Gustatory Regulation of Physiology and Longevity in *Drosophila melanogaster*

by

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I was told at the start of graduate school that, by its end, I would be jaded with science, sick of my project, and have a love/hate relationship with my mentor and thesis committee. None of this turned out to be the case. I wish I could credit some personal attribute to this reality or point to a line of good luck, but it turns out that the bulk of the reason was due to a different kind of line – that of mentors, colleagues, friends, and family who invested their time in my success. It seems like it should be more complicated than that. It really isn't.

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Table of Contents

List of Figures

List of Tables

Abstract

Gustatory Regulation of Physiology and Longevity in *Drosophila melanogaster*

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Chemosensory perception is critical for the assessment of an organism's nutritive environment, allowing for an appropriate physiological response to a given – and often changing – set of stimuli. Broad perturbations of chemosensation in model organisms regulate lifespan, presumably due to alterations in physiological homeostasis after integration through the central nervous system [CNS]. A detailed understanding of the underlying circuitry which coordinated this control, though critical for its targeting in order to maximize organismal health, remained largely unknown.

Here, we utilized the gustatory system of *Drosophila melanogaster* as a model by which to study this regulation. We found that loss of function of individual gustatory genes caused significant alterations in lifespan in a bidirectional manner and across diverse taste modalities. We focused our studies on two of these mutants – the long-lived *ppk28* water-sensing mutants and the short-lived *Gr5a* sweet receptor mutants – to determine the underlying mechanisms responsible for longevity modulation.

In *ppk28* mutants, we initially found substantial and longitudinal augmentation of both carbohydrate and lipid stores. We utilized genetic epistasis and immunohistochemical analyses to elucidate the underlying endocrine control of this lifespan extension, and determined that the glucagon-like adipokinetic hormone [AKH] was specifically upregulated and required for increased longevity. Furthermore, we found extended lifespan to be *dFoxO*-dependent. Additionally, we employed several physiologic assays to demonstrate that *ppk28* mutants adaptively respond to lack of water gustatory information via these mechanics, altering nutrient metabolism to promote a physiological state conducive to both the production of metabolic water and increased longevity.

In *Gr5a* mutants, we found that both whole-organism and hemolymph levels of selectively trehalose – the ligand for the *Gr5a* receptor – were increased, while levels of other carbohydrates remained unchanged. qPCR analysis of transcript levels of critical genes in the trehalose synthesis pathway led us to propose that this increase is due to upregulation of *de novo* trehalose synthesis. Further, we present evidence suggesting that *Gr5a* mutants may increase action of insulin-like peptide signaling, potentially as a response to augmented hemolymph trehalose levels, a modulation congruous with a short-lived phenotype.

These studies uncovered, in the greatest detail yet attained, how discrete gustatory information is capable of regulating *Drosophila* lifespan through the modulation of CNS-derived endocrine molecules responsible for control of nutrient homeostasis. The

resultant metabolic switches which occur from the transduction of taste information through this circuitry, in turn, determine health and lifespan status of the organism. They also posit the intriguing hypothesis that loss of the ability to sense a metabolically important molecule in the environment is sufficient to induce a physiological shift conducive to generating increased internal amounts. This work is thus important not only for the understanding of the basic biology of sensory signaling, but also how this signaling is capable of regulating organismal long-term health and lifespan.

Chapter 1: Sensory Signaling Systems and the Regulation of Aging

Chapter 1.A. Introduction

No organism lives in complete isolation – its existence is unavoidably intertwined with its environment. Signals emanating from these surroundings provide information critical for the organism's health, and, ultimately, survival. Neurosensory systems serve to relay these inputs to the CNS, where they are decoded into appropriate behavioral and physiological outputs. Necessarily, many are both rapid and acute to ensure a correct response in both advantageous and potentially harmful situations. As such, the internal effects of this integration are metabolically consequential. The difference in the sum total of a given organism's perceptive experiences, then, provides a point of variation in physiological state of organisms, even within species. Thus, though aging is a complex phenomenon, sensory signals represent one of the most potentially influential elements of its control.

Individual taxa support similar modes of sensory perception. The roundworm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and human *Homo sapiens*, for instance, all maintain specialized cells devoted to recognition of common peripheral stimuli, including chemicals, light, mechanical force, and temperature. Though the details and complexity of these systems vary, their common function suggests that the study of sensory input in diverse species can be applied broadly.

Ongoing work manipulating sensory input independent of other regulatory factors, such as dietary intake, has uncovered multiple modalities capable of modulating lifespan. Chemosensory cues, both olfactory and gustatory, have been found to elicit significant longevity effects in both *C. elegans* and *D. melanogaster*, with limited yet intriguing data in humans suggesting gustatory cues are sufficient to induce metabolic and behavioral change. Additionally, light and temperature stimuli have been found to influence aging in worms and flies. These data have further uncovered that peripheral inputs modulate longevity in a bidirectional manner, even within modalities, with specific cues negatively or positively affecting lifespan.

The internal mechanisms responsible for transducing sensory data into longevity-modulating metabolic shifts represent an obvious progression in understanding this regulation. Endocrine signaling pathways, especially those related to nutrient-sensing and response to dietary intake, have been hypothesized as particularly critical. These networks have proven extensively capable of modulating aging, and they provide a reasonable connection between sensory input and regulation of physiology. Indeed, initial work in this vein has revealed a regulatory role for endocrine signaling pathways in transducing sensory information into longevity-altering changes in physiological state. As further studies uncover circuitry in greater detail, it is likely that complex interactions between multiple affected pathways will need to be parsed out. Of particular necessity will be defining both the neural control of these signaling pathways as well as the transcriptional regulatory networks and specific tissues targeted by these pathways through which they exert their effects.

The utilization of simpler model systems, including *Drosophila*, lies at the leading edge of these studies. This has served as a necessary step for rapid determination of conserved mechanics of sensory-mediated networks modulating aging. These data will, in turn, inform study in more complex mammalian systems. The fundamental principles collected across species will plausibly be highly relevant in determining interventional strategies for augmenting the duration of human health.

Chapter 1.B. Peripheral Systems of Sensory Perception

All organisms maintain methods of recognizing their external milieu. Though sensory organs amongst eukaryotic model organisms are morphologically dissimilar, classes of sensation are largely overlapping in nature. Indeed, worms, flies, and mammals all harbor neural sensory cells that work to recognize a diverse array of stimuli. This reality stems from the necessity to invoke both behavioral and physiological modulations required for basic survival, responses that occur as a result of sensory neural input. For instance, the ability to distinguish between attractive and aversive chemical stimuli is critical for an organism to pursue nutritive food sources and potential mating partners and to avoid toxic compounds and non-productive mating. A functional thermosensory response allows for the avoidance of possibly harmful environments of extreme heat or cold. Appropriate recognition of light inputs sensitizes an organism to circadian cues, potentially phototoxic environments, and, for some, visualization of the external world. Diverse external mechanical forces confer a wide range of sensory experiences, such as touch, hearing, balance, and perception of humidity. There are, of course, key differences in sensory recognition. Worms, for instance, can perceive electrical changes (Sukul and Croll, 1978), flies are capable of recognizing magnetic fields (Dommer et al.,

2008), and mammals maintain a vestibular sense of balance and body movement (Khan and Chang, 2013).

In addition to broad – and somewhat expected – functional conservation, studies of the molecular mechanisms of these sensory systems in worms, flies, and mammals have begun to uncover striking similarities in the transduction of comparable classes of environmental signals. In this vein, the TRP superfamily of cation channels represents a major contributor, involved either directly or indirectly with multiple types of sensory recognition (Montell, 2011). For example, the TRPA1 channel alone is critical for the perception of multiple noxious stimuli, including cold temperature in worms (Xiao et al., 2013) and mammals (Schepers and Ringkamp, 2009), as well as noxious chemicals in flies (Kim et al., 2010) and mammals (Kwan et al., 2006). TRP channels also play critical roles in mechanotransduction across species, including sensitivity to touch (Christensen and Corey, 2007). Seven transmembrane receptors, too, work to transduce environmental cues, notably of both volatile [odorants] and non-volatile [tastants] chemicals. A series of large seven-transmembrane GPCR families broadly mediate chemosensation in *C. elegans*, including the seven transmembrane (*str*) and *str*-like groups (Robertson, 1998). In flies and mammals, olfaction is likewise negotiated by a class of seven transmembrane receptors, named olfactory receptors (Buck and Axel, 1991; Couto et al., 2005; Sato et al., 2008). Gustation in these species, in addition to seven transmembrane gustatory receptors, also involves the function of ion channels, including the sodium epithelial channel [ENaC] family (Cameron et al., 2010; Yarmolinsky et al., 2009). The evidence of conservation of both functional and molecular structure, then, strongly indicates that manipulation of a given sensory

system in one model organism should help to uncover fundamental principles concerning the regulatory consequences of environmental information across species.

Though there is less segregation present in the worm, in both flies and mammals, separate receptor and cell populations recognize distinct sensory modalities and relay information to discrete areas of the CNS. In *Drosophila*, gustatory receptor neurons map to the subesophageal ganglion, whereas olfactory receptor neurons map to the mushroom body (Montell, 2009; Ramaekers et al., 2005). So too, in mammals, do gustatory and olfactory inputs map to spatially separated areas of the brain (Galindo et al., 2012; Murthy, 2011). Indeed, knowledge of required brain regions may be coupled with neural networks previously shown to integrate through these areas [e.g., circuits of olfactory learning and memory through the mushroom body of *Drosophila* (Davis, 2011)], to inform mapping studies. Nonetheless, the segregation of subsequent effectors that are sensitive to these signals is currently poorly understood. Do two, or more, types of sensory cues ultimately enact the same downstream circuitry and physiological consequences? If so, where do points of intersection lie? Uncovering of such nodes of convergence would have the benefit of focusing efforts at targeting a small set of factors capable of advantageous physiological effects to maximize healthy lifespan, and away from the need to manipulate a large number of different sensory inputs. Furthermore, the hedonic value of environmental cues [e.g., the enjoyment that accompanies the taste of good food] could remain intact.

Chapter 1.C. Environmental Sensing and the Regulation of Aging

The initial step in determining which neural networks had the potential to influence organismal aging began with the identification of the regulatory capabilities of the most upstream portions of these circuits – the sensory inputs themselves.

Chemosensory perception represents both the longest and most well-studied of the sensory modalities in the role of longevity modulation. The ability to distinguish between attractive and aversive chemical stimuli is critical for an organism to pursue nutritive food sources and potential mating partners and to avoid toxic compounds and non-productive mating. Of the 302 neurons present in *C. elegans* hermaphrodite, 60 are ciliated and dedicated to the recognition of external sensory cues, with males possessing 52 extra ciliated sensory cells largely required for mating behavior (White et al., 1986). 32 of these sensory cells detect chemical signals (Whittaker and Sternberg, 2004), including individual gustatory and olfactory stimuli, as well as the osmotic concentration of these molecules. Flies also use specialized neuronal cells to detect a wide array of both volatile and non-volatile chemicals. Odorants are recognized by a superfamily of seven transmembrane odorant receptors (ORs) expressed in specialized neurons (ORNs). These cells express the obligate odorant coreceptor – *Or83b* – in combination with various other ORs (Benton, 2008). The recognition of tastants are also largely dependent on a superfamily of seventransmembrane molecules, named gustatory receptors (GRs), expressed in peripheral sensory neurons (GRNs) located on the labellum, legs, and wing margins. GRs are responsible for the gustatory response to sweet, bitter, and pheromone compounds (Montell, 2009). Additionally, members of the *pickpocket* (*ppk*) family of ion channels are located in these same cells and required for water (Cameron et al., 2010) and pheromone (Thistle et al., 2012) recognition in the

adult, and located in the larval terminal organ and critical for salt sensing (Liu et al., 2003). Very recent work has uncovered the role of ionotropic receptors [IRs] in chemosensory perception, with an IR64a/IR8a complex required for the function of acid sensing ORNs and IR76b tuned toward high salt gustatory stimuli in adult GRNs. Similar to chemosensation in flies, humans utilize specified cell types expressing smell or taste sensors to recognize chemical compounds. Recognition of volatile compounds closely resembles both the worm and fly with olfactory sensory neurons lining the nasal cavity expressing diverse olfactory receptors (Gaillard et al., 2004). Taste receptor cells (TRCs) covering the tongue and soft palate and express a smaller set of heterodimeric taste receptors or ion channels (Yarmolinsky et al., 2009), and differ slightly from worm and fly taste-recognition cells in that, instead of existing as neurons per se, TRCs are epithelial cells which are innervated by afferent nerve fibers which serve to transduce each sensory signal to the CNS.

The first study to demonstrate that sensory perception was capable of modulating lifespan found was performed using *C. elegans* and showed that impairment of sensory ciliary structure or signaling abilities extended lifespan (Apfeld and Kenyon, 1999). Additional work demonstrated that both olfactory and gustatory chemosensory inputs were capable of altering worm lifespan, and, furthermore, that this regulation could occur in a bidirectional manner, depending on the sensory neuron or neurons ablated (Alcedo and Kenyon, 2004). The conservation of the regulatory nature of chemosensory cues was established with the discovery that *Drosophila* olfactory perception, broadly, had a negative effect on lifespan (Libert et al., 2007). In this work, it was found that genetically inhibiting the capacity to smell through a loss of function mutation in the

Or83b olfactory coreceptor required for most olfactory input positively influenced longevity. Congruously, this study showed that environmental stimulation of the olfactory system mitigated lifespan extension upon dietary restriction (Libert et al., 2007). A later study identified CO_2-sensing olfactory neurons expressing the olfactory receptor *Gr63a* as negative regulators of lifespan, uncovering a discrete regulatory olfactory input (Poon et al., 2010). Work determining if sensory regulation of lifespan extended to the gustatory system in flies was the focus of this thesis work and is described in detail in subsequent sections. In addition to these studies, concomitant work in the lab showed that *ppk23*, a contact gustatory pheromone sensor in *Drosophila* (Thistle et al., 2012), is required for proper male courtship and decreases male lifespan when mutated (Gendron et al., 2013). This finding was in line with those in the worm showing that pheromone-sensitive chemosensory inputs proved capable of orchestrating substantial changes in lifespan, as neurons which recognize dauer pheromone mediate lifespan extension upon stimulation (Kawano et al., 2005), and a diffusible male-specific signal or set of signals leads to hermaphrodite shortevity (Maures et al., 2013). In particular, *daf-37*-expressing ASK neurons, which perceive the small molecule component of dauer pheromone ascaroside#2, have been specifically implicated (Ludewig et al., 2013).

Though a direct link to control of aging has not been shown for mammalian populations, there is intriguing evidence that suggests that activation of the human chemosensory system, independent of caloric consumption, can enact substantial metabolic change. Though the mechanistic basis is still largely unknown, separate studies utilizing endurance athletes have shown a performance increase for both cyclists (Carter et al.,

2004) and runners (Rollo et al., 2008) given a low-percentage carbohydrate-containing mouth rinse over those given plain water, though neither were able to ingest any additional calories. Such data present the possibility that certain manipulations of the gustatory system may enhance metabolic physiology in a way conducive to augmenting physical performance, and, perhaps, long-term health without the potentially negative effects of additional caloric intake.

Other sensory modalities have since been connected to control of organismal lifespan. The lifespan-extending effect of lowering body temperature in both poikilotherms and homeotherms (Conti, 2008) was long attributed to a “slowing down” or “speeding up” of organismal biochemistry. Work using *C. elegans*, however, has suggested that this phenomenon is, at least in part, a regulated process mediated by the recognition of surrounding temperature by thermosensory neural inputs. The strongest evidence for this assertion has been the finding that longevity conferred by cold temperatures is regulated by TRPA1-expressing cold-sensing neurons and intestinal tissue of worms (Xiao et al., 2013). In addition, an earlier study had suggested that the shortevity that comes as a consequence of higher environmental temperature was regulated by the bilateral AFD thermosensory neurons, with genetic mutation or ablation of these cells leads to an even shorter lifespan at high, but not low temperatures (Lee and Kenyon, 2009). Congruously, work has shown that even a small reduction in core body temperature can extend lifespan of homeothermic mice (Conti et al., 2006), suggesting that control of aging via thermosensory signals may likewise extend to mammals. Specific work into the possible neural basis of this control, however, has not been performed.

The possibility that photosensory stimuli may also generate substantial effects on lifespan has likewise begun to be explored. Work using *D. melanogaster* suggests that increasing photoperiod has a negative effect on longevity whereas decreasing photoperiod increases fly lifespan (Shostal and Moskalev, 2012). Relatedly, deviation from a 24-hour period, as well as disruption of the critical circadian rhythmicity gene *Bmal1* in mice decreases longevity (Kondratov et al., 2006; Libert et al., 2012), though the negative consequences of loss of circadian rhythmicity are often thought to be due to maladaptation to the surrounding environment, rather than modulation of the aging process *per se*. Again, however, the sensory neurons required for this potential regulation remain to be determined.

Together, these studies have established that multiple types of sensory inputs can have differential effects on longevity, and they suggest that, inasmuch as it is regulated by external cues, the lifespan of an organism may be determined by the sum total of its given environmental stimuli. They have also provided a jumping off point – or, rather, a series of jumping off points – with which to begin determining the mode of action by which these signals exert their potent effects.

Chapter 1.D. Mechanisms of Sensory-mediated Lifespan Regulation

Behavioral responses to varying sensory information have been well-characterized and are often intuitive – a worm will display a stereotyped set of movements when touched on the nose (Kaplan and Horvitz, 1993), a fly will extend its proboscis to a favorable food source (Dethier, 1976), a human will blink its eye in response to bright light (Yates and Brown, 1981). Unlike behavioral responses, however, the type and duration of

expected physiological responses to environmental cues, though likely a causative agent in determining lifespan, is somewhat less intuitive. Though the study of sensory regulation of lifespan is still quite nascent, initial work has begun to shape a general paradigm by which sensory inputs are capable not only of enacting behavioral changes but also sustained metabolic responses capable of modulating the aging process. Motivated, perhaps, by the considerable amount of previous work positing the role of endocrine signaling pathways themselves in driving alterations in longevity (Barzilai et al., 2012), this work has been largely targeted to the level of these pathways, particularly those involved in nutrient sensing, in the worm and fly model systems.

The insulin/IGF signaling pathway is critical to the maintenance of metabolic homeostasis and represents the most thoroughly studied endocrine network in the context of aging regulation. Indeed, the first long-lived genetic mutants were identified in *C. elegans*, and discovered to be the homologs of PI3-kinase and the insulin receptor, both components of the worm's insulin-like signaling pathway (Friedman and Johnson, 1988; Kenyon, 2011; Kenyon et al., 1993). This paradigm has held remarkably constant across species, with manipulations that decrease but do not abrogate insulin-like signaling found to extend lifespan from worms to mammals (Tatar et al., 2003). Though lifespan data are lacking in humans, lowered levels of IGF signaling have been shown to greatly decrease the incidence of cancer and diabetes (Guevara-Aguirre et al., 2011; Steuerman et al., 2011), suggesting a positive role for reduction of this pathway in maintaining overall health.

The preponderance of evidence demonstrating the ability of insulin/IGF signaling to regulate lifespan tipped this pathway as likely to be involved in sensory modulation of

aging. Indeed, the *C. elegans* homolog of the FoxO transcription factor [*daf-16*], the major downstream target of the worm's insulin-like signaling pathway, was found to be required for the effects on lifespan due both to broad ablation of chemosensory structures, as well as individual gustatory inputs, though only partially responsible for those coordinated by discrete olfactory neurons (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999). Likewise, the *C. elegans* insulin receptor homolog, [*daf-2*] was also implicated in at least part of this control (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999). *daf-16*, in concert with protein kinase C [PKC] has also been found to comprise a part of the lifespan regulatory axis downstream of neuronally-expressed cold-activated TRP channels mediating the longer life conferred by stimulus by colder temperature cues (Xiao et al., 2013). Prior to this thesis work, the criticality of the FoxO transcription factor in chemosensory-mediated longevity control in the *Drosophila* system had been examined only in the case of long-lived *Or83b* mutants. Paralleling the worm, longevity modulation due to olfactory cues shows at least partial independence of dFoxO as the longevity-extending *Or83b* mutation does not require its function (SD Pletcher, unpublished data), revealing the necessity of other downstream effectors in this regulation. Apart from control by chemosensory inputs, the increased fly lifespan due to a decreased photoperiod is also potentially dependent on the *dFoxO* transcription factor (Shostal and Moskalev, 2012).

It was not so surprising that a signaling pathway sensitive to dietary intake was also sensitive to information from the chemosensory inputs that perceive the organism's nutritive environment. As such, other nutrient-sensitive pathways have been investigated as necessary constituents of longevity circuits headed by chemosensory

signals. Our work here has found that glucagon/glucagon-like signaling has opposite effects on lifespan in the *Drosophila* system, where it regulates both carbohydrate and lipid metabolism (Lee and Park, 2004). Ubiquitous expression of the fly glucagon homolog, adipokinetic hormone [AKH] had been found to extend lifespan (Katewa et al., 2012). It was tantalizing to hypothesize that the insulin and glucagon-like signaling pathways may have antagonistic effects on lifespan as well as nutrient metabolism and that control of longevity via sensory circuits may work through the modulation of these pathways in opposing directions. Rigorous testing of this model, however, remains to be performed.

The target of rapamycin [TOR] signaling network is sensitive to and responsible for regulating levels of protein and has been heavily implicated, mainly through its inhibition, in lifespan extension in invertebrate systems, as well as the amelioration of aging-related phenotypes in mammals (Stanfel et al., 2009). There has been to date, however, no direct evidence linking the TOR pathway as a component of lifespan-regulating sensory circuits.

Signaling pathways with less direct roles in nutrient-sensing have also been determined to serve as intermediaries between sensory input and lifespan control. For example, the control of short lifespan at higher ambient temperatures in *C. elegans* relies upon the activation of the *daf-9*/*daf-12* sterol hormone pathway (Lee and Kenyon, 2009). Likewise, the *Drosophila* homolog of mammalian general reward-intergrating Neuropeptide Y pathway – Neuropeptide F [Npf] – is required for the longevity and metabolic effects mediated by pheromone-receptive *ppk23* taste inputs (Gendron et al., 2013). Additionally, the sirtuin proteins, extensively researched for their role in

promoting lifespan extension, have also been linked to this regulation. The *C. elegans* sirtuin SIR-2.1 has been reported to increase longevity when overexpressed to varying extents, although the details of this relationship remain controversial (Burnett et al., 2011; Rizki et al., 2011; Tissenbaum and Guarente, 2001). A recent study has suggested that *daf-37*-expressing pheromone-sensing neurons require SIR-2.1 to increase lifespan after stimulation of worms with the dauer-inducing small molecule ascaroside#2 (Ludewig et al., 2013), providing indication that sensory inputs may also impinge upon sirtuin-mediated lifespan alteration.

It is tempting to posit that modulation of longevity due to environmental manipulations, rather than a change in the ability to perceive this environment *per se*, may require intact sensory function and may exert its effect through similar downstream regulatory networks. Dietary restriction, classically defined as a reduction of caloric intake while avoiding malnutrition, has been the most robust environmental manipulation determined to regulate lifespan across species (Fontana et al., 2010). So broad is the evidence that several groups have promoted variations of this regime as a favorable lifestyle choice (Cava and Fontana, 2013). Consistent with a critical role for sensory perception in this phenomenon, there is evidence suggesting that chemosensory recognition of a restricted or enriched food source plays a role in mediating its longevity effects. In the fruit fly, olfactory cues from yeast, the major dietary protein source, are sufficient to partially mitigate lifespan extension on nutrient dilution (Libert et al., 2007). Similarly, lifespan extension granted by adult-onset bacterial deprivation in worms is reduced by solely the presence, and not actual consumption of a bacterial lawn (Smith et al., 2008). These results suggest that the decline in longevity due to an enriched diet may be in

part mediated at the level of sensory perception of that food source. Whether the converse is true – that is that sensory perception also plays a role in the lifespan extension due to a calorically reduced diet – remains to be seen, as does the identification of specific neurons and signaling systems involved. For instance, though the ASI chemosensory neurons in *C. elegans* have been implicated as being required for this extension, this is likely separate from their role in actual chemosensation (Bishop and Guarente, 2007). Nevertheless, the phenomenon of dietary restriction potentially provides a discrete variation of a general theme – that a given environmental stimulus elicits potent physiological changes through its recognition by sensory mechanisms.

Chapter 1.E. The Utility of the *Drosophila* System for Mapping Neural Circuits

Collectively, these studies highlight the primacy of the role that alteration of critical signaling pathways, including many with endocrine function, plays in sensory-mediated control of longevity. This modulation, then, serves to sensitize animals to a given stimulus and to enact appropriate behavioral and metabolic responses. Over the lifetime of the organism these responses have profound effects on the rate at which it ages. Together, the extent and function of pathways implicated in dictating the translation of sensory input into physiological changes argue for a complex set of lifespan-modulating circuits tuned toward a given set of sensory information, rather than a limited and more general regulatory response. Strictly defining the cellular and molecular connections responsible for transducing a sensory stimulus into a longevity-modulating physiological response, however, has not been addressed well.

Defining the neurobiological framework of longevity regulation via sensory inputs necessitates complex genetic and functional strategies. First, it is necessary to be able to directly activate or inhibit candidate neural populations to test their requirement in a given circuit. Second, it requires genetic reagents capable of establishing neuronal connections to determine the relationship between required sets of neurons. The elucidation of the mechanisms of accompanying physiological modulations, on the other hand, requires the ability both to measure changes in signaling pathways after circuit activation as well as to restrict these networks spatially and temporally. Indeed, combinations of these strategies have been utilized to map in varying detail neural circuits responsible for predominantly behavioral responses in worms, flies, and mammalian systems. Of these systems, *C. elegans* boasts the greatest cellular refinement, with its entire constituency, including neural networks, completely resolved (White et al., 1986). This has led to the elucidation of circuits involved in, among other things, locomotion (Piggott et al., 2011), mate-searching (Barrios et al., 2012), and feeding coupled with a fat storage response (Greer et al., 2008). Though of obvious importance in determining basic regulatory principles, the relative simplicity of the worm's nervous system – consisting of 302 neural cells (White et al., 1986) – underscores the somewhat limited application of these findings. The mammalian nervous system, too, has begun to be interrogated to establish functional connections responsible for various responses. This has been aided greatly by the expansion of genetic and optogenetic reagents allowing for more specific control of neurons. As such, partial circuits have been mapped relating regulatory neurons to behaviors such as feeding (Atasoy et al., 2012; Carter et al., 2013; Wu et al., 2012) and anxiety (Jennings

et al., 2013). As opposed to the worm, it is the complexity of the mammalian nervous system, with its tens of millions of neurons (Williams, 2000), that is a potentially limiting factor in the feasibility of defining detailed neural mechanics. Furthermore, the average lifespan of mammalian models are an order of magnitude greater than worm and fly systems, an especially pertinent consideration when elucidating networks regulating organismal longevity.

Of the common model systems, then, *Drosophila* has perhaps the highest utility in generating both an explicit and relevant picture of the neural and physiological control points underlying sensory control of aging. Not only is the mean lifetime of a fly relatively short, but the complexity of the *Drosophila* nervous system – consisting of approximately 100,000 neurons – is amenable to establishing precise neural connections while still maintaining broad architectural conservation with its mammalian counterparts. Indeed, work detailing, for example, feeding (Marella et al., 2012) and mating (Rezaval et al., 2012) regulatory circuits have been performed in detail in the fly. Several tools facilitate this type of investigation. Furthermore, the greatest detail of longevity-modulating circuits to date has come from utilizing the *Drosophila* gustatory system. First, the *Drosophila* system is well-suited for large-scale screens to identify circuitry constituents, even for longevity as an output phenotype (Linford et al., 2013). Second, the *Drosophila* system affords the ability to modulate cellular activity with meticulous specificity. An unmatched complement of transgenic lines allowing for the restriction of an effector gene to specific neuronal cell populations in flies have been generated, including a number of extensive collections (Dietzl et al., 2007; Pfeiffer et al., 2008). These, in turn, can be used in combination with lines capable of modulating

neuron function, including the activating ion channels *TRPA1* (Hamada et al., 2008) and *NaChBac* (Ren et al., 2001) as well as the hyperpolarizing *Kir2.1* ion channel (Baines et al., 2001) and dominant-negative vesicular transport inhibitor, *shibire* (Kitamoto, 2001). The subset of affected neurons can further be specified through the use of intersectional strategies between multiple such binary systems, including the Gal4/UAS, LexA/LexOp, and Q systems (Brand and Perrimon, 1993; Lai and Lee, 2006; Potter et al., 2010). Furthermore, as yet another layer of regulation, these manipulations in many instances can be temporally controlled through the use of drugs or temperature (Roman et al., 2001). Third, innervations between neurons can be elucidated via direct imaging – utilizing, for example, the GFP Reconstitution Across Synaptic Partners [GRASP] technique (Feinberg et al., 2008) – with the subsequent use of electrophysiological assays helping to parse out functional relationships. Together, these applications will permit the uncovering of the neural basis of the regulation of longevity via discrete sensory inputs. Determination and measurement of the downstream physiological changes that accompany activation of these circuits will be motivated by the types of neurons and associated signaling pathways implicated in these control networks and serve to fully characterize the entirety of this regulation.

Given the promising yet preliminary evidence for its role in lifespan regulation, and the utility of the *Drosophila* system for mapping neural circuits, this work focused on utilizing the gustatory system of *Drosophila* as a model by which to define sensory-mediated regulation of organismal aging. In addition to garnering novel insight into the control of the aging process, we also hoped, along the way, to uncover fundamental principles of systems of sensory recognition as well as neural control of endocrine signaling.

