the disorders of sleep behavior that can potentially accelerate disease progression and reduce independence. Together, our work supports a key role for sensory perception in the regulation of complex behaviors that serve to support the normal functions of the organism, and it serves as an essential step in understanding how organisms process information from their environment.

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