Chapter 2: Regulation of Lifespan by the *Drosophila* Gustatory System

Chapter 2.A. Introduction

Precisely defining sensory-mediated longevity circuits required the focus on a discrete sensory modality and model organism, for which we chose the gustatory system in the fruit fly, *Drosophila melanogaster*. Several motivations, as previously discussed, prompted this selection. First, the fruit fly had been successfully utilized to map diverse neural circuits (Gordon and Scott, 2009; Rezaval et al., 2012). Second, there was a growing body of evidence for the conserved regulation of longevity by chemosensory systems in both *C. elegans* and *D. melanogaster* (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999; Libert et al., 2007; Poon et al., 2010). In particular, we were prompted by work from Joy Alcedo's lab showing that broad perturbations of the *Drosophila* gustatory system significantly altered lifespan. Mutation of the critical transcription factor *pox neuro* [*poxn*] changes all *Drosophila* gustatory peripheral sensory neurons into those with mechanosensory function, essentially eliminating taste input (Boll and Noll, 2002). Addition of *poxn* as well as different regions of its enhancer restored function of differing subsets of these inputs, dependent on the portion of the enhancer region included (Boll and Noll, 2002). Using this strategy, they observed that loss of function of *Drosophila* gustatory inputs could alter lifespan in a bidirectional manner, subject to the given genetic manipulation (Ostojic et al., 2014). Intriguingly, the *dFoxO* transcription factor was found to be required by those inputs which shortened lifespan, reminiscent of the

dependence of the worm homolog of the FoxO transcription factor for a portion of regulation of *C*. *elegans* longevity by gustatory sensory neurons. The precise regulatory inputs responsible for lifespan modulation, however, were unknown.

As such, we collaborated with the Alcedo Lab to perform a longevity screen of genetically controlled, single gustatory gene loss of function mutants which spanned the range of taste modalities within the *Drosophila* system. Significant alterations in lifespan in mutants as compared to background controls suggested that the mutated gene represented a specific input capable of modulating *Drosophila* longevity. These “hits” were tested in both labs using two different food sources as a way to boost our confidence in the likelihood of having uncovered a real effect. Inputs of interest were verified by performing genetic rescue experiments to ensure the lifespan effect was indeed due to the genetic mutation of interest. Candidate inputs which had been verified were then able to be used as the basis for subsequent studies characterizing the downstream mechanisms responsible for their associated lifespan effects.

Chapter 2.B. Materials and Methods

Drosophila Stocks. *Gr33a*, *Gr66a*, and *Gr93a* mutants (+; *Gr33a* $^{\Delta}$; +, +; +; *Df*[*3L*] ex83, +; +; *Gr93a* $^{\Delta}$), the *ppk28*-containing duplication line (*Dp(1;3)DC320*), and the (+; *Gr66a-Gal4*; +) and (+; *UAS-Gr66a*; +) lines were acquired from the Bloomington Stock Center. *ppk28* mutants (*ppk28* $^{\Delta}$; +; +) were a gift from Kristin Scott. *Gr5a* mutants (*Gr5a* $^{\Delta}$; +; +), and (w; *Gr5a-Gal4*; +) and (+; +; *UAS-Gr5a*) lines were a gift from Anupama Dahanukar. *Gr64* mutants (+; +; *Gr64* $^{\Delta}$) were a gift from Hubert Amrein. All lines were backcrossed

at least six generations to a background strain of w^{1118} [Vienna *Drosophila* Resource Center] (*w*; +; +).

Lifespan Analyses. All lifespan experiments were performed at 25ºC in a humidity-controlled incubator with a 12:12 light:dark cycle. For experiments performed in the Pletcher Lab, fly embryos were collected and adult flies reared according to a protocol optimized by our lab (Linford et al., 2013). Experiments were flipped every two to three days with data collected using software developed by our lab [SD Pletcher]. For experiments performed in the Alcedo Lab, newly eclosed flies were allowed to age for 2h and adult virgin males and virgin females were separated, 8-12 per vial. Survival was checked three times per week.

Food Recipes. Pletcher Lab experiments were performed on food containing 10% sucrose and 10% yeast with the exception of *Gr64* mutant lifespan experiments where these concentrations were 30% and 5%, respectively. Alcedo Lab experiments were performed on food containing 10% yeast, 7.5% dextrose, 5.5% cornmeal, and 1% flour, supplemented with a drop of yeast paste on top of the food.

Statistical Considerations. Log-Rank Analysis with associated p-value was used to determine statistical significance between mutant and control lines.

Chapter 2.C. Identification of Discrete Gustatory Signals Capable of Regulating Lifespan

To identify individual regulatory gustatory inputs, we measured lifespan of both male and female flies on either a standard SY10% food source [Pletcher Lab] or an enriched food source [Alcedo Lab] (see Chapter 2.B.). We found that mutants for the widely-expressed bitter taste receptor *Gr66a* (Moon et al., 2006) showed significantly extended mean longevity in both males and females on both food types, whereas loss of function of the more sparsely-expressed *Gr33a* bitter taste receptor (Moon et al., 2009) induced a smaller extension in females and a slight decrease in male longevity. Loss of function of the bitter taste receptor *Gr93a* (Lee et al., 2009) had little to no effect on lifespan. Conversely, sweet taste receptor mutants decreased mean lifespan, including significant shortevity in female *Gr5a* and *Gr64* mutants (Dahanukar et al., 2007; Slone et al., 2007) and a smaller decrease in *Gr5a* mutant males. Finally, we observed that water-sensing *ppk28* mutants (Cameron et al., 2010) were significantly long-lived. Importantly, the magnitude and direction of lifespan alterations were generally consistent between labs [summarized in Table 2.1., with I Ostojic and TP Chan].

These results clearly showed that individual gustatory inputs indeed are capable of regulating *Drosophila* lifespan (and that others are not), that these regulatory inputs span multiple taste modalities, and that these effects are bidirectional. As described, our most significant hits were long-lived *Gr66a* bitter receptor [Figure 2.1.] and *ppk28* water sensor mutants [Figure 2.2.], inferred thus to be negative regulators of lifespan, and short-lived Gr5a sweet receptor mutants [Figure 2.3.], inferred thus to be a positive regulator of lifespan. A concurrent study in the lab further showed that pheromone-sensing *ppk23* mutants (Thistle et al., 2012) were also short-lived (Gendron et al., 2013). With these candidate regulatory inputs in tow, we next performed genetic rescue

experiments to pinpoint the gustatory gene mutations as the true cause of the observed longevity modulations.

Chapter 2.D. Verification of Candidate Regulatory Gustatory Inputs

To substantiate the claim that taste mutants were long or short-lived due to loss of function of the specific gustatory gene in question, we performed rescue experiments to restore gene expression in each mutant background. For long-lived *ppk28* water-sensing mutants, we utilized a genomic construct containing the endogenous *ppk28* locus whereas, for long-lived *Gr66a* mutants as well as short-lived *Gr5a* mutants, we took advantage of the *Gal4-UAS* bipartite expression system (Brand and Perrimon, 1993). These studies, as well as those executed for the remainder of this thesis work, focused on female flies as they showed the most significant and robust lifespan effects.

Lifespan extension in *ppk28* mutants was indeed rescued through the introduction of a 102 Kb duplicated portion of the X-chromosome containing the *ppk28* endogenous locus (shortened to *Dp*[*320*]) by creating (*w*,*Δppk28*;;*Dp*[*320*]) flies (Venken et al., 2010), suggesting that increased longevity in these flies is due to loss of *ppk28* function [Figure 2.4.]. Similarly, re-expression of *Gr5a* in *Gr5a*-expressing cells in a *Gr5a* mutant background by generating (*w*,*ΔGr5a*; *Gr5a-Gal4*/+; *UAS-Gr5a*/+) flies (Dahanukar et al., 2007) also abrogated the lifespan effect in these mutants – in this case, extending lifespan to control levels [Figure 2.5.] – suggesting this lifespan effect, too, was due to the lack of function of the gustatory gene in question. Interestingly, a similar strategy to rescue expression of *Gr66a* in a *Gr66a* mutant background by synthesizing (*w*; *Gr66a*; *Gr66a-Gal4*/*UAS-Gr66a*) did not diminish increased longevity in these flies [Figure 2.6.].

We reasoned that there were two possibilities for this result. First, *Gr66a* re-expression levels were not strong enough using the Gal4 and UAS transgenic lines, rendering the rescue flies, for all intents and purposes, mutants. Second, as the *Gr66a* mutation is in reality a deficiency line (*Df*[*3L*] ex83) missing portions of two neighboring genes, the lifespan extension observed in these flies may be actually due to loss of function of one of these neighboring genes. From these results, we moved forward to dissecting the underlying neural circuitry responsible for the long-livedness of *ppk28* water-sensing mutants and the short-livedness of *Gr5a* sweet taste receptor mutants.

Mutant	Control	N		Mean Lifespan		±Lifespan	P-value [L-R]
		Mut	Ctrl	Mut	Ctrl		
Female							
w, *Gr5a*$^{\Delta}$	*w*	225	251	52.74	64.93	-18.93%	0
w, *Gr5a*$^{\Delta}$	*w*	297	280	51.55	70.52	-26.90%	0
w, *Gr33a*$^{\Delta}$	*w**	71	54	51.42	48.05	7.01%	0.0741
w, *Gr33a*$^{\Delta}$	*w*	247	250	69.11	58.23	18.71%	0
w, *Gr64f*$^{\Delta}$	*w***	295	272	36.72	45.90	-20.00%	0
w, *Gr66a*$^{\Delta}$	*w**	42	59	58.90	47.28	24.58%	0
w, *Gr66a*$^{\Delta}$	*w*	251	250	75.78	58.23	30.14%	0
w, *Gr93a*$^{\Delta}$	*w**	58	59	44.95	47.28	4.93%	0.0090
w, *ppk28*$^{\Delta}$	*w**	56	54	54.31	48.05	13.03%	0.0003
w, *ppk28*$^{\Delta}$	*w*	241	246	74.79	55.97	33.63%	0
Male							
w, *Gr5a*$^{\Delta}$	*w*	246	248	60.38	64.21	-5.96%	0.0285
w, *Gr5a*$^{\Delta}$	*w*	294	284	57.79	66.60	-13.23%	0
w, *Gr33a*$^{\Delta}$	*w**	68	68	49.88	55.23	- 9.69%	0.0038
w, *Gr33a*$^{\Delta}$	*w*	241	250	65.04	62.32	- 4.36%	0.4320
w, *Gr66a*$^{\Delta}$	*w**	42	46	62.00	47.76	29.82%	0
w, *Gr66a*$^{\Delta}$	*w*	237	250	69.11	62.32	10.90%	0.0060
w, *Gr93a*$^{\Delta}$	*w**	51	46	46.52	47.75	- 2.58%	0.3384
w, *ppk28*$^{\Delta}$	*w**	68	65	61.58	55.23	11.50%	0.0165
w, *ppk28*$^{\Delta}$	*w*	245	243	72.09	61.72	16.80%	2.45×10^{-5}

Table 2.1. Summary of Lifespan Analyses of Single Gustatory Gene Mutants. Population size [”N”] and mean lifespan values [in days] of single gustatory gene mutant flies and their background controls. p values determined by Log Rank [”L-R”] analysis. *=Zurich food; **=S30Y5 food.

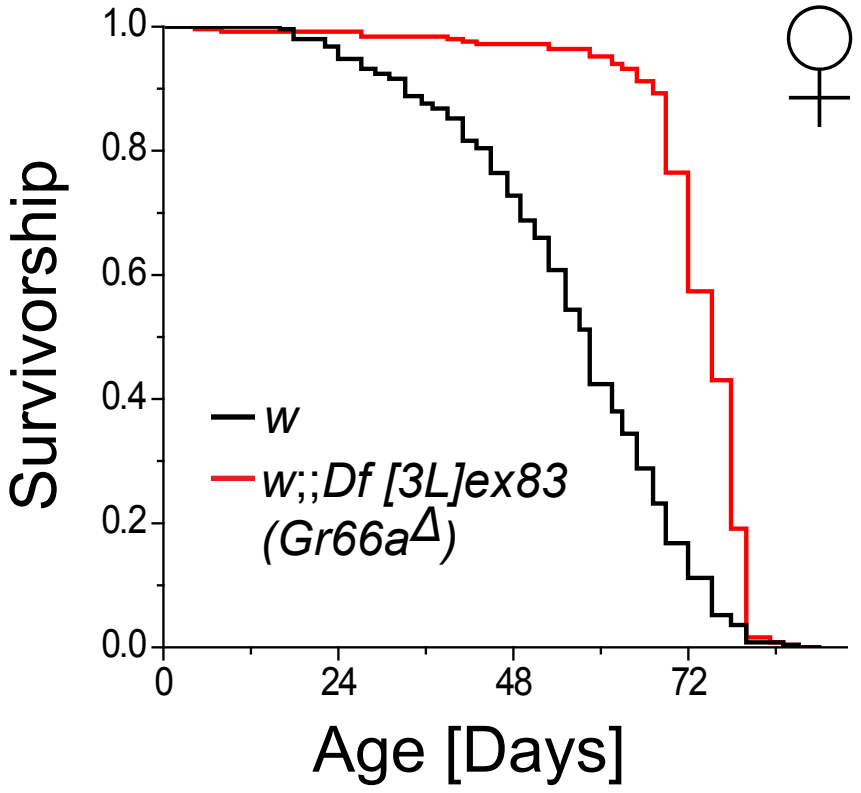


Figure 2.1. Loss of *Gr66a* Function Extends Lifespan. Survivorship curves for female flies carrying a deletion including the endogenous *Gr66a* locus (*w*;;*Gr66a*$^{\Delta}$) and their background controls (*w*) [30.14% mean lifespan increase; n=251 (*w*;;*Gr66a*$^{\Delta}$) and n=205 (*w*)]. p<1x10^-6 via Log-Rank analysis.

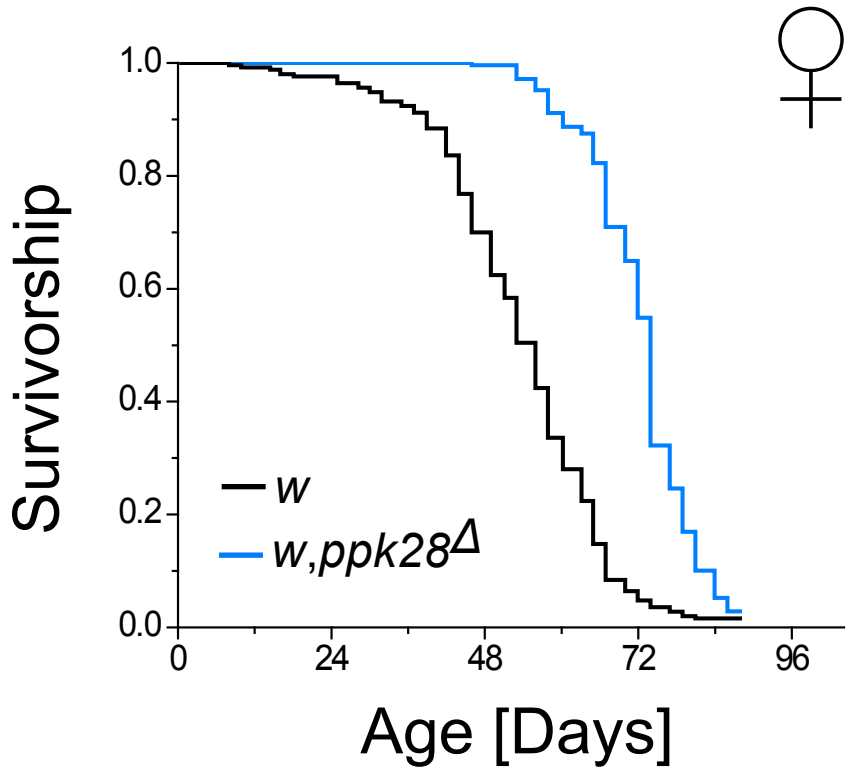


Figure 2.2. Loss of *ppk28* Function Extends Lifespan. Survivorship curves for female flies carrying a deletion including the endogenous *ppk28* locus (*w*,*ppk28*$^{\Delta}$)and their background controls (*w*) [33.63% mean lifespan increase; n=241 (*w*,*ppk28*$^{\Delta}$) and n=246 (*w*)]. p<1x10^-6 via Log-Rank analysis.

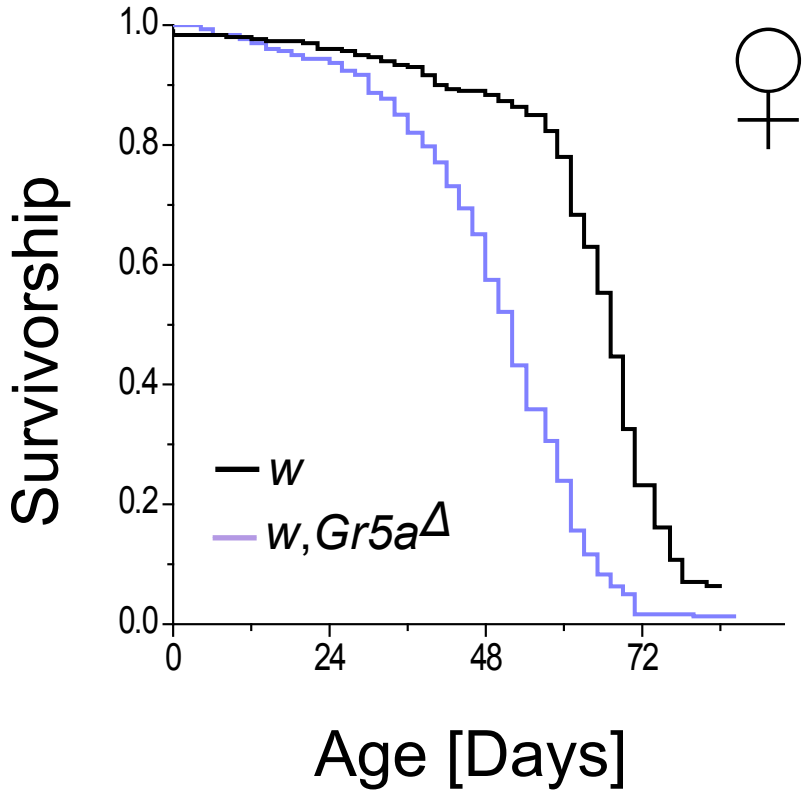


Figure 2.3. Loss of *Gr5a* Function Decreases Lifespan. Survivorship curves for female flies carrying a deletion including a portion of the endogenous *Gr5a* locus (*w*,*Gr5a*$^{\Delta}$) and their background controls (*w*) [26.90% mean lifespan decrease; n=297 (*w*,*Gr5a*$^{\Delta}$) and n=280 (*w*)]. p<1x10^-6 via Log-Rank analysis.

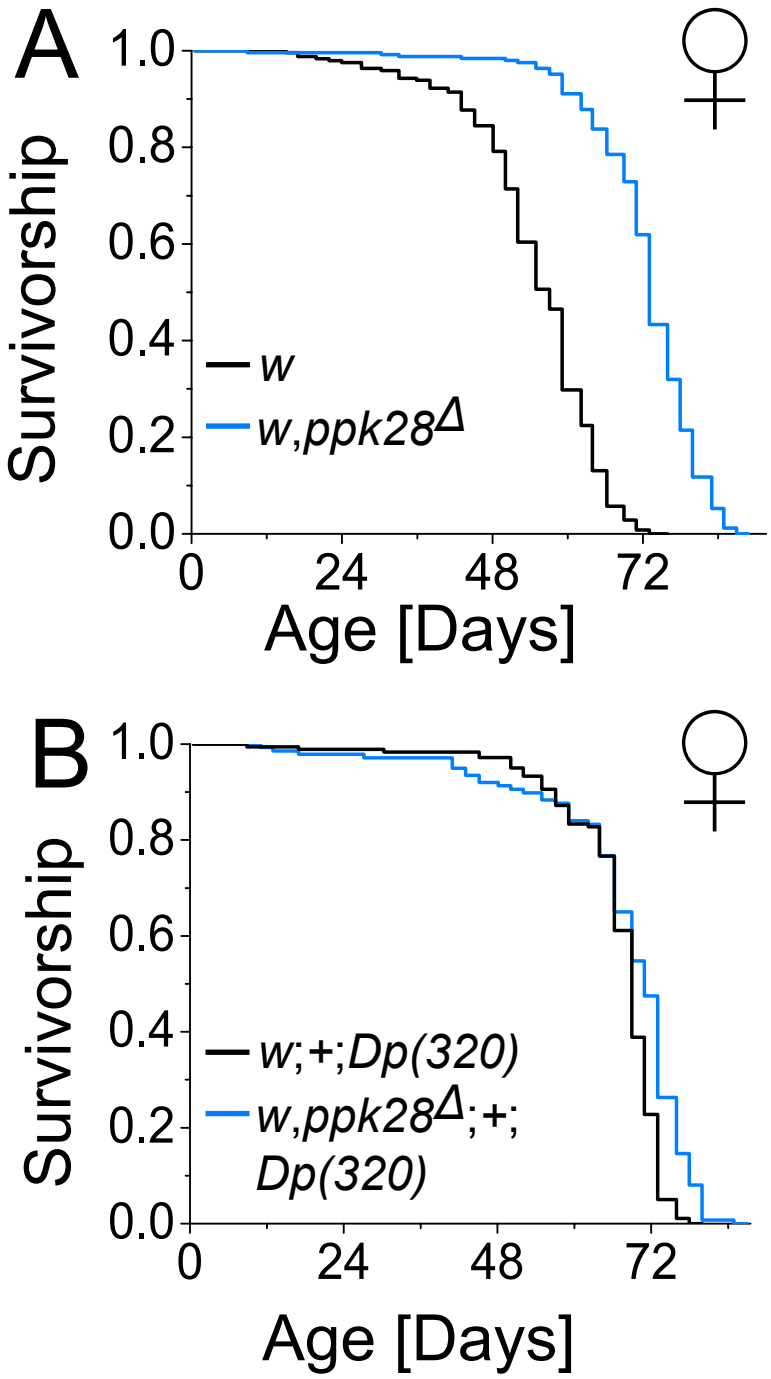


Figure 2.4. Introduction of Genomic Construct Containing *ppk28* Locus Abrogates Extended Longevity of *ppk28* Mutants. Survival curves for female *ppk28* deletion mutants (*w*,*ppk28*$^{\Delta}$) and background controls (w) (n=245 (w); n=247 (*w*,*ppk28*$^{\Delta}$) – mean lifespan increase of 30.54%) (A) as compared to survival curves representing the addition of a genomic region containing the endogenous *ppk28* locus into both *ppk28* mutant (*w*,*ppk28*$^{\Delta}$;+;*Dp(320)*) and control (*w*;+;*Dp(320)*) backgrounds (n=180 (*w*;+;*Dp(320)*); n=137 (*w*,*ppk28*$^{\Delta}$;+;*Dp(320)*) – mean lifespan increase of 1.65%) (B) on SY5% food. p<1x10^-6 for by Log Rank analysis for significant lifespan extension in (A).

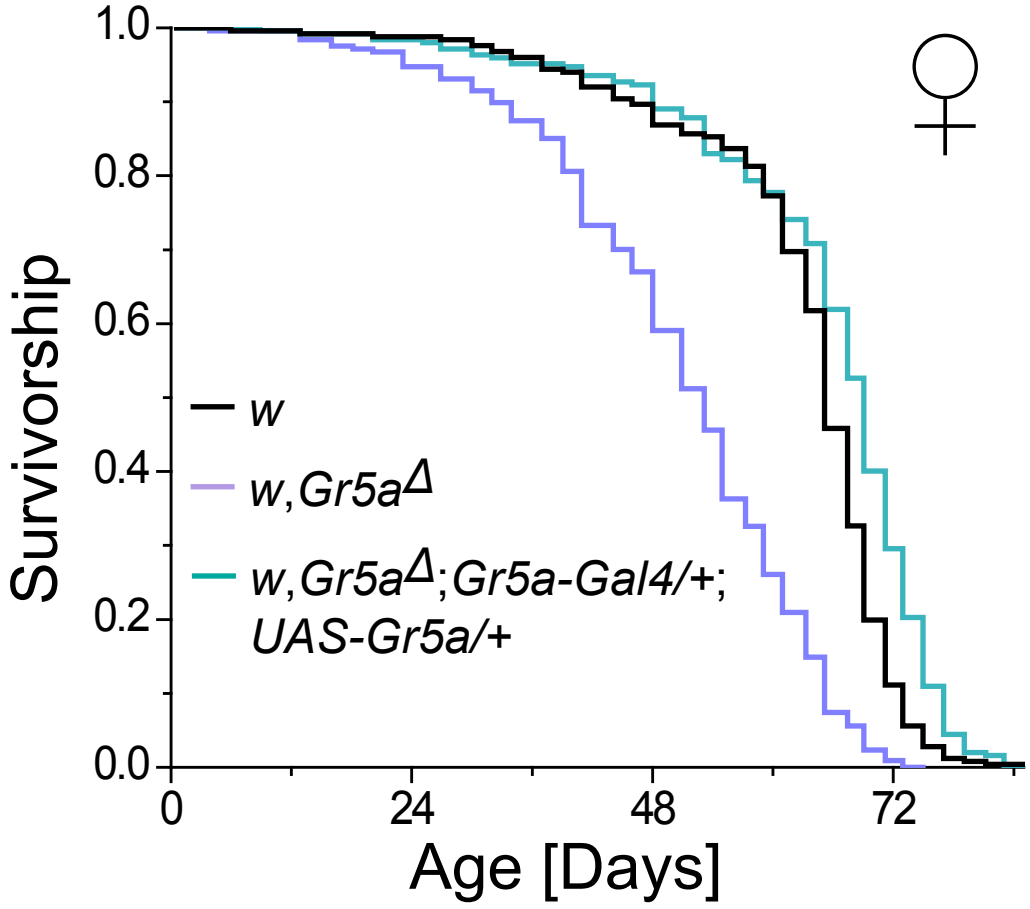


Figure 2.5. Re-expression of *Gr5a* in *Gr5a*-expressing Cells Rescues Shortened Lifespan of *Gr5a* Mutants. Survival curves for female *Gr5a* deletion mutants (*w*,*Gr5a*$^{\Delta}$), background controls (*w*) and genetic rescue (*w*,*Gr5a*$^{\Delta}$;*Gr5a-Gal4*/+;*UAS-Gr5a*/+) flies. (n=225 (*w*,*Gr5a*$^{\Delta}$); n= 251 (*w*); n=247 (*w*,*Gr5a*$^{\Delta}$;*Gr5a-Gal4*/+;*UAS-Gr5a*/+) – mean lifespan decrease of 18.77% between mutants and controls; mean lifespan increase of 3.39% between control and rescue flies). p<1X10^-6 by Log Rank analysis for mutant and control comparison.

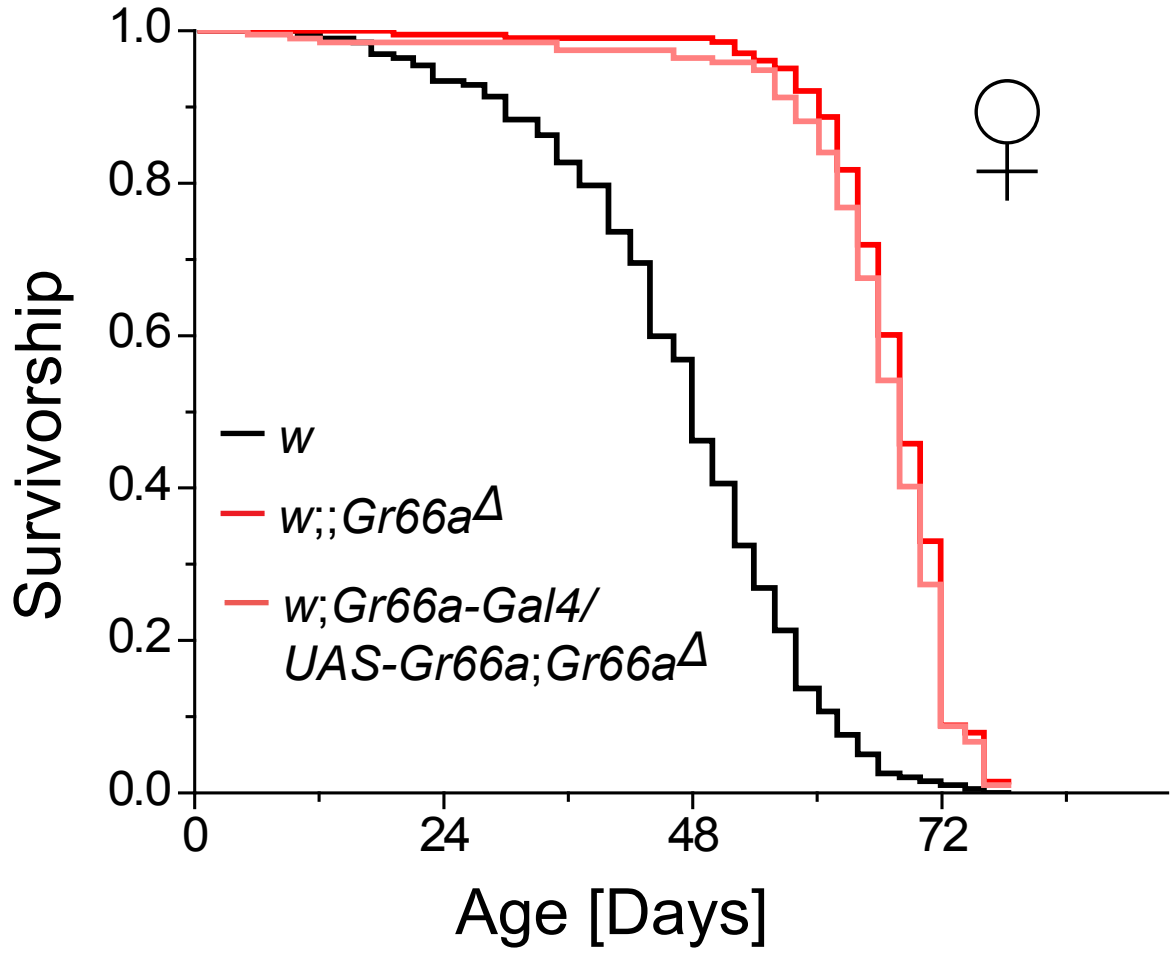


Figure 2.6. Re-expression of *Gr66a* in *Gr66a*-expressing Cells Rescues Shortened Lifespan of *Gr66a* Mutants. Survival curves for female *Gr66a* deletion mutants (*w*;;*Gr66a*$^{\Delta}$), background controls (*w*) and genetic rescue (*w*;*Gr66a-Gal4*/*UAS-Gr66a*;*Gr66a*$^{\Delta}$) flies. (n=200 (*w*;;*Gr66a*$^{\Delta}$); n= 197 (*w*); n=192 (*w*;*Gr66a-Gal4*/*UAS-Gr66a*;*Gr66a*$^{\Delta}$) – mean lifespan increase of 41.12% between mutants and controls; mean lifespan decrease of 2.18% between control and rescue flies). p<1X10^-6 by Log Rank analysis for mutant and control comparison.

Chapter 3: Dissection of a Neural Circuit Modulating *Drosophila* Longevity and Physiology Mediated by the Gustatory Water Sensor *ppk28*

Chapter 3.A. Introduction

Broad perturbation of the *Drosophila* gustatory system convincingly demonstrated this sensory modality as an influential modulator of longevity (Ostojic et al., 2014), and our initial studies provided multiple individual inputs capable of bidirectionally altering lifespan [Section 2.C.]. The most significant lifespan extension in any line tested was that of flies mutant for *ppk28*, an osmosensitive ENaC family ion channel expressed in water-sensitive GRNs and both required and sufficient for the fly's gustatory response to a water stimulus (Cameron et al., 2010). We thus utilized these mutant flies to uncover how this specific input negatively regulates lifespan, and, moreover, how solely inaccurate prediction of an organism's gustatory environment can prove conducive to a longer life.

Water is a substantial component of an organism's diet and essential for its survival. Indeed, water intake is regulated to ensure proper osmotic homeostasis, and whole organ systems are devoted to maintaining its balance. A gustatory response to water is found not only in flies and other insects (Evans and Mellon, 1962; Fujishiro et al., 1984), but also in mammals such as rats (Lindemann, 1996; Watson et al., 2007). As such, we hypothesized that altering the ability of an organism to elicit this gustatory response could modulate endocrine signaling pathways, enacting significant physiological

changes which, over the lifetime of the fly, extended its lifespan. To test this hypothesis, we performed a number of physiological assays which tested various parameters of nutrient homeostasis and associated survival and behavioral phenotypes. We also probed candidate signaling pathways known to coordinate metabolic physiology in the fly to determine the potential endocrine basis of this increased longevity. From these data, we sought to gain insight into the link between induced physiological state and its positive effect on lifespan.

Chapter 3.B. Materials and Methods

Drosophila Stocks. For background controls, we used w^{1118} (*w*; +; +) (VDRC) or (*yw*; +; +) (Bloomington Stock Center) lines. Transgenic flies included ($ppk28^{\Delta}$; +; +) (Gift from K. Scott), (+; +; *Dp(1;3)DC320*) (Bloomington Stock Center), (+;*UAS-dTRPA1*;+) (Gift from P. Garrity), (+;*AkhR*-;+) (Gift from R. Kühnlein), and (+;+;*dFoxO*(*Δ94*)) (Gift from L. Partridge).

Survival Analyses. Lifespan analyses were performed using an empirically optimized protocol established by our lab and facilitated by the use of an RFID-based tracking system and associated statistical software (dLife) developed by our lab (Linford et al., 2013). Flies were fed *ad libitum* throughout adult life. Flies were reared and housed at 25ºC in a 12:12 light-dark cycle for non-temperature sensitive experiments. For all temperature-sensitive UAS-dTRPA1 manipulations, flies were raised at 23ºC until eclosion, with half placed at 29ºC throughout adulthood and the other half remaining at 23ºC (permissive temperature control).

For desiccation resistance, approximately two week old flies were placed in vials containing ~1 cm drierite (anhydrous calcium sulfate; W.A. Hammond Drierite Company) and allowed to desiccate at room temperature.

For starvation resistance, approximately two week old flies were placed in vials containing 1% agar and maintained at 25ºC with vials changed daily.

Nutrient Level Assays. For all nutrient measurements, female flies were frozen at -80ºC and homogenized in groups of five in 200uL PBS+0.05% Triton X-100 (PBST) (IBI Scientific). Samples were centrifuged at 1,000 rpm for 1 minute to settle debris. All reactions were read with Synergy2 plate reader (BioTek).

For TAG measurement, 5uL of homogenate was placed in 150uL warmed Infinity Triglycerides reagent (Thermo Scientific), mixed briefly after five minute incubation at room temperature, and read at 520nm compared to glycerol standards.

For glucose measurement, 10uL of homogenate was placed in 150uL warmed Infinity Glucose reagent (Thermo Scientific), mixed briefly after 30 minute incubation at 37ºC, and read at 340nm compared with glucose standards.

For protein measurement, 2uL of homogenate was placed in 200uL [1:50] 4% Cupric Sulfate/BCA Solution (Novagen), mixed briefly after 30 minute incubation at 37ºC, and read at 562nm compared with BSA standards.

Negative Geotaxis Assay. Flies maintained on SY10% food were placed in empty vials and forced to the bottom with four hard taps. Flies were allowed to climb for 2 seconds and then photographed. Images were analyzed using Climber Software (developed by S. D. Pletcher) to quantify distance climbed.

Immunohistochemistry. Adult female flies were reared as described then dissected at approximately two weeks of age and stained with a dAKH-specific antibody (gift from J. Park). Adult female flies were dissected in PBS solution and tissue fixed in 4% formaldehyde for at least 2 hours. Samples were then washed five times and then incubated in PBS+0.05% Triton-X (PBST) for at least 2 hours. PBST was removed and followed by incubation in primary antibody (rabbit-anti dAkh 1:600 in PBST) for at least 8 hours. Tissue was again washed five times and then incubated in PBST for at least two hours. PBST was removed followed by incubation in secondary antibody (AlexaFluor (Invitrogen) 594-anti rabbit 1:500 in PBST) for at least three hours. Samples were washed five times and incubated in PBST for at least two hours. PBST was once more removed with samples then placed in Vectashield mounting media with DAPI (Vector Labs) for at least one hour. Samples were then mounted and visualized on Olympus FluoView 500 Laser Scanning Confocal Microscope at 20x magnification. Starved flies were placed on 1% agar vials for 14 hours prior to dissection.

Wet and Dry Mass Calculations. Groups of 10 female flies were weighed to determine wet mass and then placed overnight at 65ºC and re-weighed to determine dry mass.

Feeding Assay. Flies were reared as described on varying food types, then transferred to food of identical nutrient composition spiked with 0.05% (w/v) blue dye (FD&C Blue #1; Spectrum Chemical), 5 flies per sample. Flies were allowed to consume dyed food for 6 hours and then immediately frozen at -80ºC. Frozen flies were homogenized in 200uL PBST, centrifuged at 1,000 rpm for 1 minute to settle debris, and then 100uL of the supernatant was read directly at 630nm (reference wavelength of 670nm) and compared to blue dye standards.

RNA Extraction and Quantitative PCR. Whole flies (for *InR*, and *thor* mRNA measures) or flies without abdomens (for *dILP2* and *dAkh* mRNA measures) were frozen at -80ºC, and total RNA was extracted from these samples using TRIzol reagent (Invitrogen) according to manufacturer's protocol. Extracted RNA from each genotype was diluted to equal concentration in RNAse-free water. RT-PCR was performed using SuperScript III First Strand cDNA Synthesis (Invitrogen) to generate cDNA, which was then quantified by real-time PCR analysis using Power SYBR Green PCR Master Mix and a StepOne Plus Real-time PCR system (Applied Biosystems). Primers used were as follows: AKH_F: GCGAAGTCCTCATTGCAGCCGT, AKH_R: CCAATCCGGCGAGAAGGTCAATTGA; dILP2_F:ATGGTGTGCGAGGAGTATAATCC, dILP2_R: TCGGCACCGGGCATG, InR_F: CGCAAAAAGAAAGCACGCGA, InR_R: TTAGTCGTCGCCGATCCCTT, THOR_F: TCCTGGAGGCACCAAACTTA, THOR_R: GAGTTCCCCTCAGCAAGCAA.

Statistical Considerations. All survivorship data was compared via Log Rank analysis between relevant genotypes. ANCOVA was used for the negative geotaxis assay and a two-way ANOVA in analysis of TAG and glucose levels. Two-sided Student's T-tests were performed for wet/dry mass calculations and qPCR results. Sample sizes and replicate numbers are explicitly stated in each figure.

Chapter 3.C. *ppk28* Loss of Function Extends Lifespan and Healthspan Independent of Feeding Behavior

To determine whether longevity extension of *ppk28* mutants was independent of a specific genetic background, we replicated the initial lifespan experiment with *ppk28* null mutants ($ppk28^{\Delta}$) backcrossed eight generations in the *w* genetic background as well as separately into the *yw* genetic background for six generations. Indeed, female *ppk28*-null mutant flies showed a significant increase in mean and maximum lifespan in both genetic backgrounds, a maximum of 43.55% increase in mean lifespan in *w* and 24.78% in *yw* [Fig. 3.1.] (see Table 3.1. for replicate experiments). Loss of *ppk28* also extended male lifespan in both genetic backgrounds, though to a lesser degree [Fig. 3.2.]. Additionally, a second *ppk28* mutant allele created via P-Element insertion into the third of four exons of the *ppk28* gene ($ppk28^{G981}$) (Bellen et al., 2004) extended female lifespan (mean lifespan increase of 13.1%) as compared to background control [Fig. 3.3]. To test whether loss of water-sensing gustatory information was also associated with an increase in overall health, we performed a negative geotaxis assay and found that *ppk28* mutants showed greater performance than background controls throughout

their lifespan [Fig. 3.4.]. Furthermore, *ppk28* mutants showed increased resistance to starvation stress [Fig. 3.5.].

Gustatory manipulations have the potential to alter food intake, and dietary restriction is sufficient to affect lifespan across model organisms (Fontana et al., 2010). Therefore, to determine whether *ppk28*-null mutants were long-lived simply because they were eating less than their background controls, we quantified feeding behavior in these flies. Our longevity assays used a sugar-yeast medium containing 10% of both macronutrients (10%SY), and food intake rates in *ppk28* mutant flies were statistically indistinguishable from control animals under these conditions at a young age and significantly increased at an advanced age [Fig. 3.6.]. Furthermore, we found that overall nutrient concentration (ranging from 5%SY-15%SY), and therefore the osmolarity of the medium, had no effect on this relationship [Fig. 3.7.]. These data suggest that *ppk28* mutant flies are not long-lived due to decreased feeding behavior.

Chapter 3.D. *ppk28* Mutants Maintain Augmented Nutrient Stores

The criticality of water for metabolic processes led us to hypothesize that water gustatory neurons may exert their regulatory effect through control of nutrient metabolism. To establish whether loss of *ppk28* input was sufficient to modulate nutrient homeostasis, we assayed whole organism levels of both carbohydrate and lipid in the long-lived *ppk28* deletion mutants. Indeed, these flies displayed both increased levels of triacylglyceride [TAG] and glucose as compared to background controls, effects that were rescued with reintroduction of *ppk28* into the mutant background [Fig. 3.8.]. Notably, we observed that aging had little effect on TAG and glucose levels as

differences among genotypes persisted throughout life. Though *ppk28* mutants were slightly heavier than background controls [Fig. 3.9.], normalization by body weight did not affect the relationship between genotypes. Furthermore, the difference in mass was not present immediately post-eclosion [Fig. 3.10.]. These data suggest that altered nutrient levels in water-sensing mutants were due to a directed switch in physiological state early in adult life rather than a loss of homeostatic control.

Chapter 3.E. Lifespan Extension of *ppk28* Mutants Requires Nutrient Homeostasis-Regulating Signaling Pathways

Having observed significant differences in nutrient levels, we next hypothesized that signaling pathways responsible for the coordination of metabolic homeostasis may be required for *ppk28*-mediated lifespan extension. In mammals, levels of glucagon and insulin are key to the coordination of carbohydrate and lipid metabolism (Jiang and Zhang, 2003). Flies maintain functionally homologous molecules to each of these hormones – the glucagon-like adipokinetic hormone (*dAkh*) (Bharucha et al., 2008) as well as eight insulin-like peptides (*dILP*s 1-8) (Brogiolo et al., 2001; Colombani et al., 2012; Garelli et al., 2012).To test the requirement of each signaling network in *ppk28*-mediated lifespan extension, we undertook an epistasis approach in which double mutant flies, functionally null for *ppk28* as well as a critical component of either pathway, were generated and assessed for lifespan. We found that the extended lifespan of *ppk28*-null flies was abrogated by the introduction of a null mutation for the *Akh* receptor (*AkhR*) (Gronke et al., 2007) into the control and *ppk28* mutant backgrounds [Fig. 3.11.]. Likewise, a similar strategy using a null mutation for the *Drosophila* homolog of FoxO

transcription factor (*dFoxO*) (Slack et al., 2011), an integral component in ILP signaling, also abolished the increased longevity found in *ppk28* mutant flies [Fig. 3.12.]. As *dFoxO* is normally active under levels of low ILP signaling, these data support a model by which lifespan extension may be due, at least in part, to reduced levels of insulin signaling. Consistent with this interpretation, we found that transcript levels of *dILP2* were decreased in *ppk28* mutants [Fig. 3.13.].

Chapter 3.F. Glucagon-like Adipokinetic Hormone Signaling is Upregulated in *ppk28* Mutants

The requirement of the AKH receptor was a surprise as its cognate hormone has been little studied in relation to aging. One prediction from the genetic data is that release of AKH neuropeptide from its site of synthesis would be increased in *ppk28* mutant flies. AKH is produced in a small subset of neurons called the *corpora cardiaca* (CC) (Lee and Park, 2004). Though *Akh* mRNA levels were not significantly different between *ppk28* mutant and control flies [Fig. 3.14.], previous work in both the locust (*Locusta migratoria*) and *Drosophila* suggest that *Akh* gene transcription is uncoupled from neuropeptide release and that the neuropeptide may be discharged in a controlled fashion from a pool of continuously synthesized protein (Diederen et al., 2002; Rhea et al., 2010). To determine whether loss of *ppk28* affected AKH release or sequestration, we directly imaged neuropeptide localization in dissected CC from adult flies stained with a dAKH-specific antibody (Lee and Park, 2004). As a positive control indicative of active AKH signaling, we used starved control flies. AKH pathway activity is inversely correlated with starvation resistance (Lee and Park, 2004), and starvation should thus

stimulate AKH release. We found that *ppk28* mutants showed increased neuropeptide staining, as compared to background controls, specifically in the axonal projections from which AKH is released to target areas. A complete lack of staining in these projections was never observed in *ppk28* mutant animals but was frequently observed in control animals [Figs. 3.15.; 3.16.]. The staining pattern of *ppk28* mutants closely resembled that of starved flies, arguing that loss of *ppk28* function activates AKH signaling.

Our results indicated that an increase in AKH signaling was induced in water-sensing mutants and was essential for lifespan extension. These data are indicative of AKH as a key effector of water taste sensation and as a cause of *ppk28* lifespan extension. If so, then increasing activity of this pathway, in the absence of sensory manipulation, should similarly increase lifespan. We therefore tested this hypothesis by conditionally expressing the activating cation channel dTRPA1 (Hamada et al., 2008) in *Akh*-ergic neurons and studying the effects of targeted neuronal activation on lifespan. Indeed, we found that transgenic flies (*Akh-Gal4;UAS-dTRPA1)* were significantly long-lived compared to genetic control animals at a temperature in which dTRPA1 is activated (29ºC) but not at a control temperature (23ºC) in which it is not [Fig. 3.17., courtesy of BY Chung]. Depleted TAG levels, which are characteristic of enhanced neuronal AKH secretion, persisted for at least 26 days in flies with activated dTRPA1, suggesting that this genetic strategy was effective in securing chronic pathway stimulation [Fig. 3.18.]. Furthermore, this manipulation increased staining in CC cell axons, consistent with findings in long-lived *ppk28* mutants [Fig. 3.19.]. Importantly, this lifespan extension could not be attributed to a reduction in food intake [Fig. 3.20.]. The overexpression of *Akh* in *Akh*-ergic neurons (*Akh-Gal4;UAS-Akh)* had no effect on lifespan [Fig. 3.21.].

This is not unexpected because of the documented uncoupling between Akh mRNA synthesis and secretion of AKH protein in insects where physiological phenotypes associated with activation of AKH signaling, including an increase in larval hemolymph trehalose, are not recapitulated by modulation of gene overexpression (Lee and Park, 2004). Together, these data suggest that neuronal modulation of *Akh*-expressing cells is required to promote physiological changes that are advantageous for maximizing health and longevity.

Chapter 3.G. Loss of the Ability to Sense External Water Increases Internal Water Stores

The physiologic effects of *ppk28* loss of function were reminiscent of adaptations used by a number of desert species that have limited access to fresh water, which use TAG lipolysis and oxidation of free fatty acids as primary sources of metabolic water production (Frank, 1988; Naidu, 2008). We considered that *ppk28* mutants, while not physically starved for water, might nevertheless induce similar physiological strategies due to its perceived scarcity. In this model, mutant flies should maintain higher levels of internal water, which in *Drosophila* can be measured by the subtraction of mass post-desiccation ("dry mass") from its initial mass ("wet mass") (Folk et al., 2001). Indeed, *ppk28* mutant flies showed an increased change in mass after desiccation than background controls [Fig. 3.22.]. Though *ppk28* mutants maintained a modest, yet significant, increase in wet mass, this is almost certainly due to augmented stores of water, as well as TAG and glucose levels, rather than an increase in gross size. Indeed, whole-organism protein levels were not significantly different between ppk28 mutants

and their background controls [Fig. 3.23.]. Furthermore, insects with higher internal water content have been found to be desiccation resistant (Gray and Bradley, 2005), and we found that *ppk28* mutants exhibited significantly increased survivorship under desiccating conditions [Fig. 3.24., with ZM Harvanek]. Consistent with a model by which internal water stores are increased through the action of the AKH signaling pathway, we found that constitutive activation of AKH-expressing cells also increased the difference between wet and dry mass [Fig. 3.25.]. Together, these results suggest that loss of *ppk28* function may drive the production of metabolic water through a glucagon-like AKH-dependent mobilization of lipid stores as a compensatory response to the absence of sensory information pertaining to accessible water.

Chapter 3.H. Perspective

Here, we exploited a specific gustatory signal – emanating from water-sensing taste neurons – as a model by which to understand sensory modulation of aging. As water is an essential component of an organism's diet and its recognition and ingestion are critical to an organism's health, we reasoned that modulation of this sensory cue alone may have significant physiological consequences. Indeed, we discovered that loss of function of the ion channel required for water taste perception – *ppk28* – extended lifespan and augmented health-related parameters (e.g., stress resistance and climbing ability). Furthermore, mutation of *ppk28* resulted in alteration of metabolic homeostasis through an increase in lipid stores and a subsequent activation of glucagon-like AKH signaling. Several observations suggested that this physiological switch increased production of metabolic water, resembling a strategy utilized by species with a severe or complete lack of access to environmental water. Compatible with this model, we find

that activation of *Akh*-ergic neurons is sufficient to increase lifespan, suggesting that signals leading to such activation are also efficacious for increasing health and longevity [Fig. 3.26.].

Though often overlooked as a principal dietary component in favor of carbohydrate, protein, or lipid, water is just as crucial to an organism's ability to maintain metabolic homeostasis and just as quintessential for its survival. Indeed, diverse organisms, including flies and mammals, commit a similar amount of sensory resources to the perception of water (Cameron et al., 2010; Gilbertson, 2002). Our studies suggest that information about external water availability transduced through *ppk28*-sensing neurons is capable of affecting its internal production and that stimulation of this system by water intake may represent an important consideration for determining dietary influence on health status.

Classic dietary restriction (DR) paradigms of carbohydrate and protein robustly increase lifespan across species, and act at least partially through sensory mechanisms independent of caloric intake (Libert et al., 2007; Smith et al., 2008). Though conclusions from previous work over the role of dietary water in mediating the *Drosophila* DR-lifespan extension axis have been mixed (Ja et al., 2009; Piper et al., 2010), our studies suggest that water restriction, inasmuch as it decreases stimulation of water-sensing neurons, may also be a viable strategy for enhancing physiological state. Given the necessity of water for metabolic reactions and the discomfort of thirst, however, of perhaps more translational relevance is the implication that the glucagon-like AKH signaling pathway is a potent modulator of health and lifespan. There is some evidence suggesting that this pathway may, in fact, impinge on the control of lifespan

via protein restriction as a recent study found that ectopic overexpression of *Akh* extends lifespan in flies which are fully fed, yet not under yeast restriction (Katewa et al., 2012). Though the ubiquitous nature of this manipulation may confound its physiological relevance, this is indicative that restriction of dietary components may converge on similar regulatory mechanisms. Congruously, levels of plasma glucagon increase under dietary restriction in mice (Ash and Merry, 2011). Dietary or pharmacological interventions that stimulate release of glucagon may, therefore, promote a healthier lifespan in mammals, without the necessity of actual restriction of dietary components.

The evidence that *ppk28*-mediated inputs possibly exert their metabolic and lifespan effects through alteration of insulin/ILP signaling suggests a potential additional layer of complexity to this endocrine control. Indeed, lifespan extension in *C. elegans* gustatory mutants requires the FoxO homolog *daf-16* (Alcedo and Kenyon, 2004), and additional work suggests a similar *dFoxO*-dependent mechanism in *D. melanogaster* (Ostojic et al., 2014). The relationship between the insulin and glucagon signaling pathways in maintaining metabolic homeostasis has been well-studied in mammalian contexts, with high levels of insulin known to inhibit glucagon release (Maruyama et al., 1984). Though this association is less well understood in *Drosophila*, dILP2-producing median neurosecretory cells and AKH-producing cells are in close proximity, with dILP2-ergic axons extending to the *corpora cardiaca* (Rulifson et al., 2002). If a similar crosstalk occurs in flies, activation of AKH release in *ppk28* mutants may be, at least in part, due to downregulation of ILP signaling, as suggested by decreased *dILP2* levels and *dFoxO*-dependence of longevity increase in these flies. As such, lifespan modulation

due to interventions reducing insulin/ILP activity may additionally need to be understood in light of their effect on glucagon-like signaling.

The significance of our findings for the enhancement of health and longevity underscore the importance of the further work that remains. For instance, the mechanisms responsible for the initial increase in TAG stores used as substrate for AKH remains unknown. Furthermore, the signal that activates AKH signaling in response to increased TAG levels has also not been determined. Finally, it remains to be discovered how an increase in AKH pathway activity is responsible for increasing health and longevity. Nonetheless, the results described here form the basis for an understanding of the dynamics of lifespan-modulating sensory signals and their means of command.

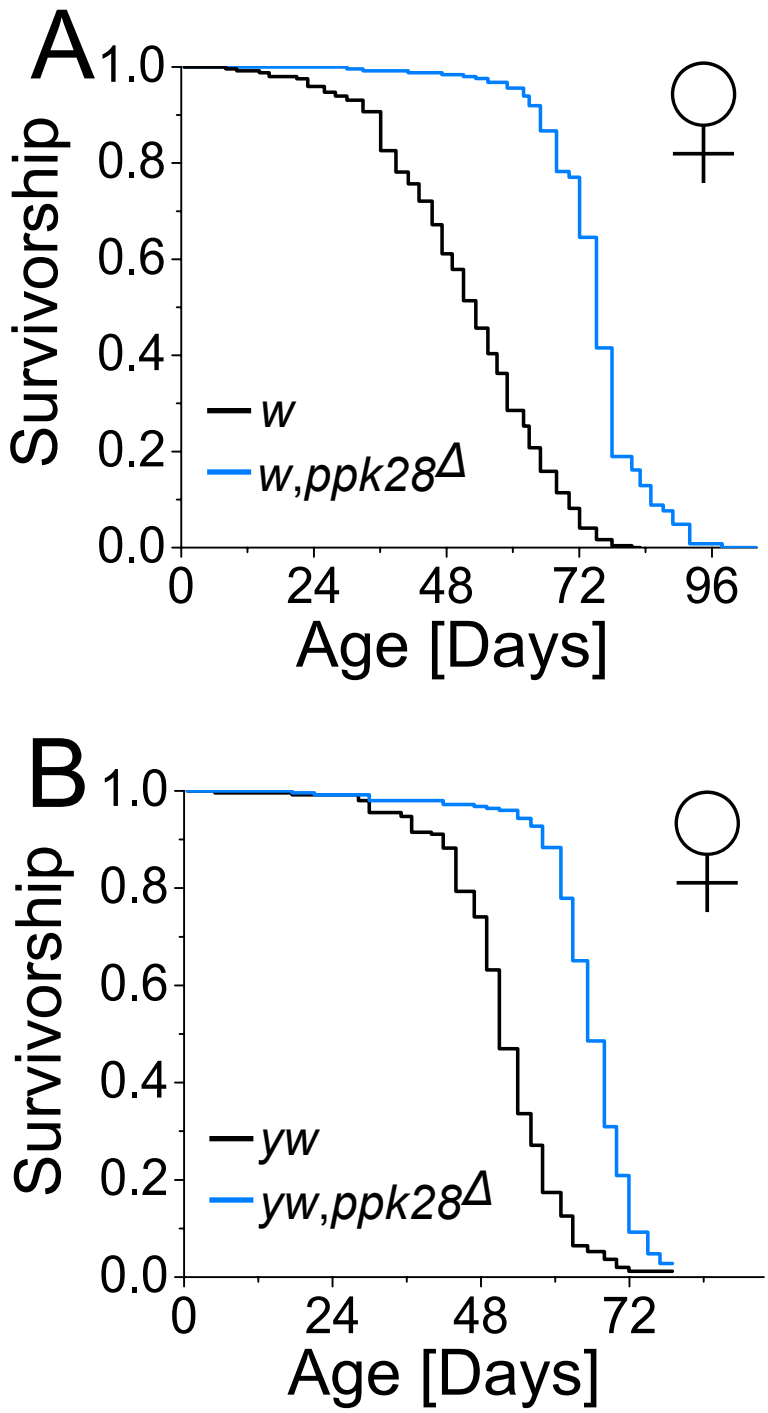


Figure 3.1. Loss of *ppk28* Function Extends Lifespan Independent of Genetic Background. Survival curves for female *ppk28* deletion mutants in both *w* (n=246 (*w*); n=248 (*w*,*ppk28*$^{\Delta}$) – mean lifespan increase of 20.68 days [43.55%]) (A) and *yw* (n=244 (*yw*); n=248 (*yw*,*ppk28*$^{\Delta}$) – mean lifespan increase of 13.48 days [24.78%]) (B) background and their corresponding background controls. p<1X10^-6 by Log Rank analysis for mutant and control comparisons.

Mutant	Control	N		Mean Lifespan		±Lifespan	P-value [L-R]
		Mut	Ctrl	Mut	Ctrl		
w, *Δppk28*	*w*	250	249	74.42	54.98	35.36%	0
w, *Δppk28*	*w*	242	252	67.38	50.37	33.77%	0
w, *Δppk28*	*w*	248	243	77.25	58.37	32.35%	0
w, *Δppk28*	*w*	241	246	74.79	55.97	33.63%	0
w, *Δppk28*	*w*	245	243	63.98	53.43	19.75%	0
w, *Δppk28*	*w*	246	247	74.76	58.18	28.50%	0
w, *Δppk28*	*w*	199	203	70.24	52.22	34.51%	0

Table 3.1. Replicate *ppk28* Mutant Lifespan Experiments. Population size [”N”] and mean lifespan values [in days] of replicate lifespan analyses of *ppk28* deletion mutant female flies (*w*,*ppk28*$^{\Delta}$) and their background controls (*w*). p values determined by Log Rank [”L-R”] analysis.

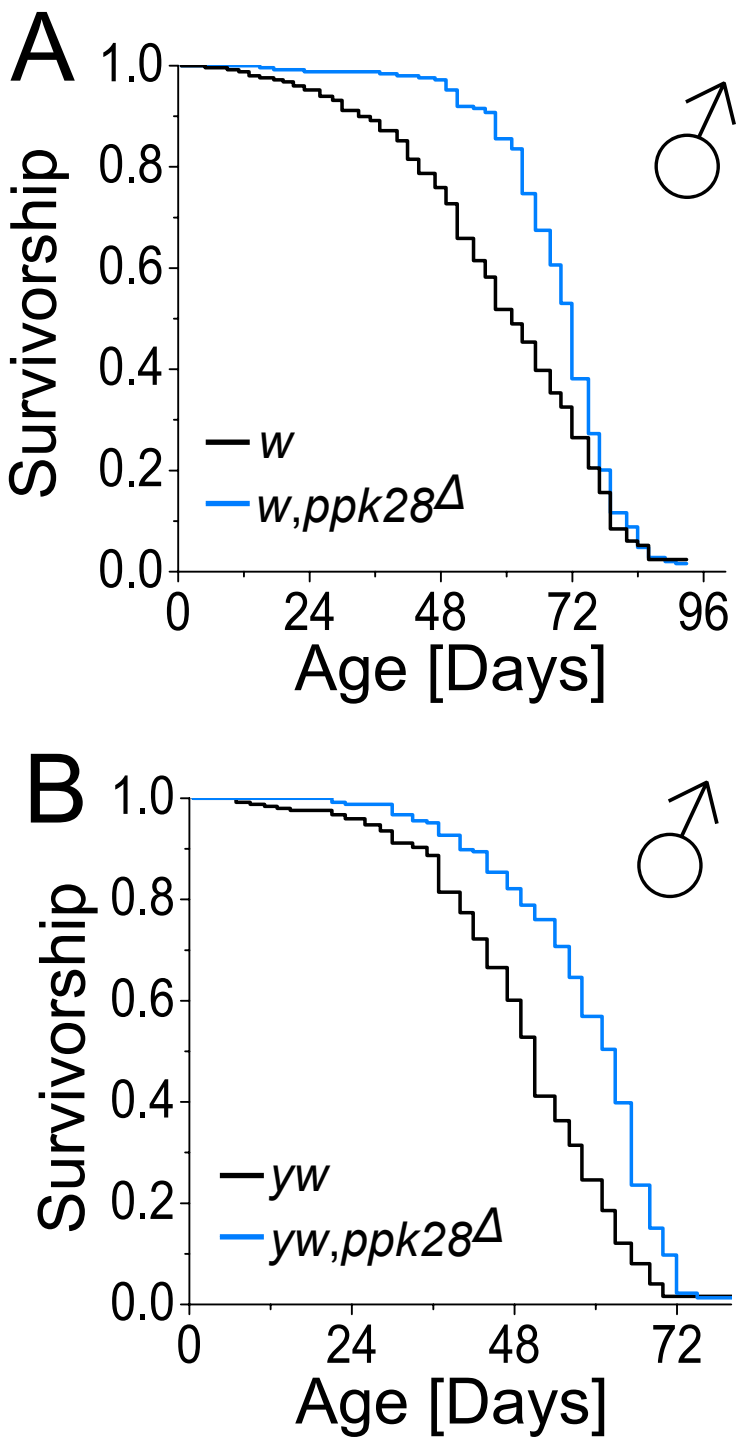


Figure 3.2. Loss of *ppk28* Function Extends Lifespan in Males. Survival curves for male *ppk28* deletion mutants (*w*,*ppk28*$^{\Delta}$) as compared to *w* (n=243 (*w*); n=245 (*w*,*ppk28*$^{\Delta}$) – mean lifespan extension of 10.37 days [16.80%]) (A) and *yw* (n=245 (*yw*); n=241 (*yw*,*ppk28*$^{\Delta}$) – mean lifespan extension of 8.87 days [16.96%]) (B) background controls. Pairwise comparisons between genotypes yielded p=2.45x10^-5 for (A) and p<1x10^-6 for (B) by log-rank test.

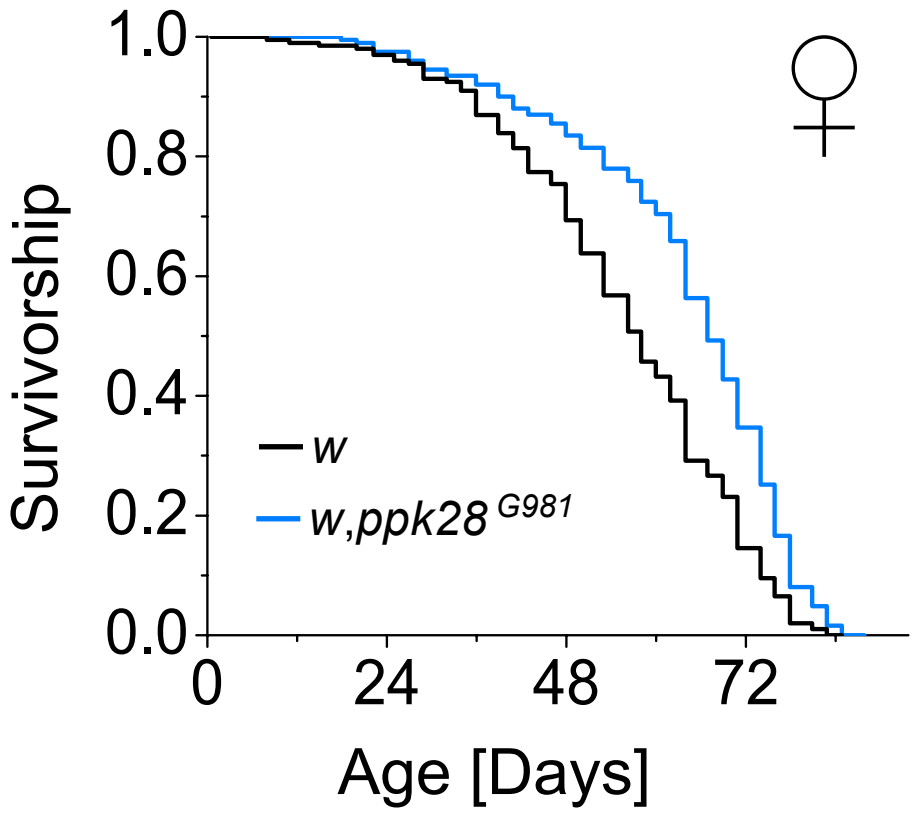


Figure 3.3. A Separate *ppk28* Mutant Allele Also Extends Lifespan. Survival curves for female *ppk28* mutants generated via P-element insertion into its coding region of (*w*,*ppk28*^{G981}) as compared to background control (*w*) (n=200 (*w*); n=201 (*w*,*ppk28*^{G981}) – mean lifespan extension of 7.68 days [13.13%]). p<1x10^-6 by Log Rank analysis.

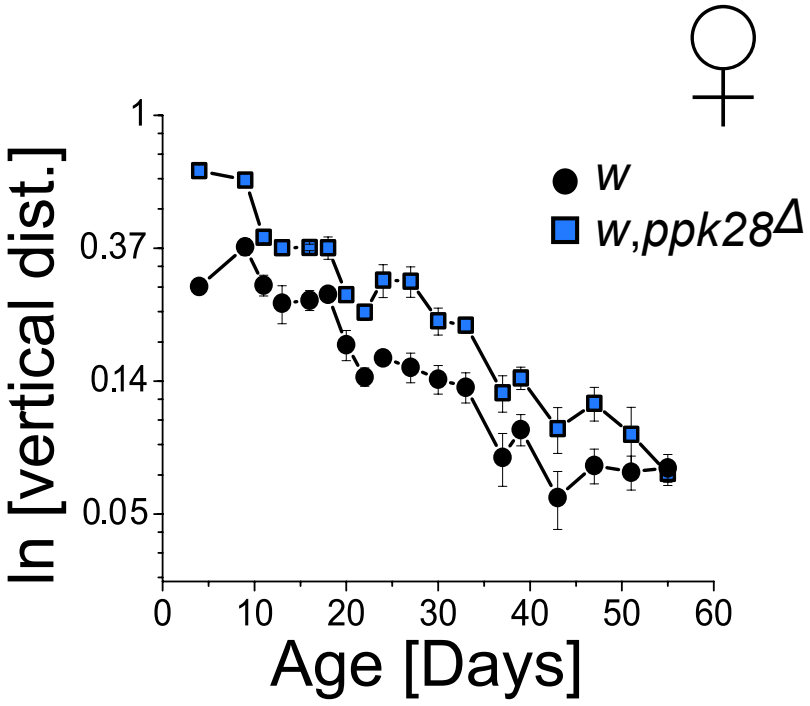


Figure 3.4. *ppk28* Mutants Show Longitudinal Increase in Climbing Performance. Analysis of vertical distance climbed in the longitudinal negative geotaxis assay of *ppk28* mutant (*w*,*ppk28*$^{\Delta}$) and background control (*w*) female flies (n=10 groups of 20 flies per genotype per timepoint). p(AGE)<1x10^-15, p(GENOTYPE)=1.13x10^-10, and p(INTERACTION)=0.031 for ANCOVA. Error bars indicate ±S.E.M.

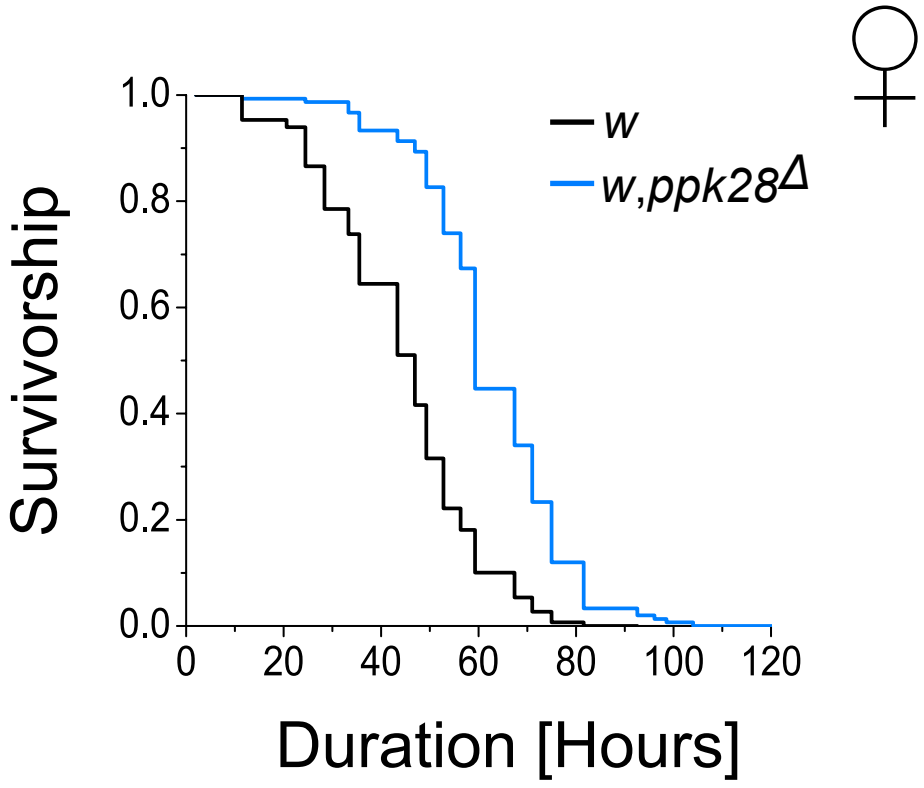


Figure 3.5. *ppk28* Mutants are Resistant to Starvation Stress. Survival curves for female *ppk28* mutant (*w*,*ppk28*$^{\Delta}$) and background control (*w*) female flies (n=149 (*w*); n=150 (*w*,*ppk28*$^{\Delta}$)) under starvation conditions.p<1x10^-6 by Log Rank analysis.

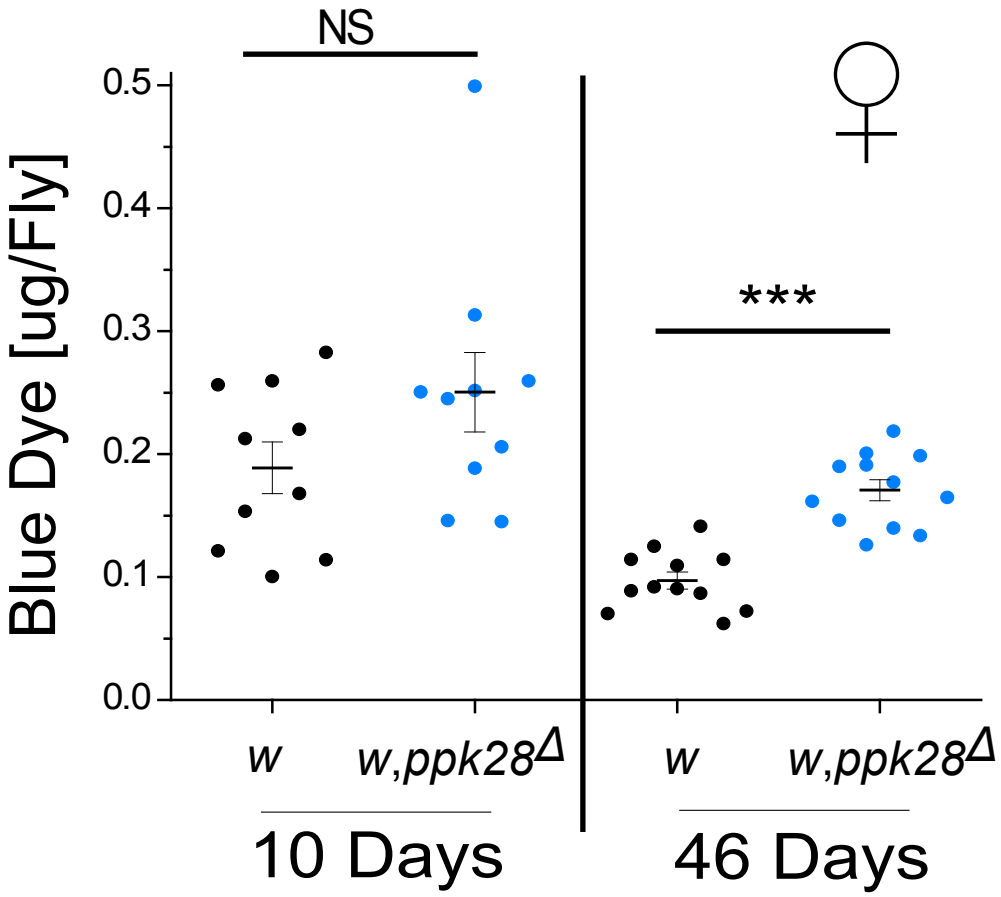


Figure 3.6. Lifespan Extension in *ppk28* Mutants is Not Due to Decreased Feeding. Total amount of blue dye consumed in a six hour period by female *ppk28* deletion mutants (*w*,*ppk28*$^{\Delta}$) as compared to background controls (*w*) on a 10% (w/v) sugar-yeast diet (SY10%) at 10 or 46 days of age (n=10 groups of 5 flies for each genotype at 10 days and 12 groups of 5 flies for each genotype at 46 days). ***= p<0.001 by two-sided Student's T-Test. Error bars indicate ±S.E.M.

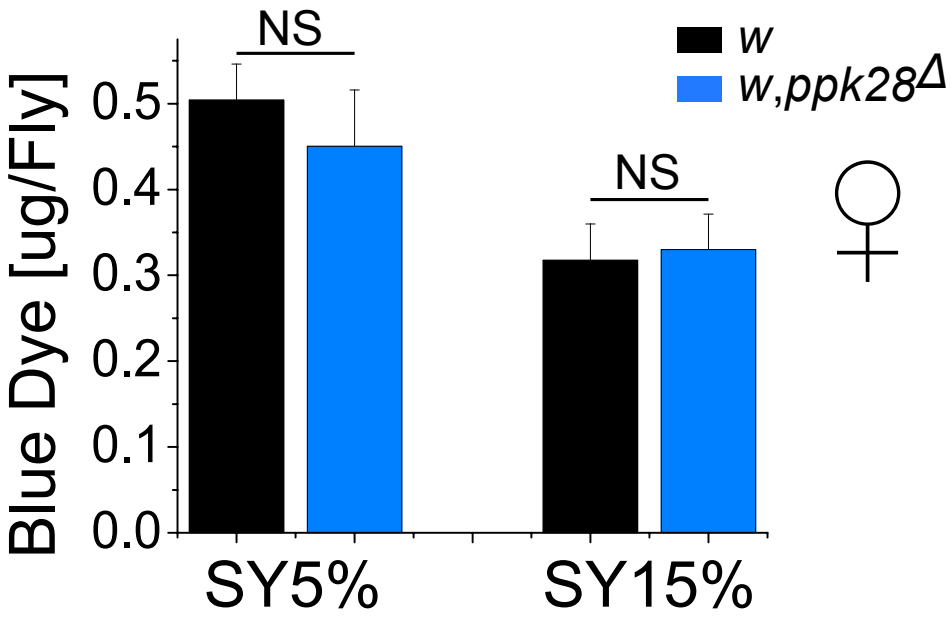


Figure 3.7. Osmolarity of Food Medium Does Not Affect Relationship in Feeding Behavior Between *ppk28* Mutants and Controls. Total amount of blue dye consumed in a six hour period by two-week old female *ppk28* deletion mutants (*w*,*ppk28*$^{\Delta}$) as compared to background controls (*w*) on on a 5% or 15% (w/v) sugar-yeast diet (SY5% and SY15%) foods (B) (n=10 groups of five flies for both foods for each genotype). Error bars indicate ±S.E.M.

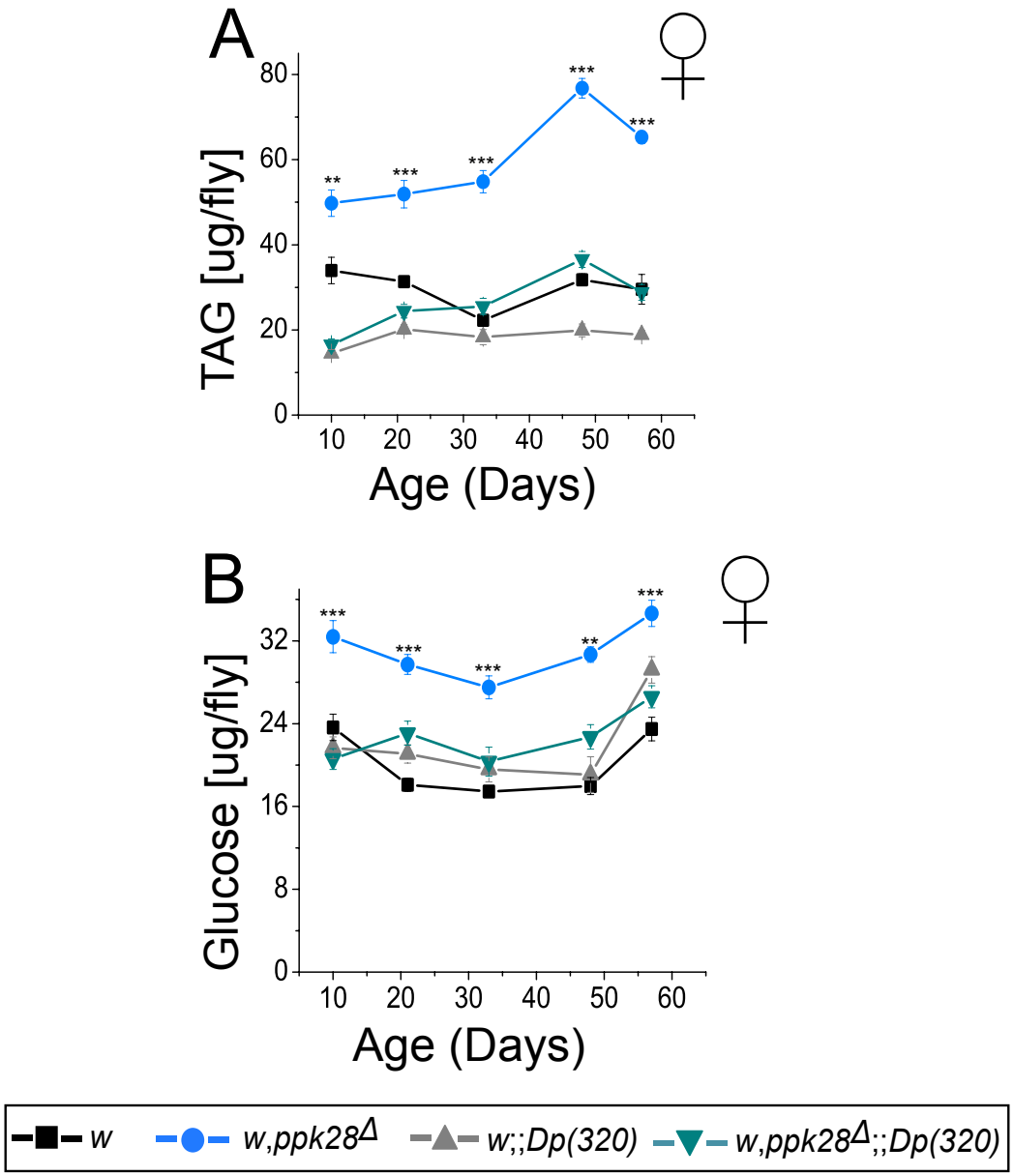


Figure 3.8. Loss of *ppk28* Function Augments Whole-organism Lipid and Carbohydrate Stores Longitudinally. Longitudinal measures of whole-fly triacylglycerides [TAG] (A) and glucose (B) in *ppk28* deletion mutant female flies (*w*,*ppk28*$^{\Delta}$) and in mutant animals also containing a ppk28 genomic rescue construct (*w*,*ppk28*$^{\Delta}$;+;*Dp(320)*), as well as their appropriate genetic background controls (*w* and *w*;+;*Dp(320)*, respectively). n=8-12 groups of 5 flies per genotype per timepoint. **=p<0.01; *** = p<0.001 for interaction term of two-way ANOVA. Error bars indicate ±S.E.M.

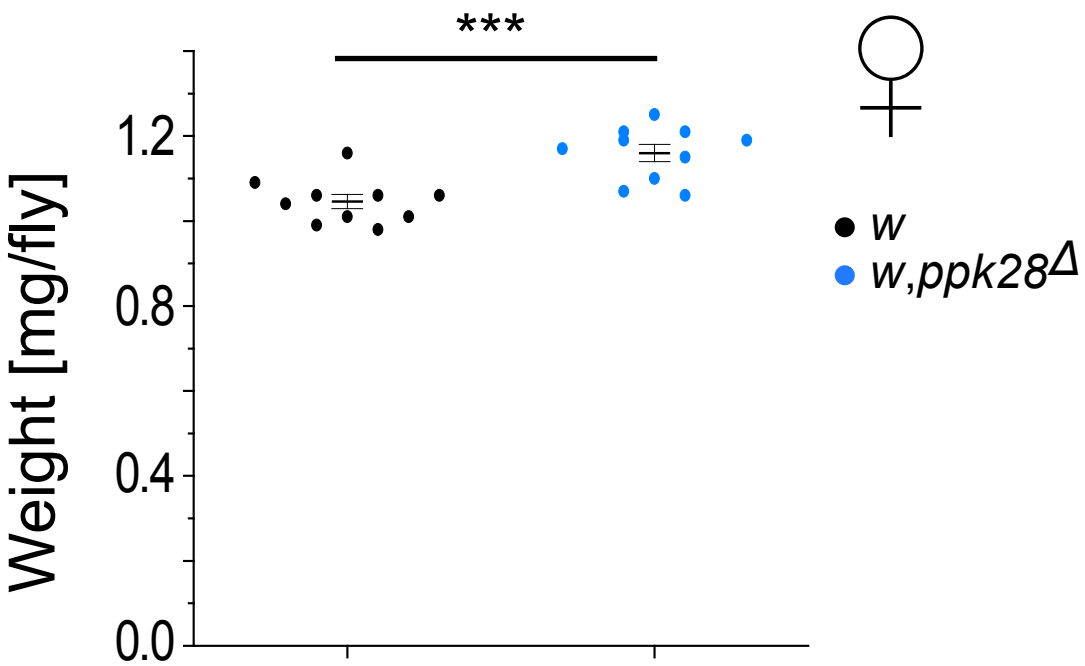


Figure 3.9. *ppk28* Mutant Adults Are Slightly Heavier Than Controls. Average weight of ppk28 deletion mutant female flies (*w*,*ppk28*$^{\Delta}$) and background controls (*w*) at 14 days post-eclosion. *** = p<0.001 by two-sided Student's T-Test. n=10 groups of 10 per genotype. Error bars indicate ±S.E.M.

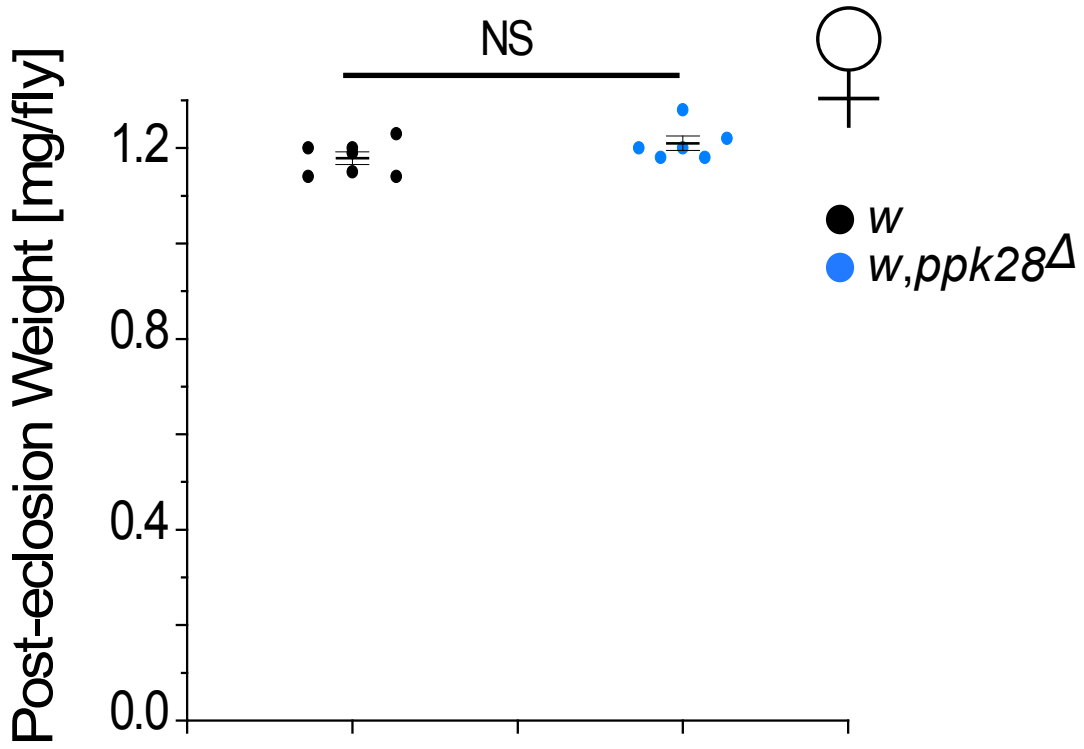


Figure 3.10. Newly Eclosed *ppk28* Mutants Do Not Weigh Significantly More Than Controls. Average weight of *ppk28* deletion mutant female flies (*w*,*ppk28*$^{\Delta}$) and background controls (*w*) at 8 or fewer hours post-eclosion. n=6 groups of 10 per genotype. Error bars indicate ±S.E.M.

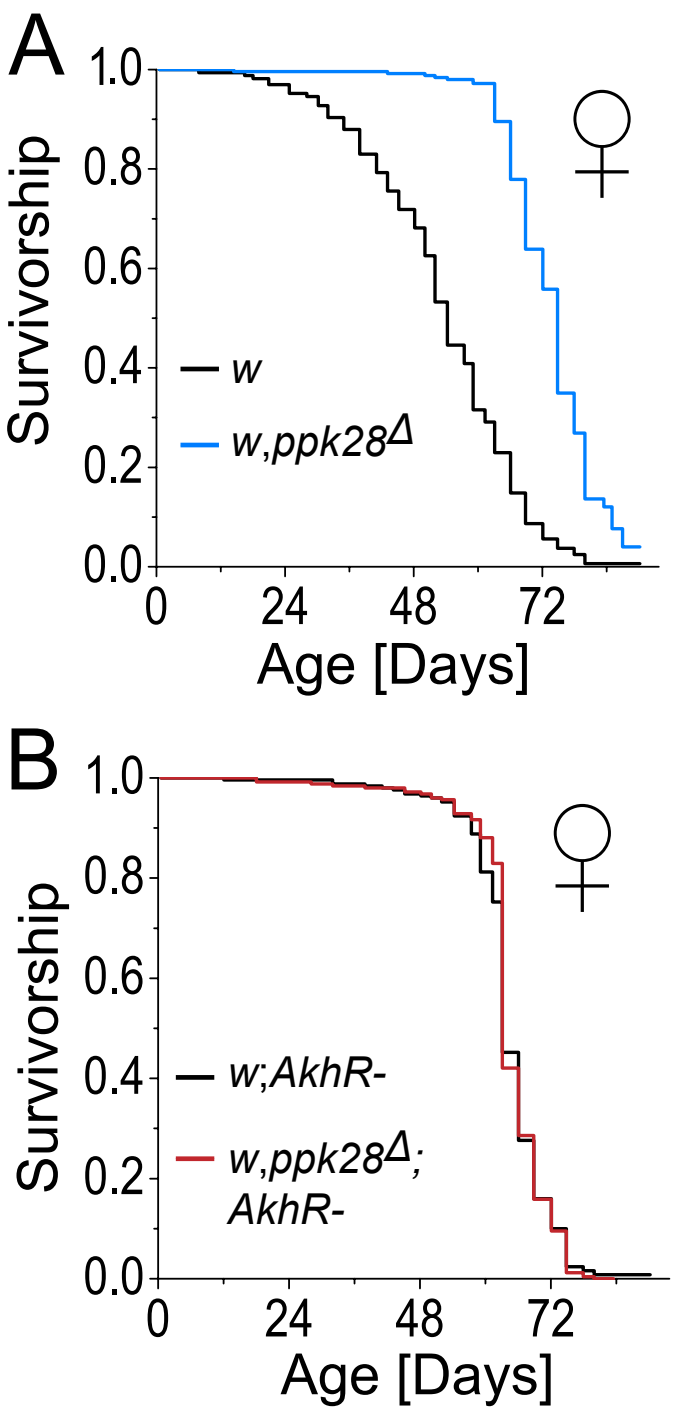


Figure 3.11. *AkhR* Function is Required for *ppk28*-mediated Longevity. Survival curves for female *ppk28* deletion mutant flies (*w*,*ppk28*$^{\Delta}$) and background controls (*w*) (n=162 (*w*); n=249 (*w*,*ppk28*$^{\Delta}$) – mean lifespan increase of 20.68 days [36.96%]) (A) and same backgrounds containing loss of function of the adipokinetic hormone receptor (*w*; *AkhR*-) and (*w*,*ppk28*$^{\Delta}$; *AkhR*-) (n=253 (*w*; *AkhR*-); n=248 (*w*,*ppk28*$^{\Delta}$; *AkhR*-) – mean lifespan increase of 0.67 days [0.99%]) (B). p<1x10^-6 by Log Rank Analysis for comparison in (A).

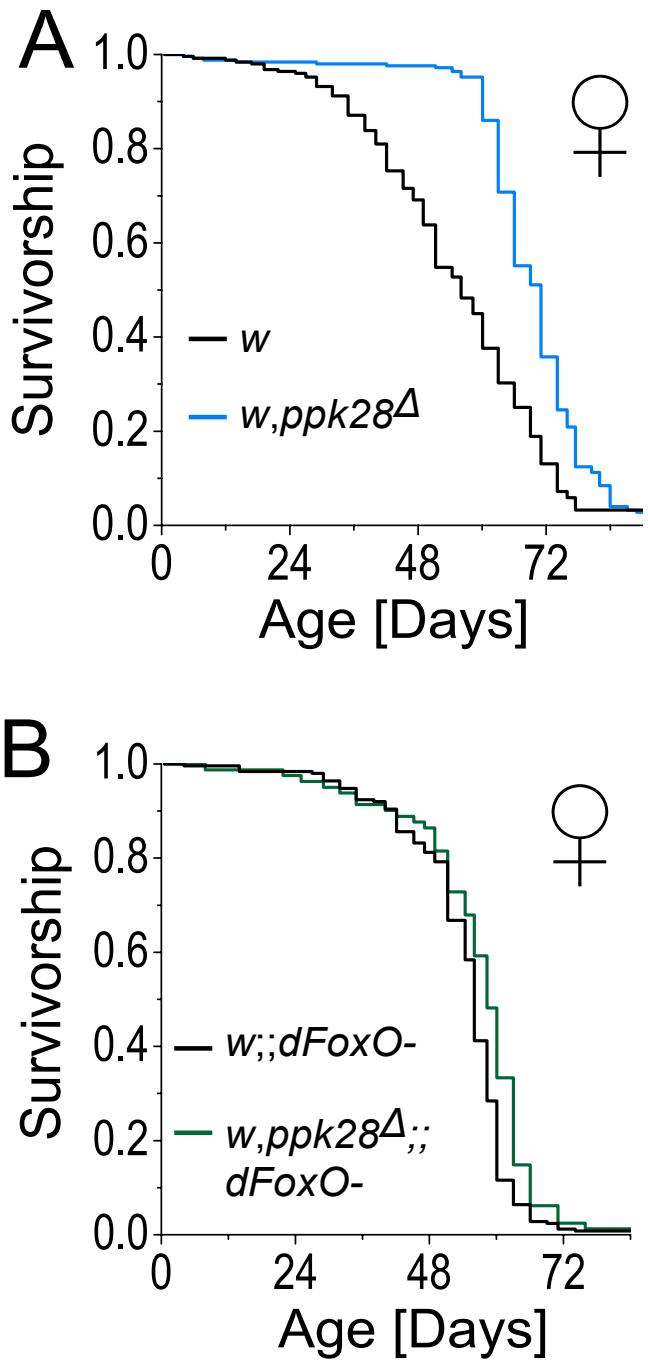


Figure 3.12. *dFoxO* Function is Required for *ppk28*-mediated Longevity. Survival curves for female ppk28 deletion mutant flies (*w*,*ppk28*$^{\Delta}$) and their controls (*w*) (n=221 (*w*); n=243 (*w*,*ppk28*$^{\Delta}$) – mean lifespan increase of 14.16 days [24.73%]) (A) and same backgrounds containing deletion of the FoxO transcription factor (*w*;;*dFoxO*- and *w*,*ppk28*$^{\Delta}$; *dFoxO*-) (n=248 (*w*;;*dFoxO*-); n=80 (*w*,*ppk28*$^{\Delta}$;;*dFoxO*-) – mean lifespan increase of 2.4 days [4.28%]) (B). p<1x10^-6 by Log Rank analysis for comparison made in (A).

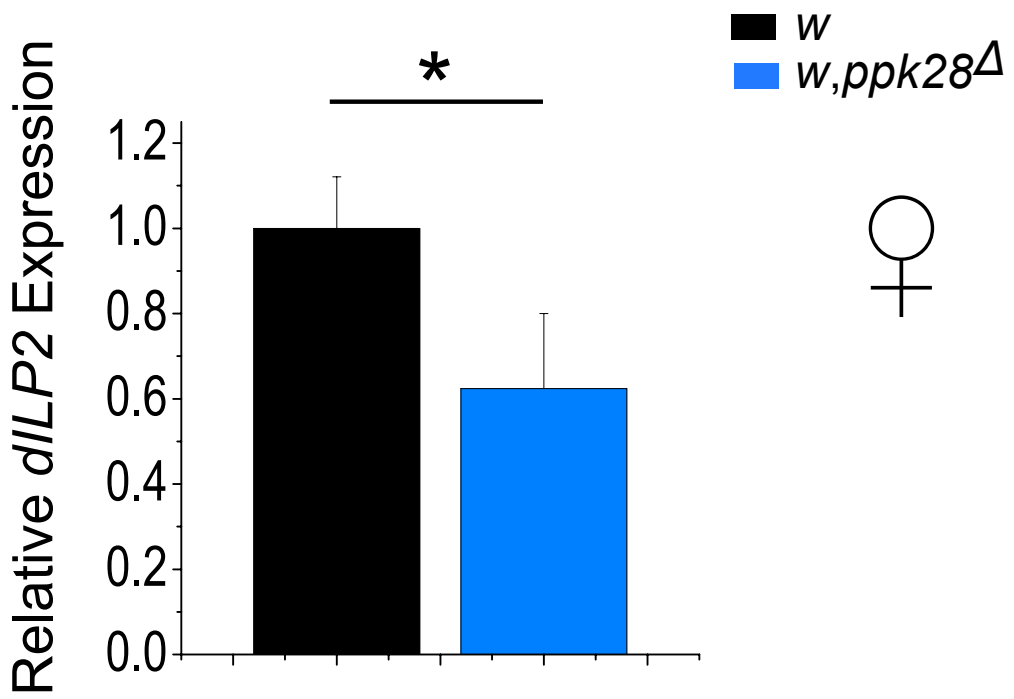


Figure 3.13. *dILP2* Levels are Reduced in *ppk28* Mutants. Relative mRNA levels of *dILP2* from quantitative PCR analysis of *ppk28* deletion mutant female flies (*w*,*ppk28*$^{\Delta}$) and their appropriate background controls (*w*). Values were normalized to *Rpl-32*, used as an endogenous control (n=3 biological replicates). *=p<0.05 by z-test. Error bars indicate ±S.E.M.

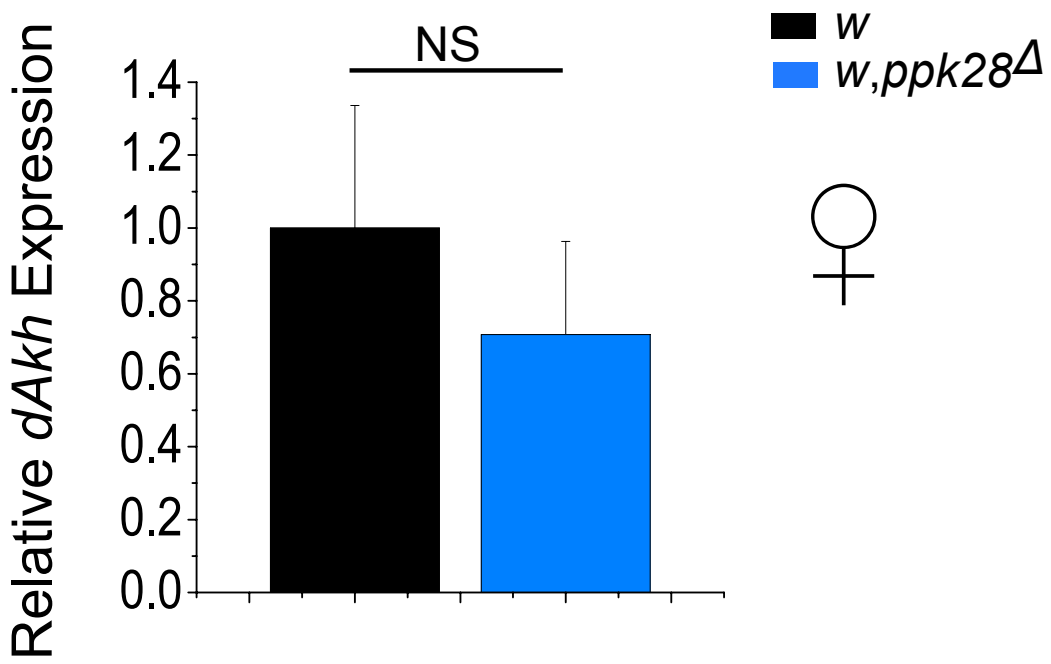


Figure 3.14. *dAkh* Levels are Not Significantly Altered in *ppk28* Mutants. Relative mRNA levels of *dAkh* from quantitative PCR analysis of *ppk28* deletion mutant female flies (*w*,*ppk28*$^{\Delta}$) and their appropriate background controls (*w*). Values were normalized to *Rpl-32*, used as an endogenous control (n=3 biological replicates). Error bars indicate ±S.E.M.

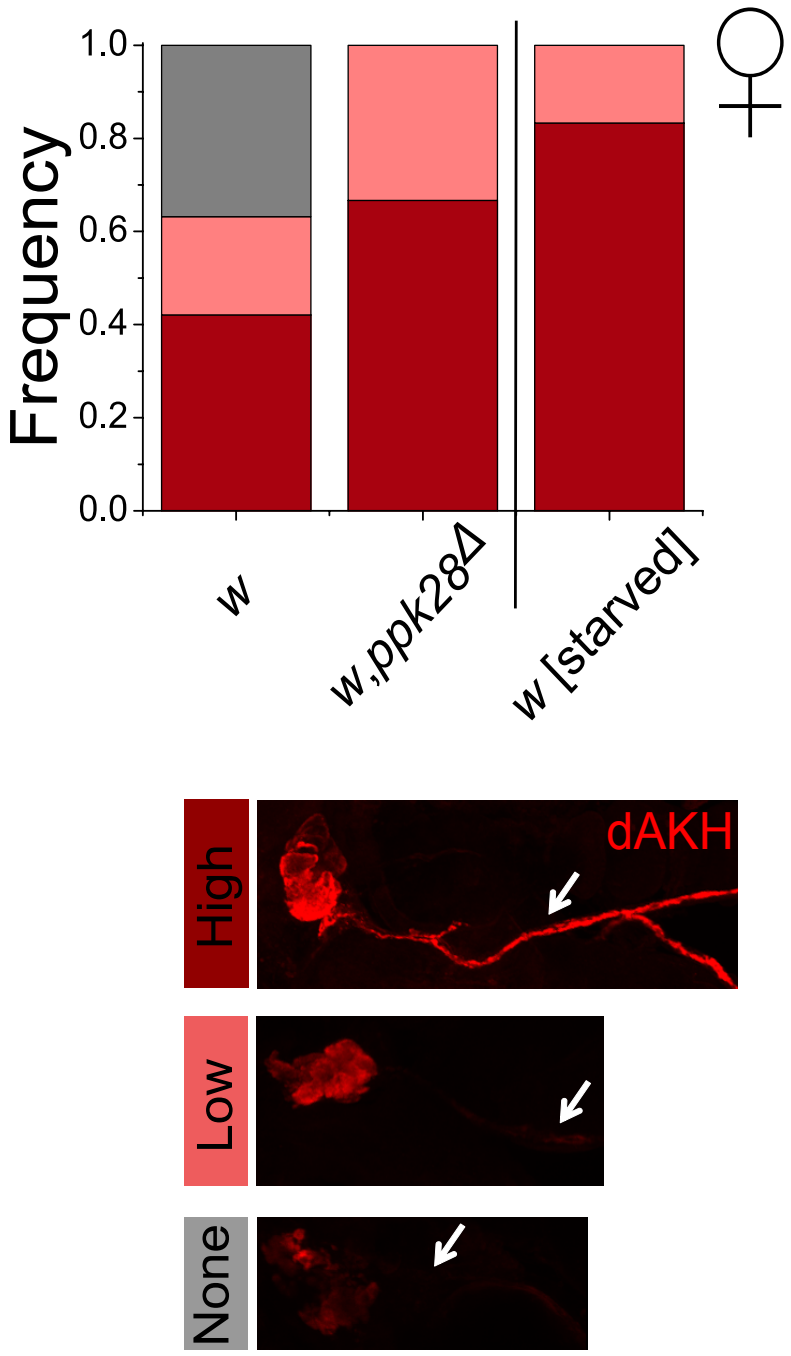


Figure 3.15. Loss of *ppk28* Increases dAKH Staining in *Corpora Cardiaca* Cells. Quantification of AKH levels in axonal projections of adult *corpora cardiaca* cells. Preparations from fully-fed female *ppk28* deletion mutant flies (*w*,*ppk28*$^{\Delta}$) as well as fed and starved control animals (*w*) were stained with α–dAKH and categorized as having high, low, or no observable AKH. A representative image from each category is shown for reference (n=19 (*w*); n=15 (*w*,*ppk28*$^{\Delta}$); n=6 (*w*,starved)).

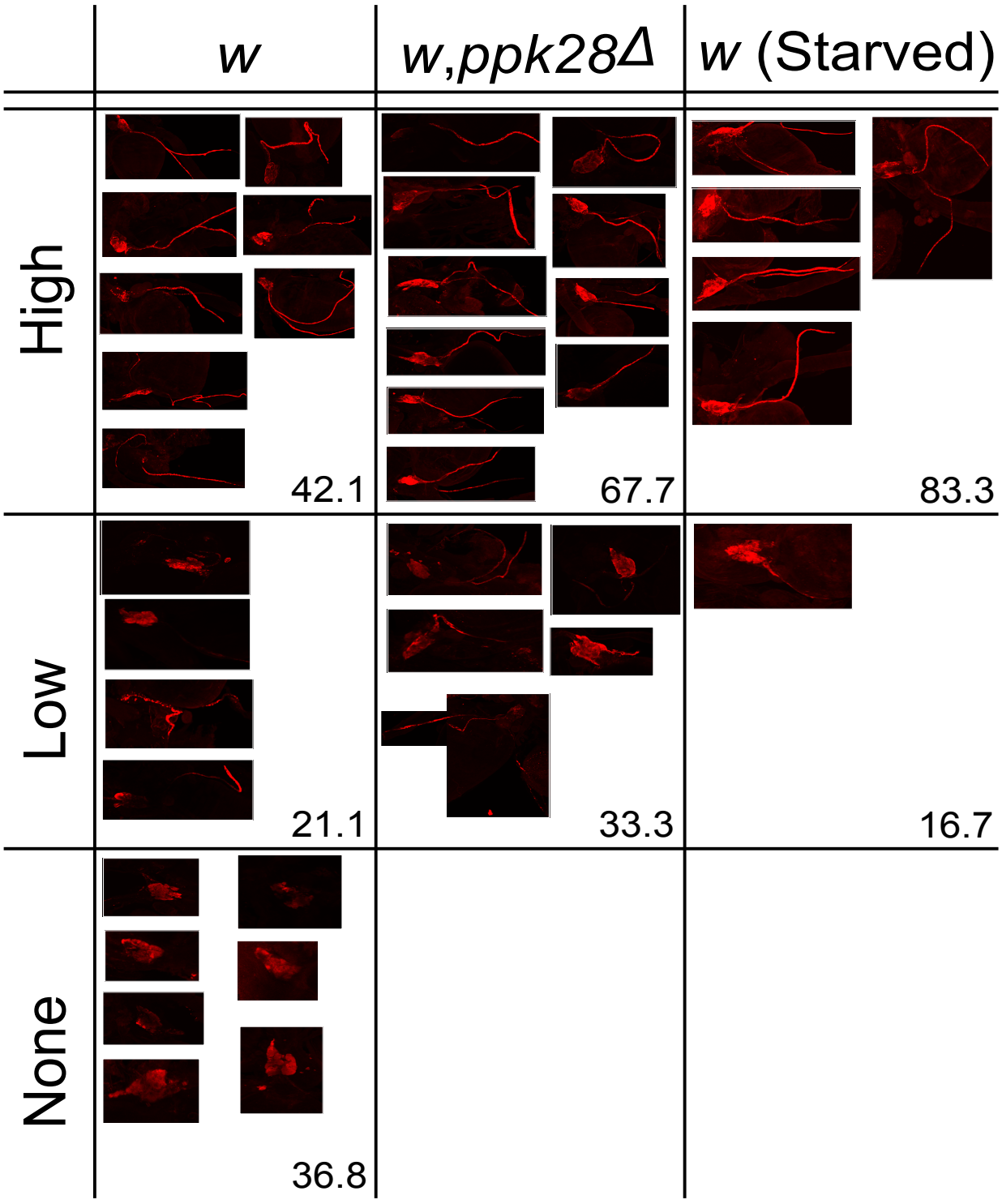

w
w,ppk28Δ
w (Starved)
High
Low
None
42.1
67.7
83.3
21.1
33.3
16.7
36.8

Figure 3.16. dAKH Staining of Fed Control and *ppk28* Mutant and Starved Control *Corpora Cardiaca* Cells. Images of adult *corpora cardiaca* cells stained with anti-dAKH antibody and grouped by intensity of staining in their axonal projections (n=19 (*w*); n=15 (*w*,*ppk28*$^{\Delta}$); n=6 (*w*,starved)). Numbers represent percentage of total genotype within each group.

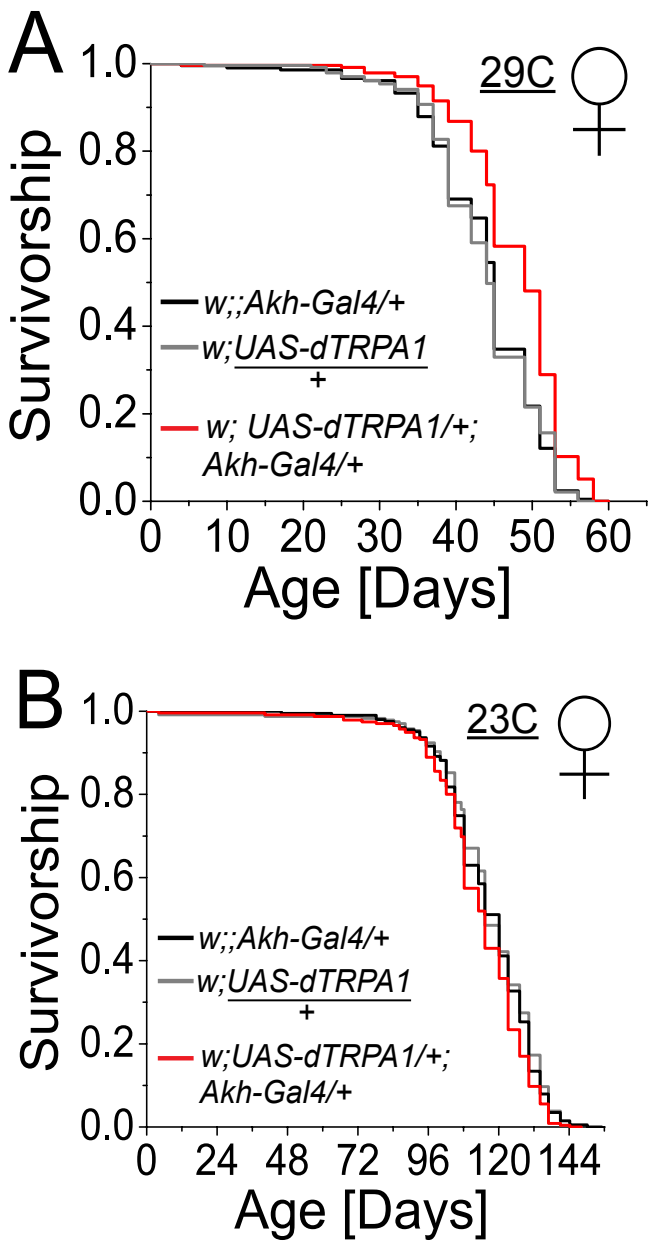


Figure 3.17. Activation of AKH-ergic Cells is Sufficient to Extend Lifespan. Survival curves for female flies with activated AKH-ergic neurons at both an activating (29°C) (A) and control non-activating (23°C) (B) temperature (neuronal activation – *w*; *UAS-dTRPA1*/+; *Akh-Gal4*/+ ; *Gal4* construct control – *w*;; *Akh-Gal4*/+ ; *UAS* construct control – *w*; *UAS-dTRPA1*/+) on SY5% food (23°C: n=202 (*w*;; *Akh-Gal4*/+); n=235 (*w*; *UAS-dTRPA1*/+); n=237 (*w*; *UAS-dTRPA1*/+; *Akh-Gal4*/+) – mean lifespan decrease of 3 days [2.50%] and 3.19 days [3.65%] as compared to *Gal4* and *UAS* construct controls, respectively; 29°C: n=207 (*w*;; *Akh-Gal4*/+); n=237 (*w*; *UAS-dTRPA1*/+); n=235 (*w*; *UAS-dTRPA1*/+; *Akh-Gal4*/+) – mean lifespan extension of 3.80 days [8.21%] and 3.74 days [8.07%] as compared to *Gal4* and *UAS* construct controls, respectively). Pairwise comparisons between statistically significantly different genotypes yielded p<1x10-6 by Log Rank analysis.

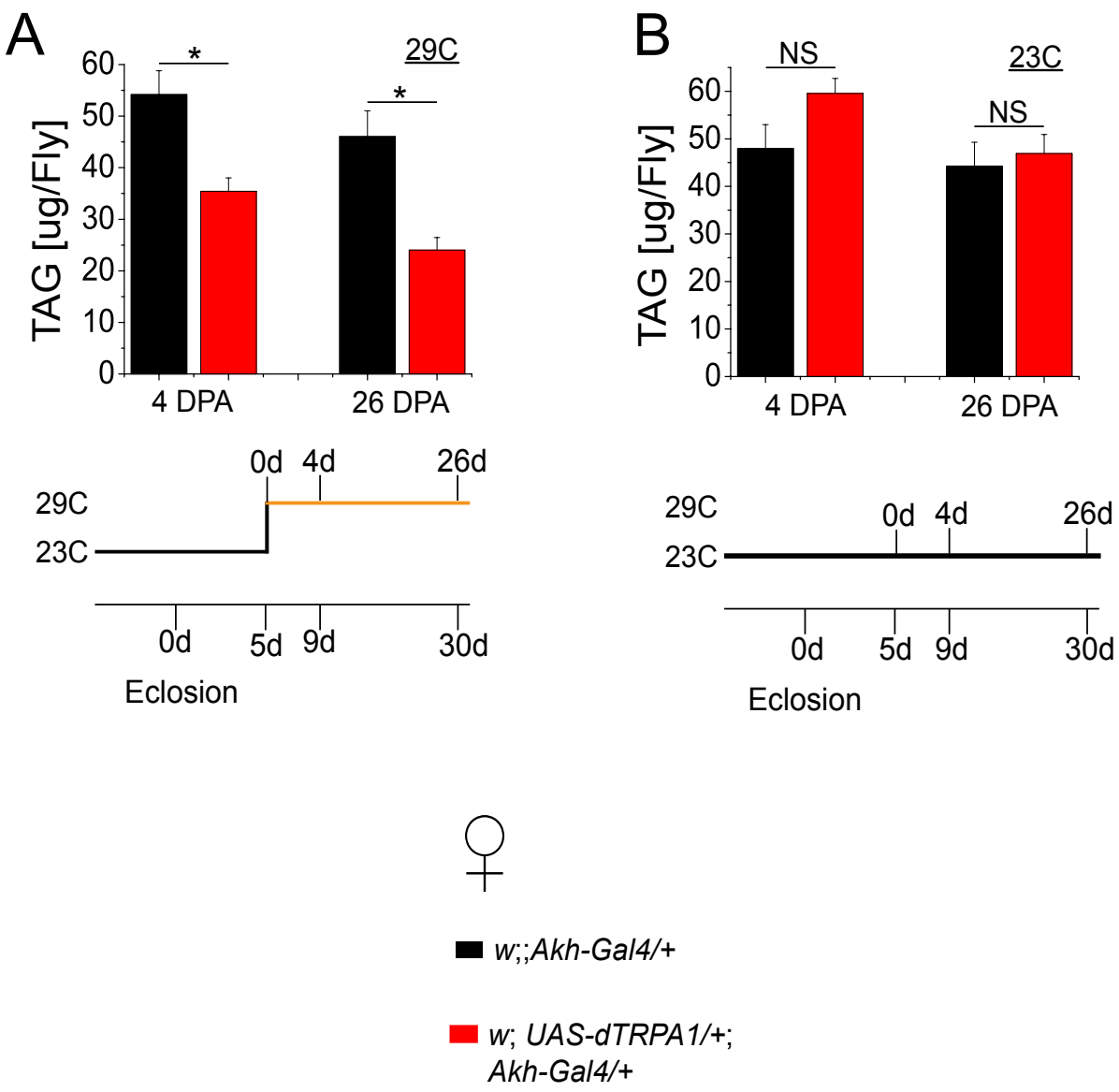


Figure 3.18. Long-term Activation of AKH-ergic Cells via dTRPA1 Results in Long-term Metabolic Response. Longitudinal measures of whole fly TAG levels of female flies with dTRPA1 expressed in AKH-ergic neurons at both an activating (29°C) (A) and control non-activating (23°C) (B) temperature (neuronal activation – *w*; *UAS-dTRPA1*/+; *Akh-Gal4*/+ ; *Gal4* construct control – *w*;; *Akh-Gal4*/+). Flies were reared at 23ºC until 5 days post-eclosion, at which point they were shifted to 29ºC. TAG measurements were made 4 and 26 days after temperature shift, which corresponded to 9 and 30 days of age – see schematics). *=p<0.05 by two-sided Student's T-Test. n=10 groups per genotype per timepoint. Error bars indicate ±S.E.M.

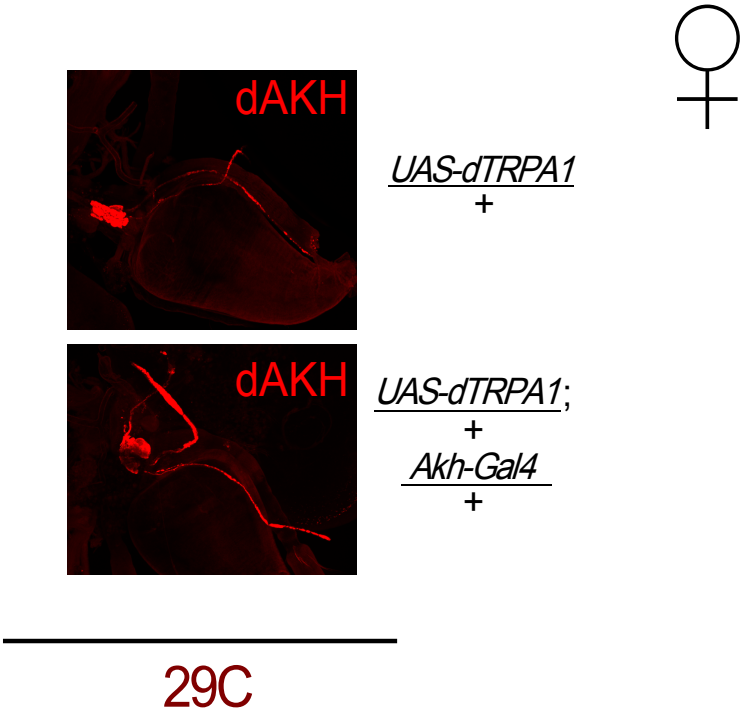


Figure 3.19. Long-term Activation of AKH-ergic Cells via dTRPA1 Results in Increased *Corpora Cardiaca* Cell dAKH Axon Staining. Representative images of adult *corpora cardiaca* cells stained with anti-dAKH antibody of approximately two-week old adult female flies with TRPA1 expressed in AKH-ergic neurons at an activating (29^{o}C) temperature (neuronal activation – *w*; *UAS-dTRPA1*/+; *Akh-Gal4*/+ ; *UAS* construct control – *w*; *UAS-dTRPA1*/+;). N=3-5 per genotype.

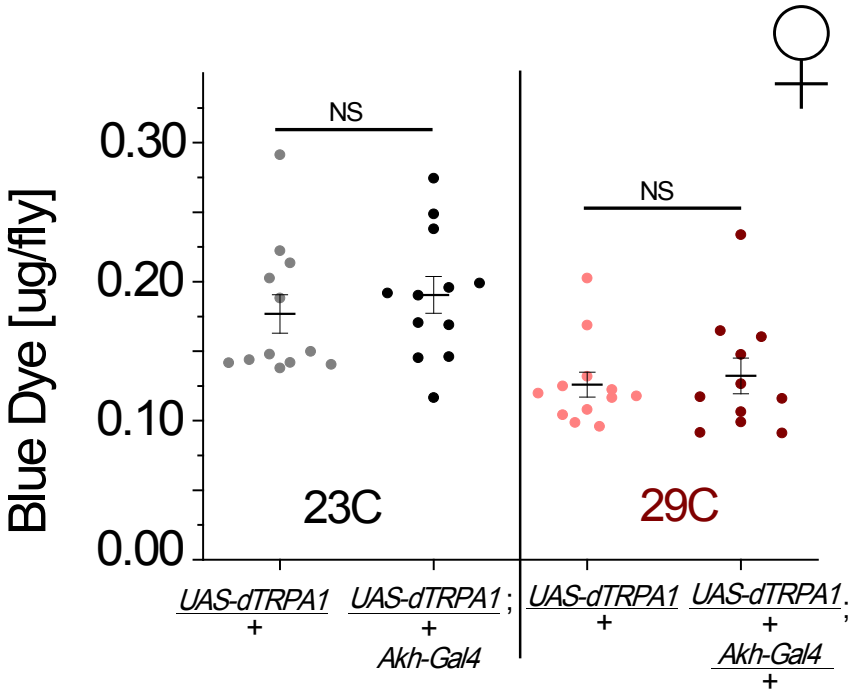


Figure 3.20. Long-term Activation of AKH-ergic Cells via dTRPA1 Does Not Alter Feeding Behavior. Total amount of blue dye consumed in a six hour period by approximately three-week old female flies with TRPA1 expressed in AKH-ergic neurons at both an activating (29^{o}C) and control non-activating (23^{o}C) temperature (neuronal activation – *w*; *UAS-dTRPA1*/+; *Akh-Gal4*/+ ; *UAS* construct control – *w*; *UAS-dTRPA1*/+;). N=11-12 groups of 5 flies for each genotype. Error bars indicate ±S.E.M.

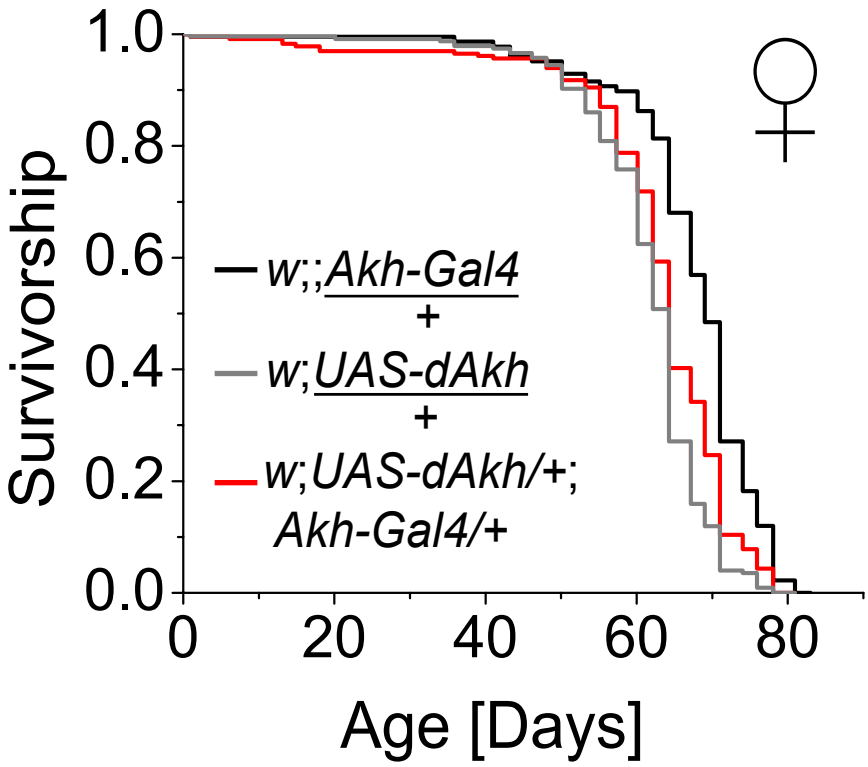


Figure 3.21. Overexpression of *dAkh* in AKH-ergic Cells is Not Sufficient to Extend Lifespan. Survival curves for female flies with overexpression of *dAkh* in AKH-ergic neurons (neuronal overexpression – *w*; *UAS-dAkh*/+ ; *Akh-Gal4*/+; *Gal4* construct control – *w*;; *Akh-Gal4*/+ ; *UAS* construct control – *w*; *UAS-dAkh*/+) (n=225 (*w*;; *Akh-Gal4*/+); n=232 (*w*; *UAS-dAkh*/+); n=231 (*w*; *UAS-dAkh*/+ ; *Akh-Gal4*/+) – mean lifespan increase of 1.44 days [2.25%] as compared to *UAS* construct control decrease of 4.60 days [6.56%] as compared to *Gal4* construct control). Pairwise comparisons between genotypes yielded p<1x10^-6 for *UAS* control and p=3.13x10^-9 for *Gal4* control by Log Rank analysis.

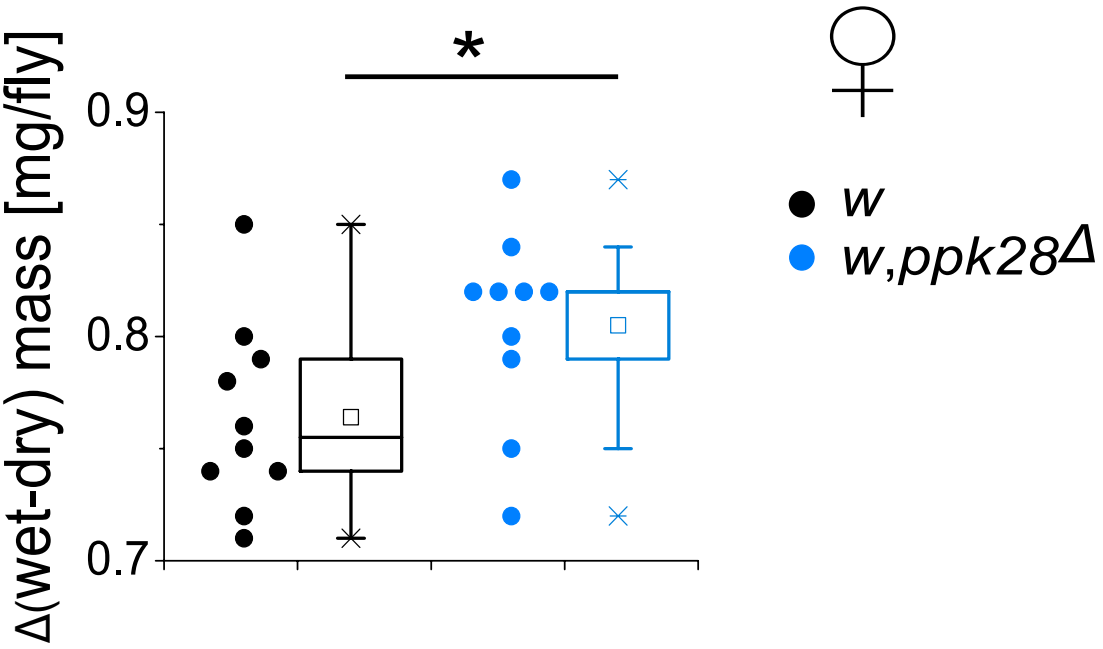


Figure 3.22. Loss of *ppk28* Function Increases Difference Between Wet and Dry Mass. Difference in wet and dry mass for *ppk28* deletion mutant background control (*w*) and *ppk28* deletion mutant (*w*,*ppk28*$^{\Delta}$) female flies (n=10 groups of 10 flies per genotype). *=p<0.05 two-sided Student's T-Test.

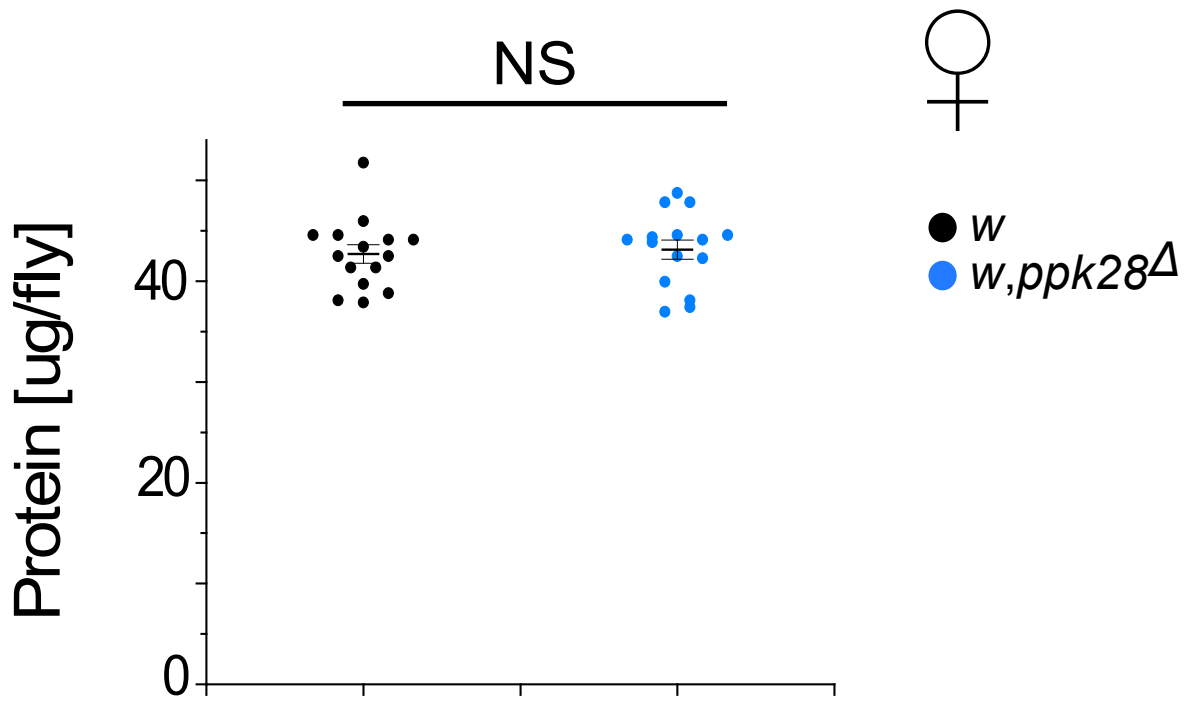


Figure 3.23. Loss of ppk28 Function Does Not Alter Whole-organism Protein Levels. Protein Levels of *ppk28* deletion mutant female flies (*w*,*ppk28*$^{\Delta}$) and background controls (*w*) at approximately two weeks post-eclosion. N=15 groups of 5 flies per genotype. Error bars indicate ±S.E.M.

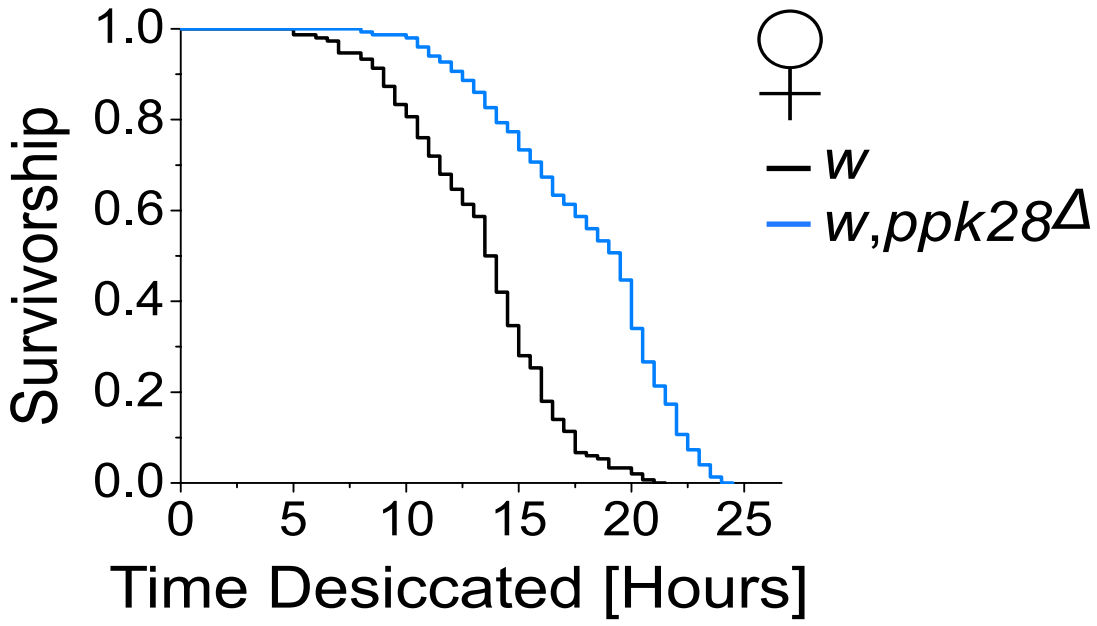


Figure 3.24. *ppk28* Mutants are Dessication Resistant. Survival curves for *ppk28* deletion mutant background control (*w*) and *ppk28* deletion mutant (*w*,*ppk28*$^{\Delta}$) female flies under desiccating conditions (n=100 flies for both genotypes). p<1x10^-6 by Log Rank analysis.

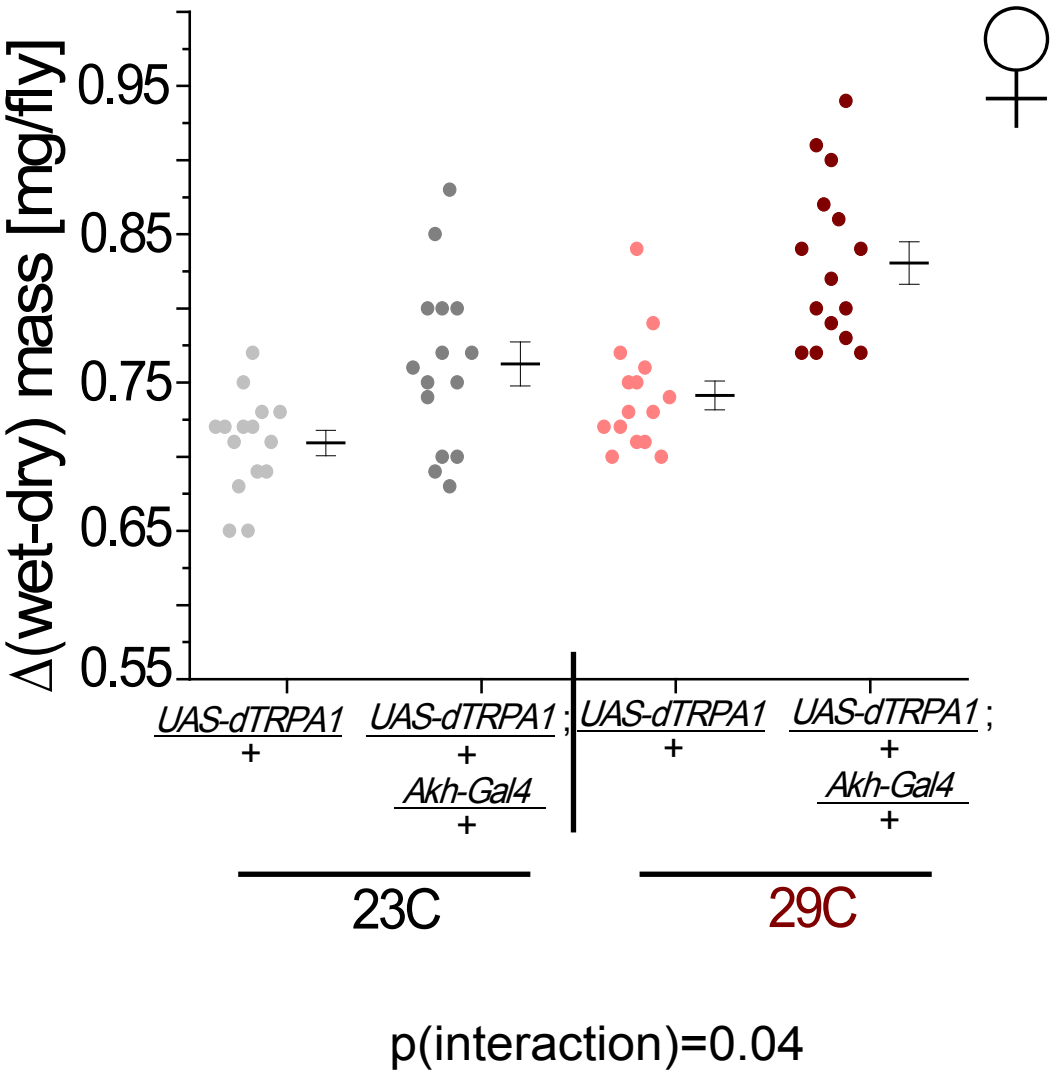


Figure 3.25. Long-term Activation of AKH-ergic Cells via dTRPA1 Results in Increased Difference in Wet and Dry Mass. Change in wet and dry mass of approximately two-week old female flies with TRPA1 expressed in AKH-ergic neurons at both an activating (29^{o}C) (p=3.1x10^-8) and control non-activating (23^{o}C) temperature (p=0.002) (neuronal activation – *w*; *UAS-dTRPA1*/+; *Akh-Gal4*/+ ; *UAS* construct control – *w*; *UAS-dTRPA1*/+;). p-values were determined by randomization using data from two replicate experiments. N=15 groups of 10 per genotype, pooled between two experiments. Error bars indicate ±S.E.M.

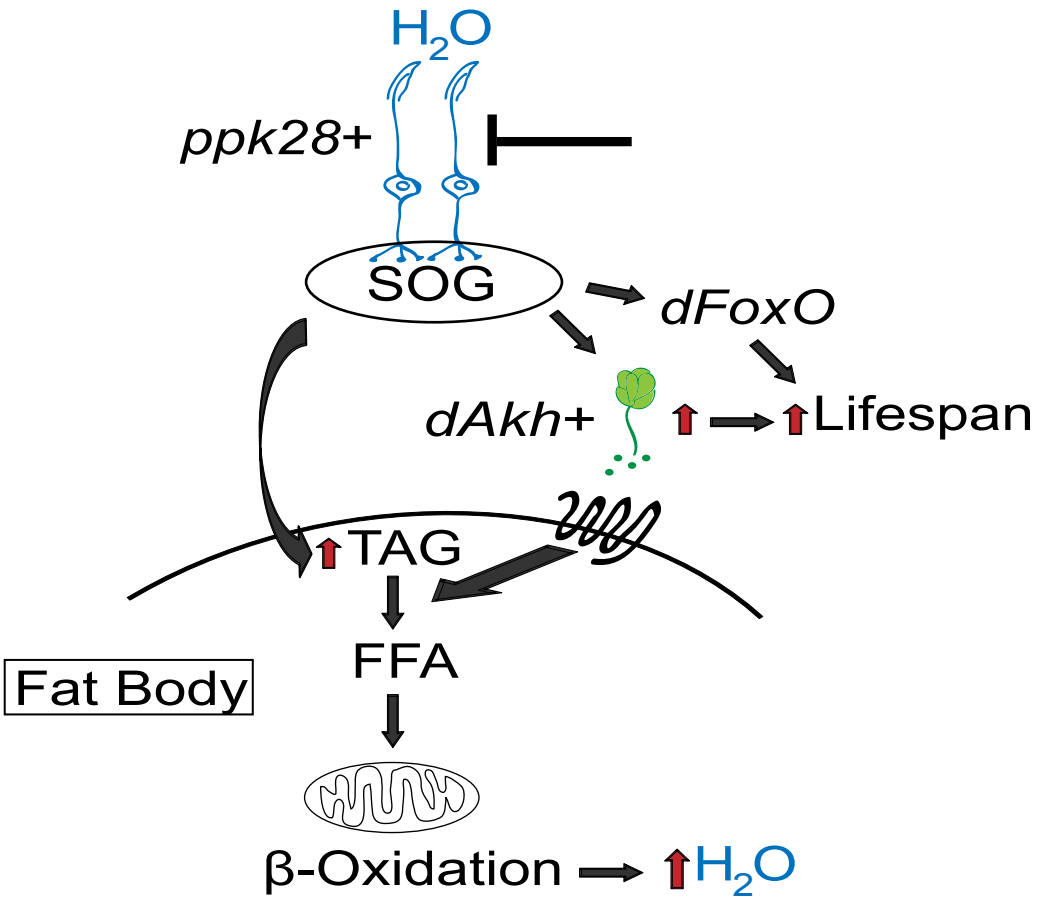


Figure 3.26. Regulation of Physiology and Lifespan Through Loss of *ppk28* Function. Model for control of metabolic homeostasis and lifespan by *ppk28*-mediated gustatory inputs.

Chapter 4: Dissection of a Neural Circuit Modulating *Drosophila* Longevity and Physiology Mediated by the Sweet Taste Receptor *Gr5a*

Chapter 4.A. Introduction

In addition to uncovering multiple long-lived gustatory mutants, our initial screen also found mutations in two gustatory genes, both sweet taste receptors, which induced shorter lifespans. One – loss of *Gr5a* – was able to be rescued [Sections 2.C. and 2.D.]. *Gr5a* is expressed in each sugar-sensitive GRN in the labellum of the fly (Marella et al., 2006) as well as in four to six GRNs in the leg tarsi (Chyb et al., 2003). *Gr5a* has been found to be specifically tuned toward the disaccharide trehalose (Chyb et al., 2003). Trehalose is a common component of the fly's natural food source – ripened or rotting fruit – as well as a metabolically important circulating molecule utilized as an energy substrate, notably for the energy-expensive process of flying (Becker et al., 1996). In addition, function of *Gr5a* is required for the activation of sugar-sensitive GRNs by other carbohydrate stimuli, including glucose (Dahanukar et al., 2007).

We utilized *Gr5a* mutants, then, with a number of motivations. First, and most obviously, we sought to determine the means by which this positive regulator of lifespan exerts its effects. Furthermore, however, we wanted to begin to understand whether gustatory circuits, though mediated by discrete initial inputs, used similar downstream mechanisms as means of control, and, if so, where these points of convergence lie.

Only in characterizing multiple regulatory gustatory networks, then, would we be able to address this question.

Our rationale and experimentation for dissecting *Gr5a*-mediated control of longevity was similar to that used for *ppk28*. Like water, carbohydrates are critical nutrients in an organism's diet and metabolism. As such, we first measured changes in *Gr5a* mutant physiology to determine the response undergone due to loss of function of this critical gustatory gene. We then examined the requirement of signaling mechanisms in this control, seeking to piece together a model by which loss of sensory input enacted change in physiological state, and, ultimately, lifespan.

Chapter 4.B. Materials and Methods

Drosophila Stocks. For background controls, we used a w^{1118} (*w*; +; +) (VDRC) line. *Gr5a* mutants ($Gr5a^{\Delta}$; +; +) were a gift from Anupama Dahanukar. Mutants were backcrossed eight generations to the control line.

Lifespan Analyses. Lifespan analyses were performed using an empirically optimized protocol established by our lab and facilitated by the use of an RFID-based tracking system and associated statistical software (dLife) developed by our lab (Linford et al., 2013). Flies were fed *ad libitum* throughout adult life. Flies were reared and housed at 25ºC in a 12:12 light-dark cycle.

Food Recipes. High trehalose lifespan experiments were performed on food containing 10% trehalose and 10% yeast (w/v) as opposed to 10% sucrose and 10% yeast (w/v).

Whole-organism Nutrient Level Assays. For all nutrient measurements, female flies were frozen at -80ºC prior to homogenization.

For TAG measurement, 5uL of homogenate from five flies in 200uL PBST spun for 1 minute at 5,000 rpm to settle debris was placed in 150uL warmed Infinity Triglycerides reagent (Thermo Scientific), mixed briefly after five minute incubation at room temperature, and read at 520nm compared to glycerol standards.

For protein measurement, 2uL of homogenate from five flies in 200uL PBST spun for 1 minute at 5,000 rpm to settle debris was diluted 1:10 and placed in 200uL BCA Protein Assay Solution (Novagen), mixed briefly after thirty minute incubation at 37ºC, and read at 562nm compared to bovine serum albumin standards.

For glycogen and glucose measurement, two aliquots of 20uL of homogenate from 2 flies in 100uL PBST spun for 1 minute at 5,000 rpm to settle debris was incubated with either 1 uL of amylogucosidase [0.5U/uL] or PBST for 1 hour at 37ºC. After a 15 minute incubation at 37ºC, 10uL from each sample was added to 150uL warmed Infinity Glucose Oxidase Reagent (Thermo Scientific), mixed briefly, and read at 340nm compared with glucose standards. Glucose levels were inferred from absorbance values of sample incubated with PBST whereas glycogen levels were inferred from absorbance values of sample incubated with amyloglucosidase after subtracting absorbance values of samples incubated with PBST.

For fructose measurement, 50 uL of homogenate from five flies in 100uL PBST spun for 1 minute at 5,000 rpm to settle debris was placed in a new centrifuge tube and spun for 10 minutes at 14,000rpm. 100uL of 6N HCl + 0.05% resorcinol (Sigma) was added and samples were again spun for 10 minutes at 14,000rpm. Samples were heated at 95ºC for 10 minutes and then 100uL read at 484nm and compared to fructose standards.

For trehalose measurement, 2 flies were homogenized in 50uL of 0.25M Na_2CO_3, spun briefly to settle debris and a 10uL aliquot taken out and heated at 95ºC for two hours. 24uL of 0.2M NaOAc and 6uL of 1N Acetic Acid were added to the sample. The sample was split into two 20uL aliquots with 1uL of trehalase added to one and 1uL of PBST to the other. After a 15 minute incubation at 37ºC, 10uL from each sample was added to 150uL warmed Infinity Glucose Oxidase Reagent (Thermo Scientific), mixed briefly, and read at 340nm compared with glucose standards. Trehalose levels were inferred from absorbance values of sample incubated with PBST subtracted from samples incubated with trehalase.

Adult Hemolymph Extraction. Adult female flies were reared as described until approximately two weeks of age. Groups of 15-20 CO_2-anesthetized flies were poked in the thorax with a large needle and placed in a .6mL microcentrifuge tube containing a small punctured hole in the bottom. The .6mL tube was placed inside of a 1.5mL microcentrifuge tube and spun at 5,000rpm for 5 minutes at 4ºC. Sample volume was measured by collecting hemolymph with a graduated capillary tube and pipetting into a

new microcentrifuge tube. Samples were diluted 1:10 in PBST and frozen at -80ºC until use.

Hemolymph Carbohydrate Measurement. Adult female flies were reared as described until approximately two weeks of age. Identical procedures to measuring whole-organism carbohydrate levels were utilized with the exception of the starting sample. For glucose and glycogen, sample was 5uL of 1:10 diluted hemolymph placed into 35uL of PBST for a total 40uL sample. For trehalose, sample was 1uL of 1:10 diluted hemolymph placed into 9uL of 0.25M Na_2CO_3 for a total 10uL sample.

Feeding Assay. Flies were reared as described on SY10% food, then transferred to food of identical nutrient composition spiked with 0.05% (w/v) blue dye (FD&C Blue #1; Spectrum Chemical), 5 flies per sample. Flies were allowed to consume dyed food for 6 hours and then immediately frozen at -80ºC. Frozen flies were homogenized in 200uL PBST, centrifuged at 1,000 rpm for 1 minute to settle debris, and then 100uL of the supernatant was read directly at 630nm (reference wavelength of 670nm) and compared to blue dye standards.

Homogenate Spike-in Assay. Whole flies were frozen at -80ºC and then homogenized in sets of 5 flies in 100uL PBST. Samples were spun briefly to settle debris and then 10uL of each sample was added to a well containing either 1mg of trehalose (Swanson Health Products) in 100uL PBST (final concentration = 10mgs/mL) or 100uL PBST only. Samples were mixed briefly and incubated for 1 hour at 37ºC. 10uL from each well was

rapidly added to 150uL of warmed Infinity Glucose Oxidase Reagent (Thermo Scientific) and incubated at 37ºC for 10 minutes. Samples were read at 500nm and amount of trehalose broken down inferred by subtracting wells with PBST only from wells with trehalose initially present.

RNA Extraction and Quantitative PCR. Whole flies were frozen at -80ºC, and total RNA was extracted from these samples using TRIzol reagent (Invitrogen) according to manufacturer's protocol. For qPCR performed on high trehalose diet-consuming flies, flies were placed on high trehalose food for one week prior to freezing. Extracted RNA from each genotype was diluted to equal concentration in RNAse-free water. RT-PCR was performed using SuperScript III First Strand cDNA Synthesis (Invitrogen) to generate cDNA, which was then quantified by real-time PCR analysis using Power SYBR Green PCR Master Mix and a StepOne Plus Real-time PCR system (Applied Biosystems). Primers used were as follows: dILP2_F:ATGGTGTGCGAGGAGTATAATCC, dILP2_R: TCGGCACCGGGCATG, tps1_F: CATGGTGGCTCTGAAGGGTA, tps1_R: TATCTGGGCTCACACCTCCA, tpp_F: CTGATCGAGACACCCTACGC, tpp_R: TCTTGGCACCCTCACCTTTG, Tret1-1_F:GAACGCGCCAAACGTATTCC, Tret1-1_R: CCAACCCAAGAACCAGCATC.

Immunohistochemistry. Adult female flies were reared as described then dissected at approximately one week of age and stained with a dAKH-specific antibody (gift from J. Park). Adult female flies were dissected in PBS solution and tissue fixed in 4% formaldehyde for at least 2 hours. Samples were then washed five times and then

incubated in PBS+0.05% Triton-X (PBST) for at least 2 hours. PBST was removed and followed by incubation in primary antibody (rabbit-anti dAkh 1:600 in PBST) for at least 8 hours. Tissue was again washed five times and then incubated in PBST for at least two hours. PBST was removed followed by incubation in secondary antibody (AlexaFluor (Invitrogen) 594-anti rabbit 1:500 in PBST) for at least three hours. Samples were washed five times and incubated in PBST for at least two hours. PBST was once more removed with samples then placed in Vectashield mounting media with DAPI (Vector Labs) for at least one hour. Samples were then mounted and visualized at 20x magnification on Olympus FluoView 500 Laser Scanning Confocal Microscope.

Trehalose Tolerance Test. Female flies between one and two weeks old were starved for 12 hours on 1% agar and then placed in a vial with filter paper soaked with a 15% trehalose solution labeled with 0.5% (w/v) blue dye (FD&C Blue #1; Spectrum Chemical). Flies were allowed to feed for 20 minutes and then scored for flies which had ingested trehalose via internal presence. Hemolymph was collected via previously described protocol at indicated timepoints post-feeding from 2-3 groups of flies per timepoint. Flies were placed into 1% agar vials immediately post-feeding until hemolymph collection. Hemolymph from each group of flies was diluted 1:10 in Na_2CO_3 and frozen at -80^{o}C until use.

Statistical Considerations. All survivorship data was compared via Log Rank analysis between relevant genotypes. Two-sided Student's T-tests were performed for qPCR results and comparison of mutant and control carbohydrate levels.

Chapter 4.C. *Gr5a* Loss of Function Specifically Increases Internal Trehalose Levels of the *Gr5a* Ligand Trehalose Via Increased Trehalose Synthesis

To begin to dissect the mechanism by which loss of *Gr5a* function induced significant shortevity, we again initially focused on potential consequential physiological modifications by measuring whole-organism levels of major nutrients. Unlike *ppk28* mutants, *Gr5a* mutants showed no significant difference in whole organism TAG levels as compared to their background controls, nor did they show a significant difference in total protein levels [Fig. 4.1.]. As the *Gr5a* receptor is required for the recognition of attractive tastants, we assayed an expanded panel of metabolically important carbohydrates. Of these, only trehalose (and not glucose, glycogen, or fructose) was altered in *Gr5a* mutants, specifically through a significant increase [Fig. 4.2.]. To establish whether this phenotype was maintained throughout the lifetime of *Gr5a* mutants, we measured trehalose levels longitudinally and found that whole-organism levels of trehalose remained high in both middle and older ages [Fig. 4.3.]. This result was intriguing for a number of reasons. First, as previously stated, trehalose is specifically recognized as a ligand by *Gr5a* (Chyb et al., 2003). This was reminiscent of *ppk28* mutants, in which a physiological shift occurred in these flies to produce internally the molecule which was not able to be sensed externally. Second, trehalose is often made analogous to mammalian blood glucose as it is an important circulating carbohydrate molecule utilized as an energy source (Reyes-DelaTorre et al., 2012). Though trehalose concentration in the hemolymph of flies is not as tightly regulated as glucose in the blood of mammals (Reyes-DelaTorre et al., 2012), it remained a formal

possibility that chronic high levels of hemolymph trehalose contributed to the shortened lifespan of *Gr5a* mutants. To test if levels of trehalose specifically in the hemolymph were increased in *Gr5a* mutants, we extracted hemolymph from two-week old adult flies and, indeed, found this to be the case. Furthermore, we found that hemolymph titers of other carbohydrates, as was the case for whole-organism levels, remained the same [Fig. 4.4.].

We reasoned that high trehalose levels could conceivably be due to increased *de novo* trehalose synthesis or to downregulation of trehalase activity. To test whether there was evidence of reduced trehalase activity in *Gr5a* mutants, we added homogenate of two-week old adult *Gr5a* mutant and control flies to a known amount of trehalose and measured the amount broken down after one hour. Using this assay, we found there to be no significant difference in trehalase activity between these genotypes [Fig. 4.5.], suggesting that this was not the cause of increased trehalose levels in *Gr5a* mutants. The enzymes required to catalyze the reactions of glucose-6-phosphate to trehalose in *de novo* trehalose synthesis in the fat body of *Drosophila* are trehalose phosphate synthase 1 [*tps1*] and trehalose phosphate phosphatase [*tpp*]. Further, newly synthesized trehalose is transported from the fat body to the hemolymph through the trehalose transporter *Tret1-1* (Reyes-DelaTorre et al., 2012). Consistent with a model by which trehalose levels are elevated due to loss of *Gr5a* function via increased *de novo* trehalose synthesis, transcript levels of all three genes are significantly upregulated in *Gr5a* mutants as compared to background controls [Fig. 4.6.]. Taken together, these results suggest that *Gr5a* mutants respond to loss of the ability to sense trehalose by increasing *de novo* trehalose synthesis.

Chapter 4.D. Hypertrehalosemia is Not Sufficient to Shorten Lifespan

Chronic high levels of circulating glucose (hyperglycemia) in humans is associated with a number of health defects, including diabetes and related kidney (Satirapoj, 2012) and heart (Rubin et al., 2012) damage. Insect hemolymph trehalose levels are not as tightly regulated as mammalian blood glucose levels, varying by as much as an order of magnitude (Reyes-DelaTorre et al., 2012), and, though hemolymph trehalose levels were significantly increased due to loss of *Gr5a* function, it was still well within this range. The possibility remained, however, that the short lifespan of *Gr5a* mutants was, at least in part, a product of longitudinally high levels of circulating trehalose. To assess whether there was a causal link between high hemolymph trehalose levels and short lifespan, we sought to induce hypertrehalosemia in both *Gr5a* mutants and controls by measuring lifespan on a high trehalose diet in which trehalose was substituted for the normal sucrose as a carbohydrate source [10% trehalose, 10% yeast]. Indeed, this diet led to increased levels of hemolymph trehalose in both genotypes as compared to those previously found on SY10% food [Fig. 4.4] and, in fact, abrogated the difference between the two lines [Fig. 4.7.]. Were hypertrehalosemia sufficient to induce shortened lifespan, we reasoned that, on this high trehalose diet, both control and *Gr5a* mutant flies should show a reduced lifespan and, furthermore, that the lifespan difference between the two genotypes should be reduced. We found, however, that there was virtually identical mean lifespans for control flies on standard [“SY”] and high trehalose food [“TY”] in concurrently running experiments [Fig. 4.8.], suggesting that simply increasing hemolymph trehalose levels is not sufficient to reduce lifespan and is not

necessarily the causal event inducing shortevity in *Gr5a* mutant flies. Interestingly, though feeding on a high trehalose diet heightened hemolymph trehalose levels in both genotypes, transcript levels of trehalose synthesis genes were still significantly increased in *Gr5a* mutants [Fig. 4.9.]. This suggested that loss of trehalose-sensing neural input drove increased trehalose synthesis independent of dietary trehalose (whereas diet-induced hypertrehalosemia may be due to, for instance, diffusion of undigested trehalose through the gut).

These data, then, argued for one of two models concerning the association between hypertrehalosemia and short lifespan in *Gr5a* mutants. On one hand, the increased hemolymph trehalose phenotype was potentially separable from the short lifespan of *Gr5a* mutants. Alternatively, it was possible that hypertrehalosemia in *Gr5a* mutant flies was initially much higher, enacting a regulated signaling response which promoted short lifespan, but which ensured that hemolymph trehalose levels, though increased, were still within an acceptable physiological range.

Chapter 4.E. *Gr5a* Mutants Show Evidence of Upregulated Insulin-like Peptide Signaling

To discern if loss of *Gr5a* function induced alterations in endocrine signaling, we focused on those responsible for the regulation of circulating carbohydrate homeostasis. In *Drosophila*, as is the case in mammals, this control is coordinated by the action of the fly's homologs of the insulin and glucagon-like signaling pathways (Becker et al., 1996; Belgacem and Martin, 2006). The glucagon-like molecule in flies is known as both

adipokinetic hormone (AKH) and hypertrehalosemic hormone (HTH), due to its ability to mobilize both lipid and carbohydrate stores (Lee and Park, 2004). Conversely, the fly insulin-like signaling pathway, mediated by eight insulin-like peptides (dILPs 1-8), have been suggested to decrease larval and adult trehalose levels, with dILP2 specifically implicated in this regulation (Belgacem and Martin, 2006; Rulifson et al., 2002), though the dynamics of this process are not perfectly fleshed out. Importantly, both have also been implicated in control of *Drosophila* longevity, with AKH signaling found to be a positive regulator of lifespan (Katewa et al., 2012; Waterson et al., 2014) and ILP signaling a negative regulator (Tatar et al., 2003).

To assess whether increased trehalose levels in *Gr5a* mutants may be due to upregulation of AKH/HTH signaling, we stained AKH-producing *corpora cardiaca* cells with an AKH-specific antibody to measure potential changes in AKH release in both *Gr5a* mutants and controls. Here, we found that axon staining in *Gr5a* mutants was not increased as compared to controls, and, if anything was decreased [Fig. 4.10.]. This finding is consistent with the proposed role of AKH in increasing rather than shortening lifespan. Finally, these may indicate that loss of *Gr5a* function leads to a severe response that drives rapid synthesis of trehalose rather than through normal means via endocrine signaling, highlighting once again the potency of signals emanating from sensory receptors.

As opposed to upregulation of glucagon-like AKH/HTH signaling, increased ILP signaling has been extensively shown to be a negative regulator of lifespan across

species, including *Drosophila*, as manipulations which reduce dILP expression or function increase longevity (Fontana et al., 2010). We speculated that *Gr5a* mutants may respond to increased synthesis and circulation of trehalose by upregulating ILP signaling, which, in turn, may have a detrimental effect on lifespan. To determine if ILP signaling was altered in *Gr5a* mutants, we measured transcript levels of *dILP2*, an insulin-like peptide, as mentioned previously, specifically connected with the control of trehalosemia. Consistent with a model by which ILP signaling was indeed heightened in *Gr5a* mutants, *dILP2* transcript levels approximately 10-fold as compared to background controls [Fig. 4.11.]. It is important to note, however, that, for a number of *Drosophila* peptides, it has been proposed that synthesis of transcript and actual translation and release of the peptide may be uncoupled.

Chapter 4.F. Perspective and Future Directions

The data from our experimentation concerning the regulation of physiology and longevity by *Gr5a*-mediated inputs thus far have been informative, but not complete. This leaves a number of models for the hypertrehalosemia and shortevity hallmarks of *Gr5a* mutants [Fig. 4.12.]. First, *Gr5a*-expressing neurons may, in parallel, activate trehalose synthesis machinery and dILP-releasing cells, uncoupling hemolymph trehalose levels and shortened lifespan. Second, loss of *Gr5a* function, in activating trehalose synthesis machinery, might induce very high hemolymph trehalose levels outside of a tolerable physiological range. This, in turn, might activate dILP2 signaling to reduce hemolymph trehalose and, subsequently, shorten lifespan. An explicit test to differentiate between these two models requires the inhibition of dILP2 release – via, for

instance, expression of the inhibiting potassium channel *Kir2.1* in dILP2-ergic cells – in *Gr5a* mutants. This, in turn, would be followed by the measurement of hemolymph trehalose in these flies as compared to *Gr5a* mutants with functional dILP2-ergic cells as well as flies with inhibited dILP2-ergic cells in a control background. A substantially higher concentration of hemolymph trehalose in flies with inhibited dILP2-ergic cells in a *Gr5a* mutant background as compared to the other two controls would present strong evidence for the latter model whereas no change in the hemolymph trehalose concentration of these flies would argue for the former. Notably, both of these models assume that circulating dILP2 levels – as are *dILP2* transcript levels – are increased in *Gr5a* mutants, which we have not yet formally tested. To analyze whether increased *dILP2* transcript levels lead to actual increased levels of circulating dILP2 peptide, we plan to utilize an ELISA-based assay recently developed by the lab of Dr. Seung Kim to measure dILP2 concentration in hemolymph extracted from both *Gr5a* mutant and control flies.

Finally, though not necessarily useful in testing these proposed models, we performed an experiment to test whether *Gr5a* mutants might show signs of insulin resistance. High levels of circulating carbohydrate coupled with hyperinsulinemia are hallmarks of Type 2 diabetics in which patients become insulin resistant and, in turn, overproduce insulin. With the major caveat that dILP2 regulation of trehalose uptake from the hemolymph is still poorly understood, we nonetheless performed a trehalose tolerance test – a modification of the standard glucose tolerance test used to measure insulin resistance in humans – to determine if *Gr5a* mutant flies showed evidence of

maintaining a diabetic-like state. We found that *Gr5a* mutant flies, after an overnight starvation and then re-feeding with a high trehalose load, took longer than controls to reduce hemolymph trehalose concentration, consistent with insulin resistance [Fig. 4.13.]. As trehalose was not directly injected into the hemolymph – a method which presented major technical difficulties – these results may also be representative simply of *Gr5a* mutants not sensing ingested trehalose and thus delaying a physiological response.

Though much work, therefore, remains, our analysis of a separate gustatory input has nonetheless provided important insight into the regulation of physiology and aging not only by *Gr5a*-mediated inputs by gustatory signals in general. We found additional evidence suggesting that loss of the ability to sense a metabolically important stimulus leads to increased internal levels of this molecule. Further, we determined that, though points of convergence between circuits may exist, discrete GRNs do not utilize identical mechanics to exert their lifespan effects. The translational implications of these findings – for instance, if modulating sweet taste perception has the potential to alter blood glucose levels or insulin signaling in humans – are intriguing and should prompt study of these mechanisms in mammalian systems.

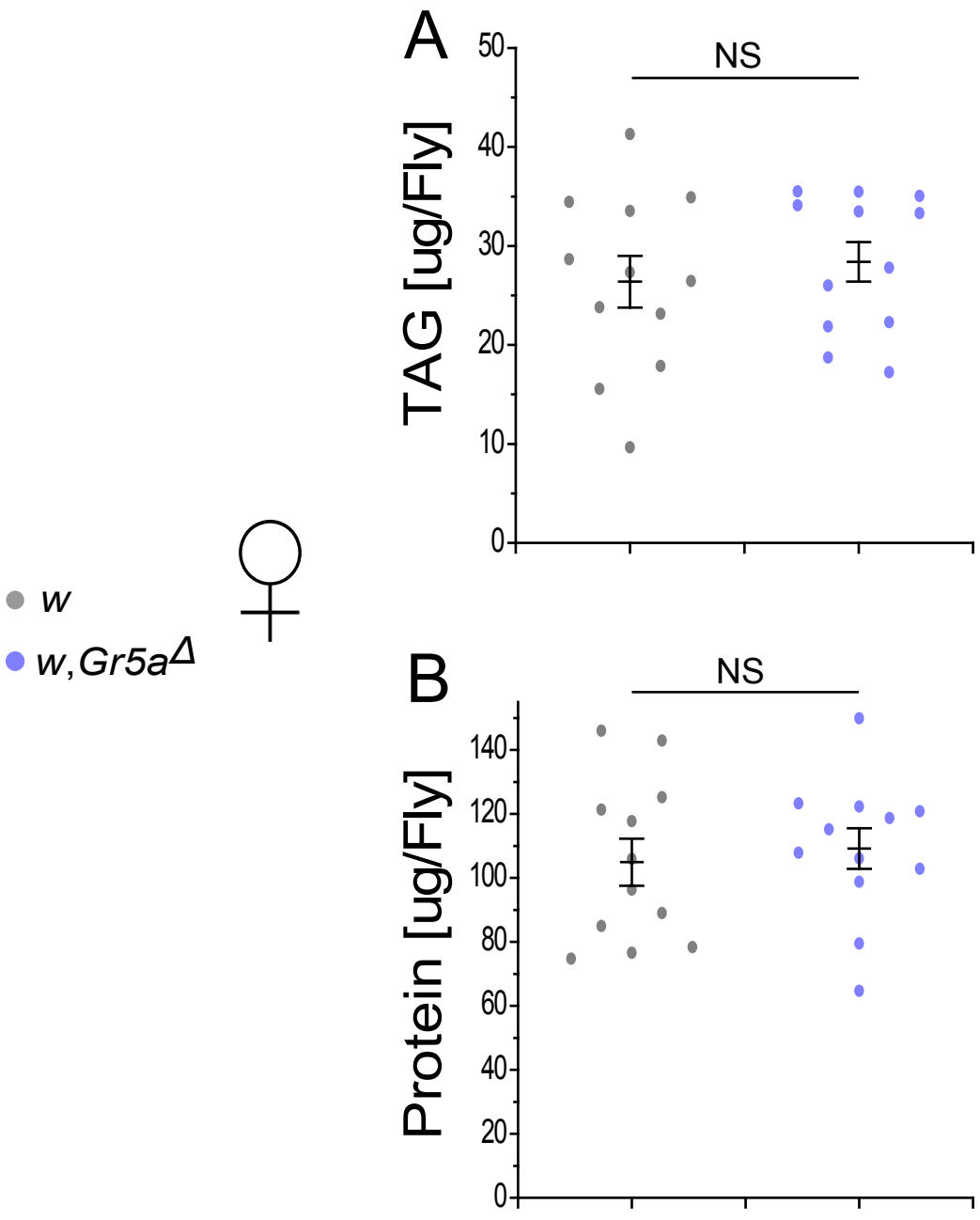


Figure 4.1. Loss of Gr5a Does Not Affect TAG or Protein Levels. Whole-organism measurements of TAG (A) and Protein (B) levels in *Gr5a* deletion mutant female flies (*w*,*Gr5a*$^{\Delta}$) and background controls (*w*). Error bars indicate ±S.E.M.

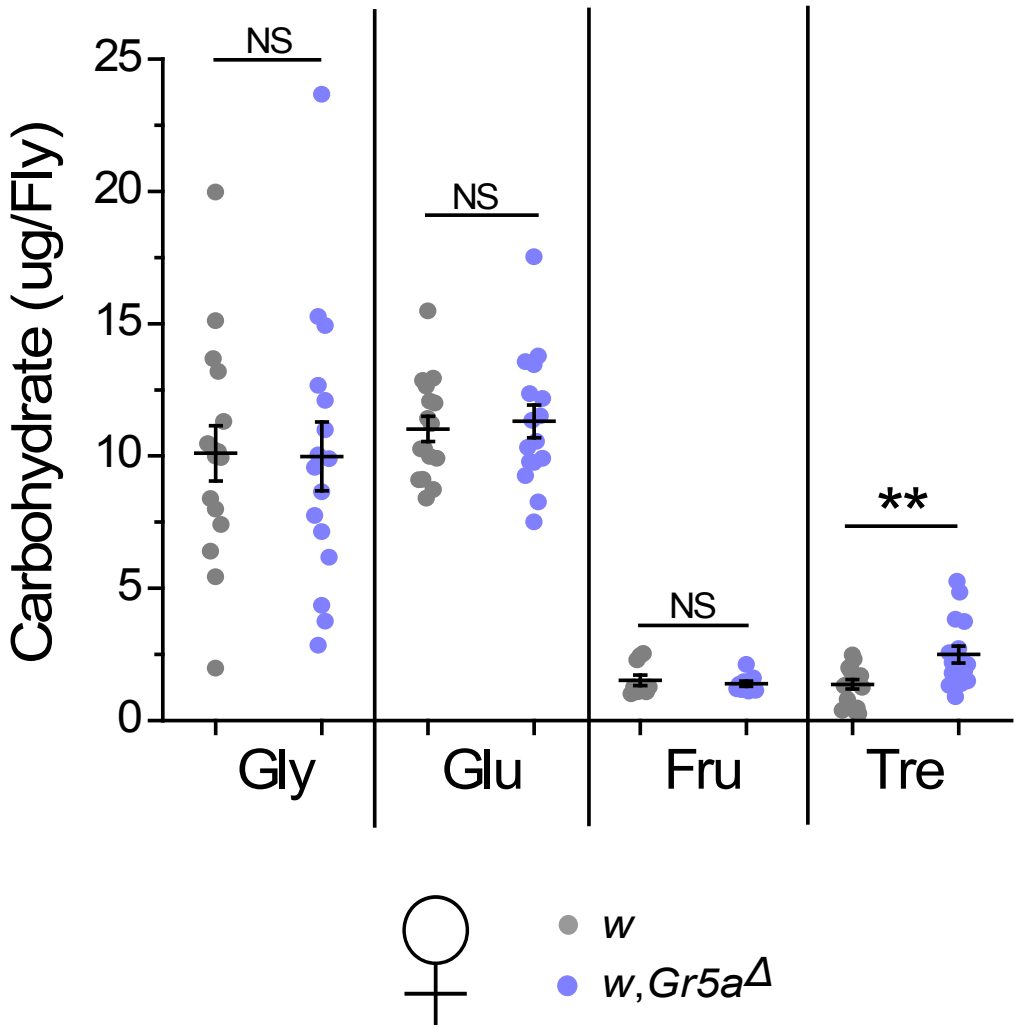


Figure 4.2. Loss of *Gr5a* Specifically Increases Whole-Organism Trehalose. Whole-organism measurements of carbohydrate levels in *Gr5a* deletion mutant female flies (*w*,*Gr5a*$^{\Delta}$) and background controls (*w*). **=p<0.01 by two-sided Student's T-Test. Error bars indicate ±S.E.M. Gly = glycogen; Glu = glucose; Fru = fructose; Tre = trehalose.

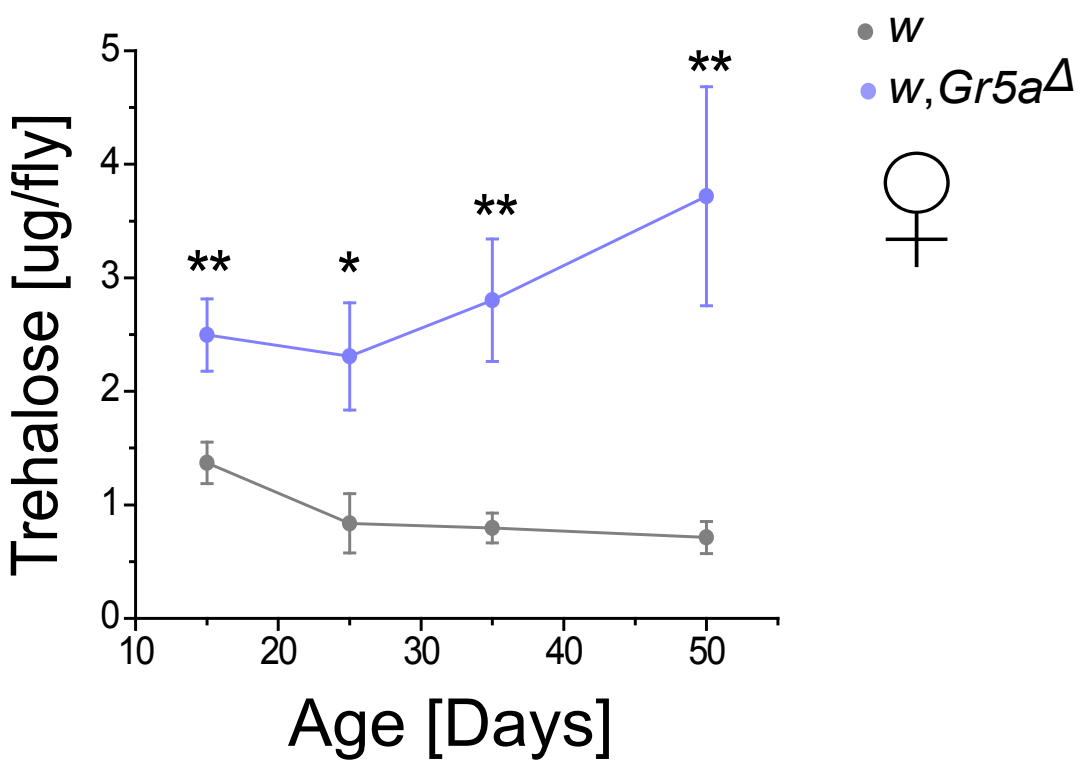


Figure 4.3. High Trehalose Levels in *Gr5a* Mutants Persist Throughout Life. Whole-organism measurements of trehalose in *Gr5a* deletion mutant female flies (*w*,*Gr5a*$^{\Delta}$) and background controls (*w*). ** = p<0.01; *=p<0.05 by two-sided Student's T-Test. Error bars indicate ±S.E.M.

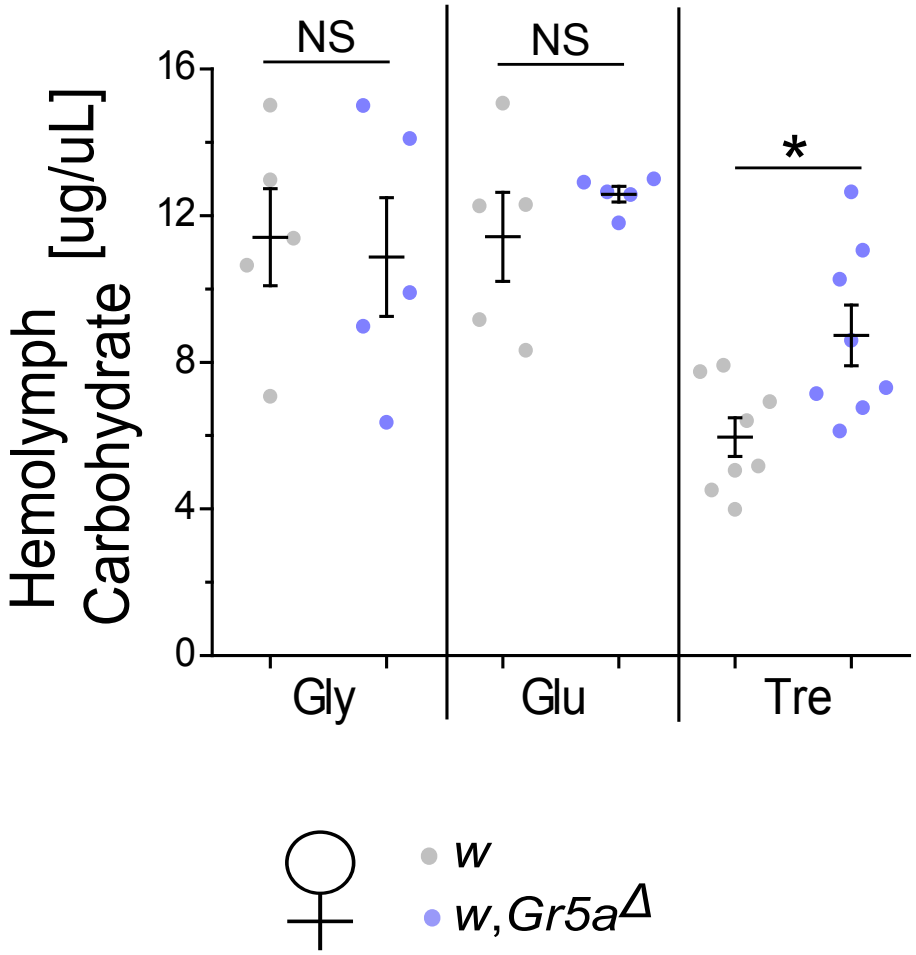


Figure 4.4. Loss of *Gr5a* Induces Hypertrehalosemia. Measurements of carbohydrate levels from hemolymph extracted from *Gr5a* deletion mutant female flies (*w*,*Gr5a*$^{\Delta}$) and background controls (*w*). *=p<0.05 by two-sided Student's T-Test. Error bars indicate ±S.E.M. Gly = glycogen; Glu = glucose;Tre = trehalose.

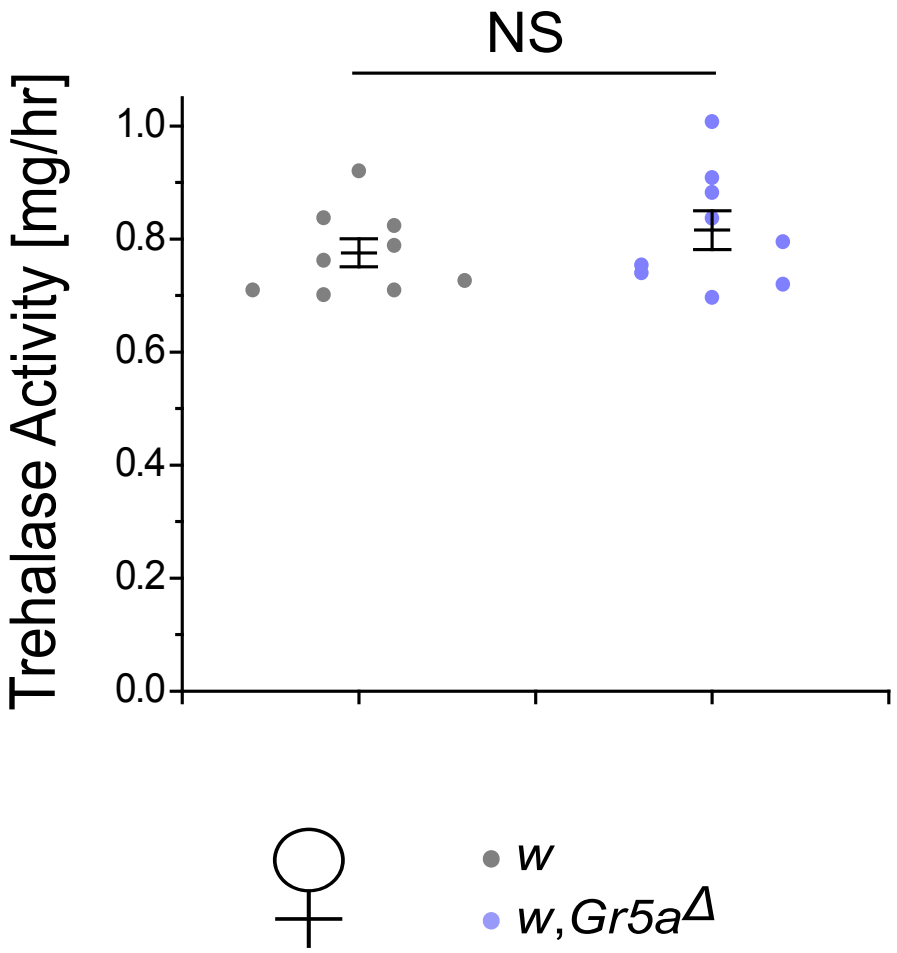


Figure 4.5. Homogenate of *Gr5a* Mutants Does Not Show Increased Trehalase Activity. Amount of trehalose converted to glucose in one hour by homogenate of *Gr5a* deletion mutant female flies (*w*,*Gr5a*$^{\Delta}$) and background controls (*w*).

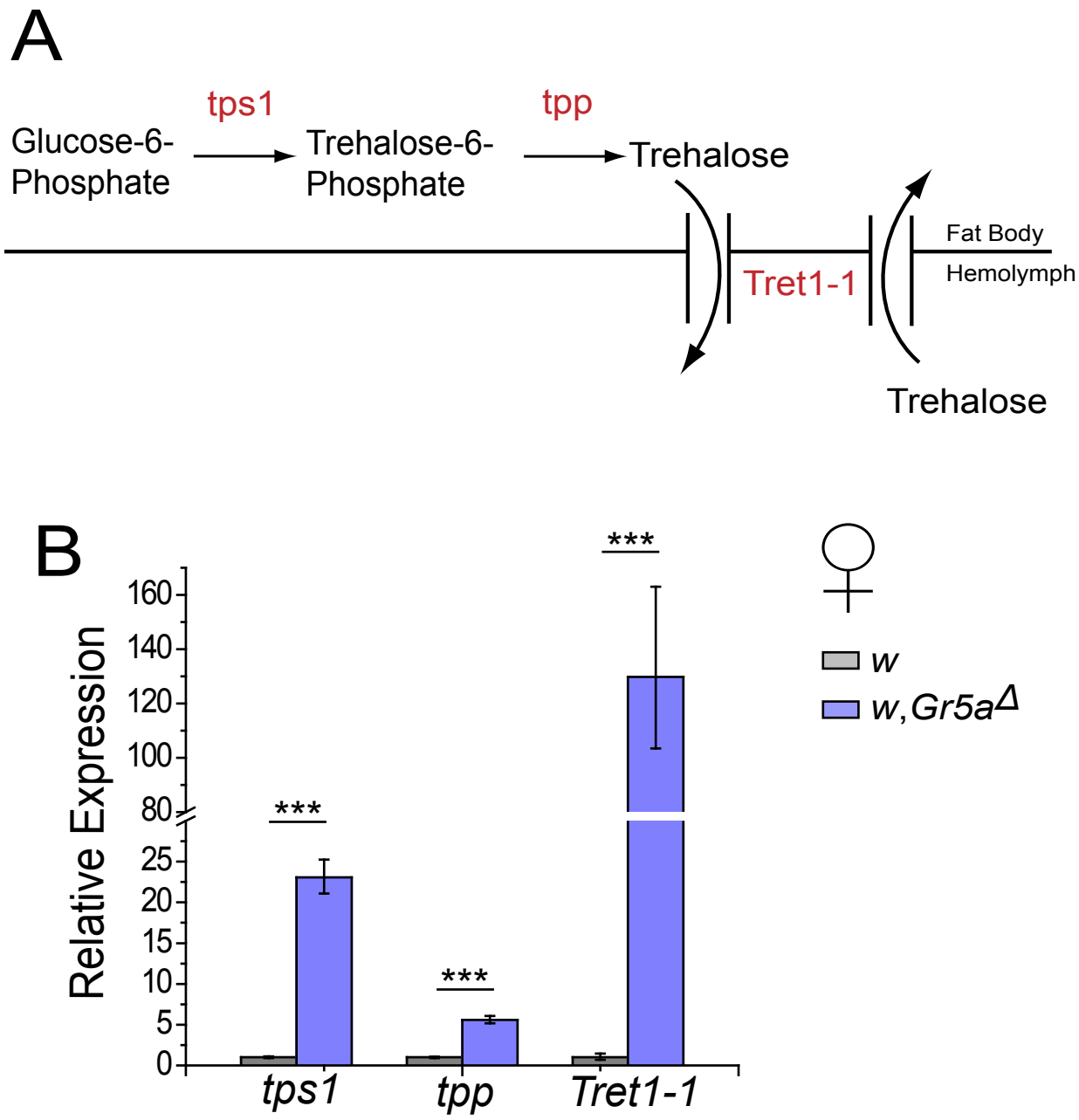


Figure 4.6. Trehalose Synthesis Genes Are More Highly Expressed in *Gr5a* Mutants. (A) Simplified pathway of trehalose synthesis in the *Drosophila* fat body. (B) Relative mRNA levels of relevant genes from quantitative PCR analysis of *Gr5a* deletion mutant female flies (*w*,*Gr5a*$^{\Delta}$) and their background controls (*w*). Values were normalized to *CaM*, used as an endogenous control (n=4-5 biological replicates). ***=p<0.001 by Student's T-test. Error bars indicate ±S.E.M.

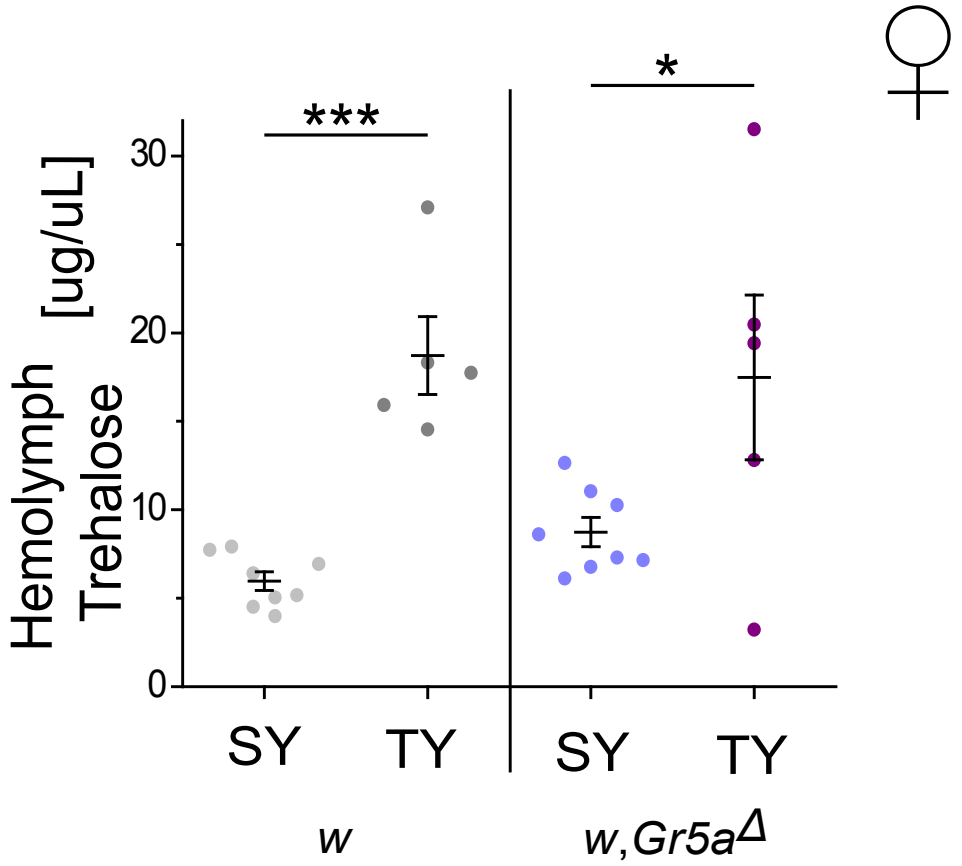


Figure 4.7. A High Trehalose Diet Induces Hypertrehalosemia. Hemolymph trehalose concentrations of *Gr5a* deletion mutant female flies (*w*,*Gr5a*$^{\Delta}$) and their background controls (*w*) on standard 10% sucrose [”SY”] or one week on 10% trehalose [high trehalose - “TY”] and yeast (w/v) diet. ***=p<0.001; *=p<0.05 by Student’s T-test. Error bars indicate ±S.E.M.

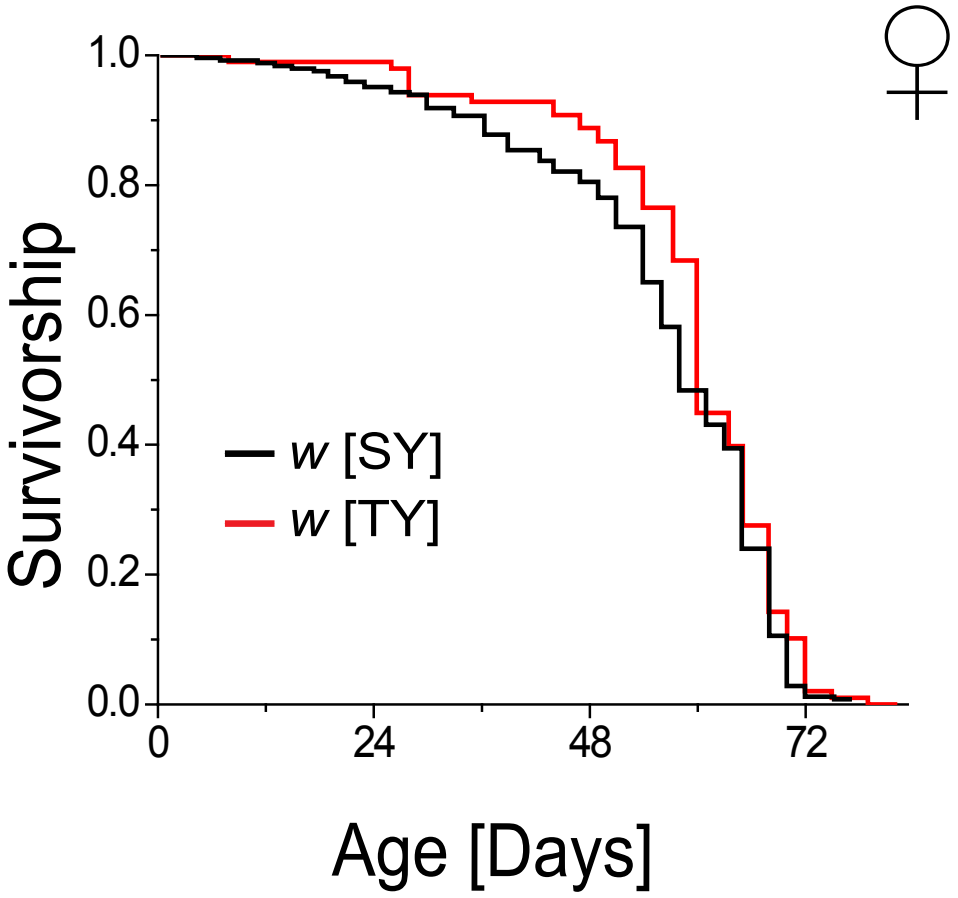


Figure 4.8. A High Trehalose Diet is Not Sufficient to Reduce Lifespan. Survival curves for control flies (*w*) on a 10% sucrose (”SY”) or trehalose (“TY”) and yeast (w/v) diet.

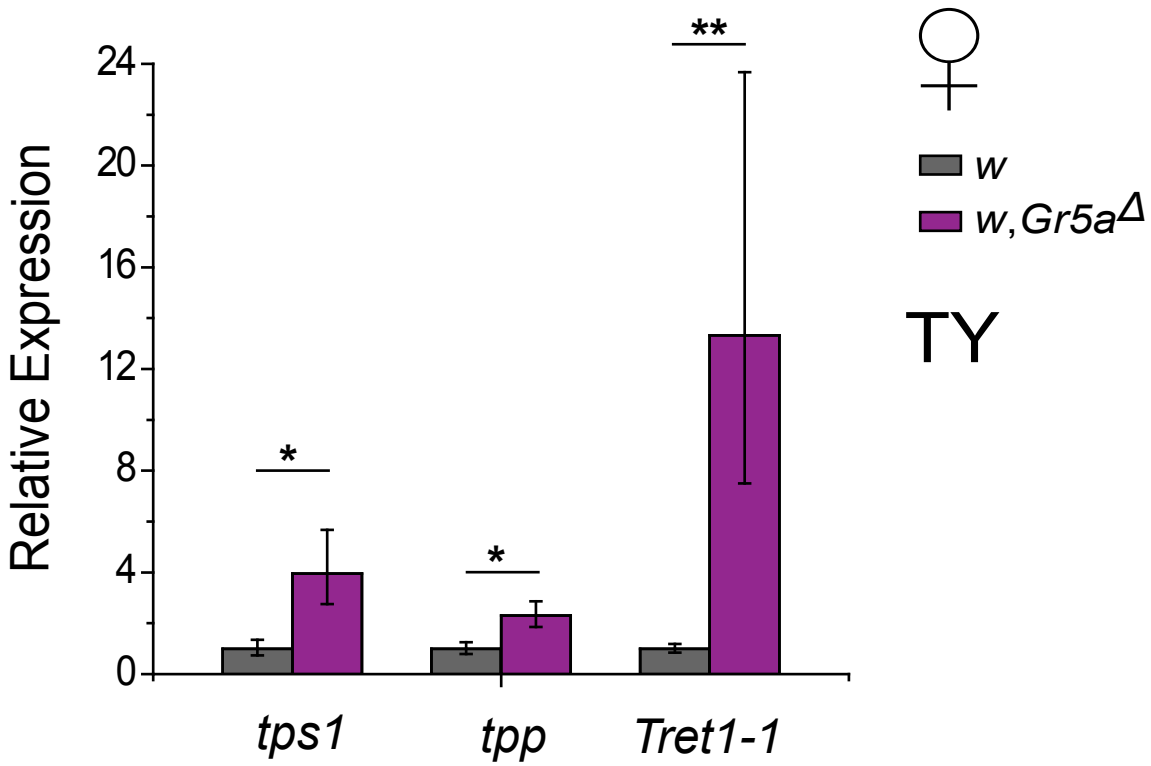
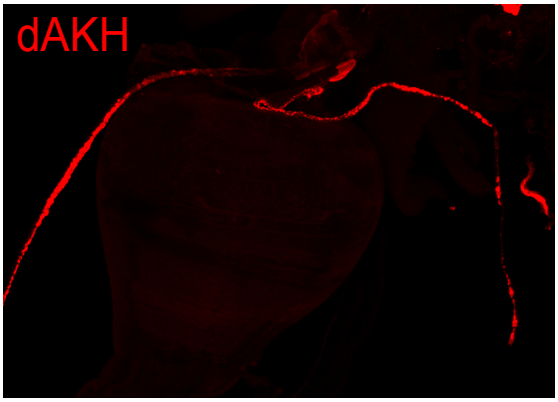


Figure 4.9. Trehalose Synthesis Genes Are Also More Highly Expressed in *Gr5a* Mutants than Controls on a High Trehalose Diet. Relative mRNA levels from quantitative PCR analysis of *Gr5a* deletion mutant female flies (*w*,*Gr5a*$^{\Delta}$) and their background controls (*w*) on a 10% trehalose ("TY") and yeast (w/v) diet. Values were normalized to *CaM*, used as an endogenous control (n=4-5 biological replicates). **=p<0.01 and *=p<0.05 by Student's T-test. Error bars indicate ±S.E.M.

w



♀

w,*Gr5a*$^{\Delta}$

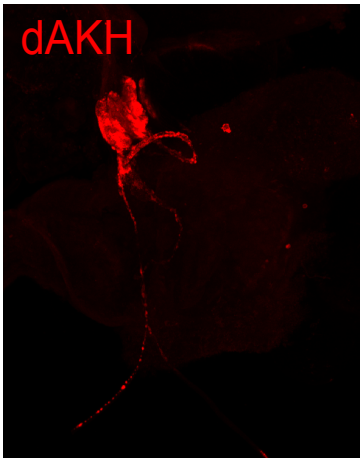


Figure 4.10. Loss of *Gr5a* Function Does Not Increase *Corpora Cardiaca* Cell dAKH Axon Staining. Representative images of adult *corpora cardiaca* cells stained with anti-dAKH antibody of approximately one-week old *Gr5a* deletion mutant female flies (*w*,*Gr5a*$^{\Delta}$) and their background control (*w*). N=4 per genotype.

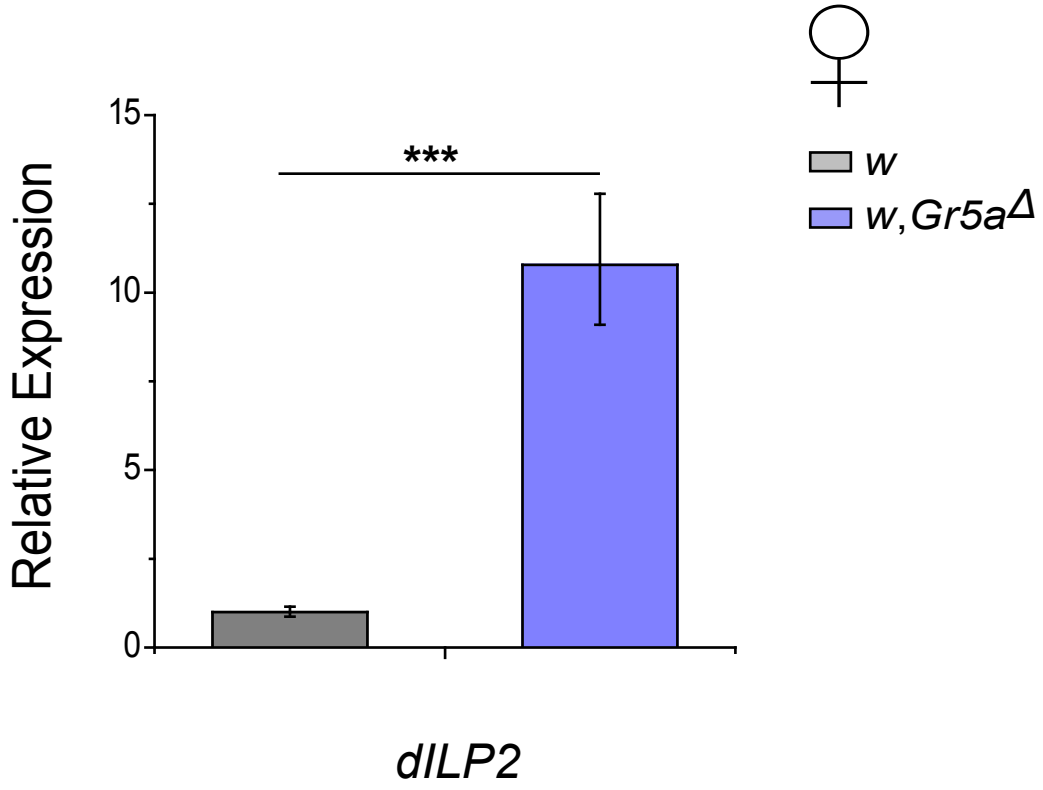


Figure 4.11. *dILP2* Transcript Levels are Increased in *Gr5a* Mutants. Relative mRNA levels from quantitative PCR analysis of *Gr5a* deletion mutant female flies (*w*,*Gr5a*$^{\Delta}$) and their background controls (*w*). Values were normalized to *CaM*, used as an endogenous control (n=4-5 biological replicates). ***=p<0.001 by Student's T-test. Error bars indicate ±S.E.M.

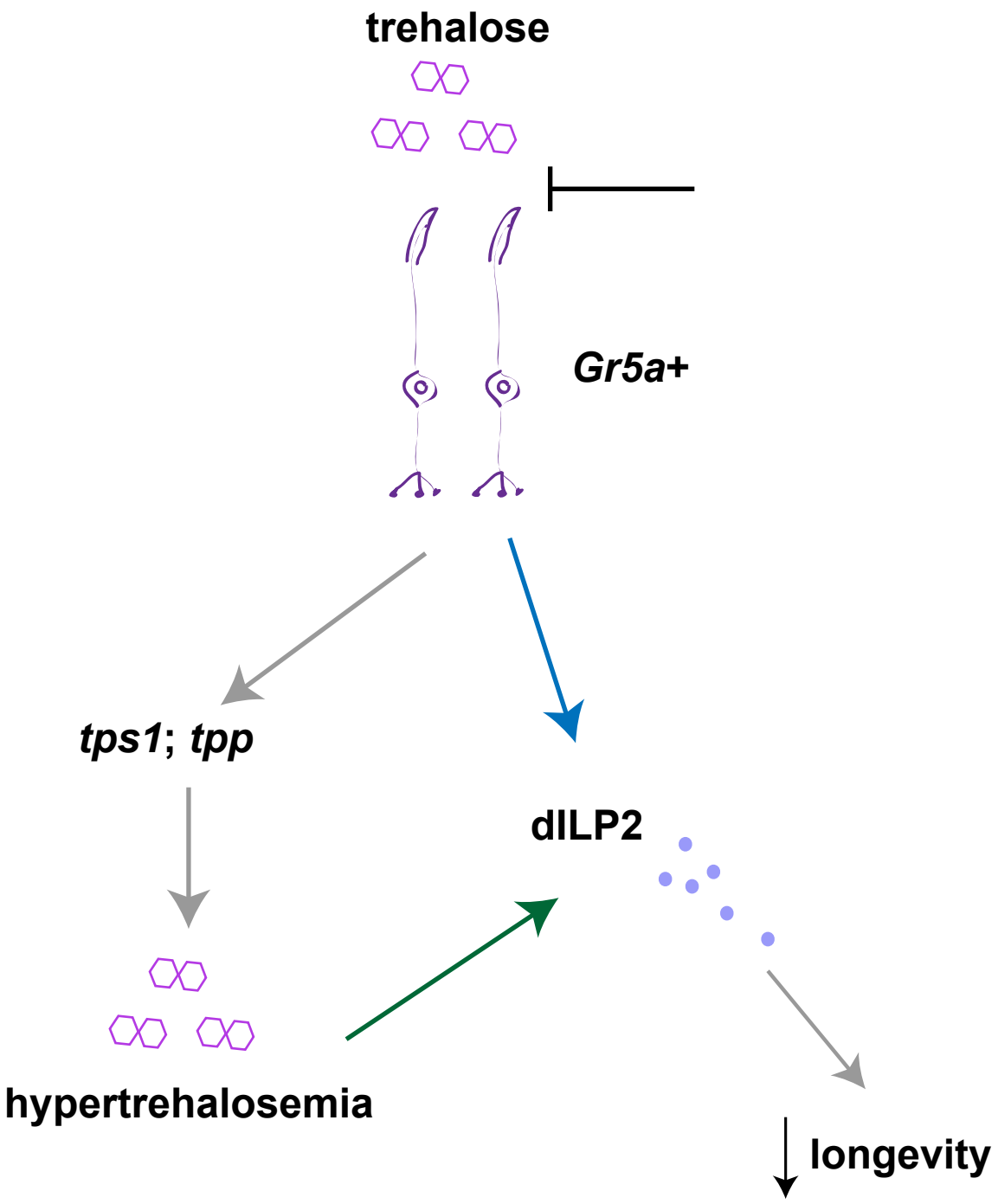


Figure 4.12. Regulation of Physiology and Lifespan Through Loss of *Gr5a* Function. Loss of Gr5a function may shorten lifespan may [blue arrow] or may not [green arrow] be independent of the hypertrehalosemic phenotype.

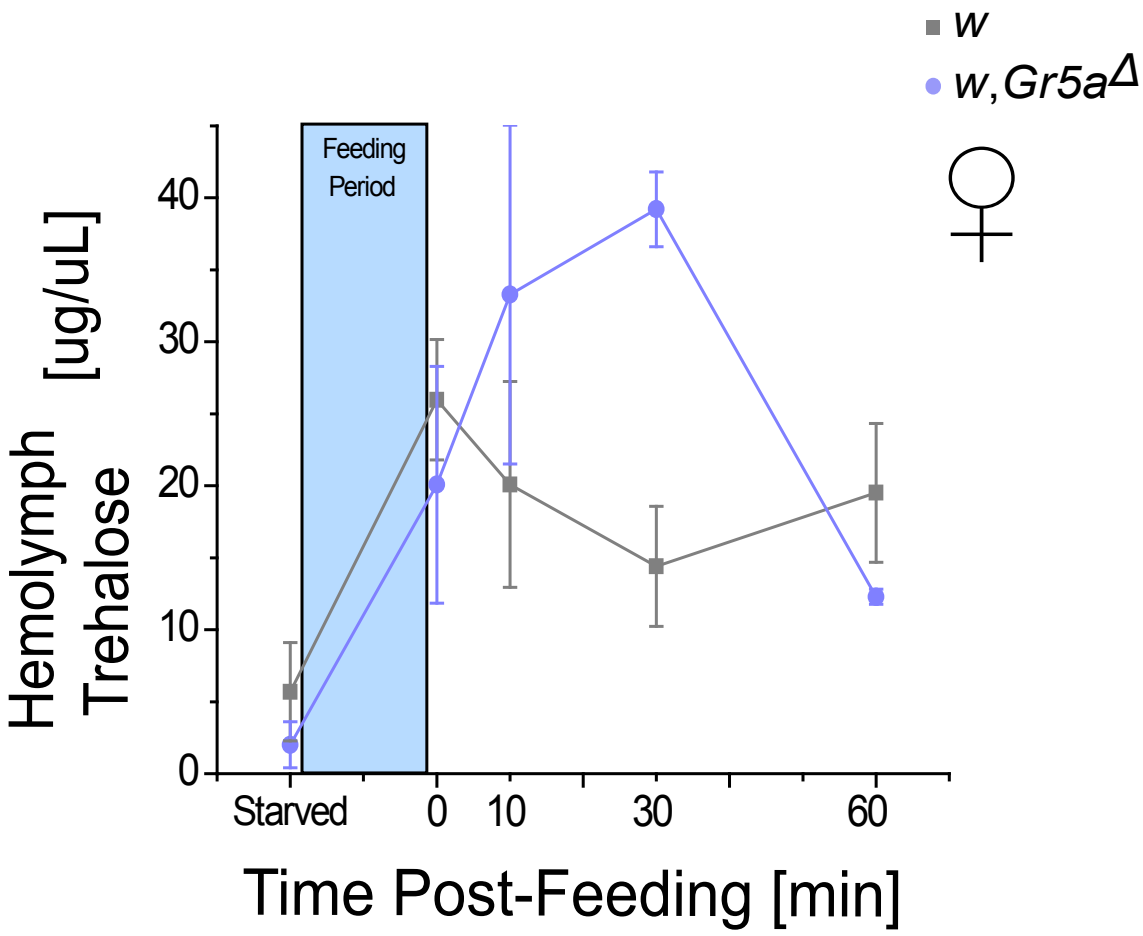


Figure 4.13. *Gr5a* Mutants May Show Delayed Ability to Respond to Ingested Trehalose. Hemolymph measurements of trehalose in *Gr5a* deletion mutant female flies (*w*,*Gr5a*$^{\Delta}$) and background controls (*w*) after starvation and feeding period at indicated time points. n=2-3 samples per time point. Error bars indicate ±S.E.M.

Chapter 5: Synthesis and Future Directions

Chapter 5.A. The Consequence of Normal and Altered Sensory Function

The broadest goal of this work was to understand how sensory input was capable of altering how quickly or slowly an organism ages. Ultimately, this research question divided into two main areas of focus – the neural (including neuroendocrine) cells responsible for this regulation, and the associated physiological changes occurring due to recognition and transduction of a given sensory cue. Maximum lifespan is a post-reproductive phenotype. Thus, it has been suggested that the selective pressure to alter allelic frequencies to maximize lifespan is likely low. Sensory systems, then, have almost certainly evolved not to enact physiological changes that increase longevity, but rather to enact those that allow for the most accurate assessment of and compensatory response to an altering environmental landscape. As such, we sought to focus our efforts first on understanding how metabolic alterations induced by sensory input might serve as a beneficial response to the organism and then how, over the lifetime of an organism, these modulations might turn out to be conducive to a shorter of longer lifespan.

Our use of the gustatory system in *Drosophila melanogaster* proved very fruitful in this regard. We used a genetic approach – the quintessence of the *Drosophila* system – in which we understood sensory regulation through disruption of normal sensory function. This provided the ability not only to piece together the circuitry responsible for a

physiological response to a given taste input, but also the consequence – both positive and negative – of altering sensory perception. In this way, we were able to answer basic questions concerning recognition of and response to a given environmental stimulus, as well as to collect information pertaining to potential targets capable of specifically positively regulating overall organismal health.

The first seminal discovery derived from our work was the uncovering of which specific gustatory inputs were positive regulators of lifespan and which were negative regulators of lifespan. We found, roughly, that sweet tastant receptors normally potentiate increased lifespan whereas bitter receptors and the water sensor *ppk28* normally potentiate decreased lifespan. Taken together with other work in our lab showing that loss of function of the pheromone sensor *ppk23* decreases lifespan, at least in males (Gendron et al., 2013), these data paint a broadstroke picture of gustatory regulation of *Drosophila* lifespan in that they give general characterization of each of the four types of GRNs residing in the taste sensilla of the fly's labellum (i.e. *Gr5a*+ sweet-sensing GRNs; *Gr66a*+ bitter-sensing GRNs; *ppk23*+ pheromone-sensing GRNs; *ppk28*+ water-sensing GRNs) [Fig. 5.1.]. It is intriguing that two of these positively affect longevity whereas two do the opposite and may speak to the tension between stimulation of differing gustatory inputs and the eventual lifespan of an organism. This picture is certainly much clearer than what was present at the start of this work in which it had been shown only that a broad disruption of function of taste perception was sufficient to alter *Drosophila* lifespan in a bidirectional manner (Ostojic et al., 2014).

We next were able to characterize the physiological changes that accompanied alteration of sensory perception itself, independent of actual environmental context – a

concept that had not been well understood. From this work, we found evidence that suggested that loss of the ability to sense a metabolically important molecule – in our case, water and carbohydrate – leads to a physiological reprogramming geared toward increased internal production of this molecule [Fig. 5.2.]. In *ppk28* mutants that had lost the ability to sense external water, this manifested itself in the increased storage and subsequent mobilization of lipid to generate increased stores of metabolic water. In *Gr5a* mutants that had lost the ability to sense external trehalose, the main circulating carbohydrate in the blood analog of *Drosophila*, we found this to occur through a substantial upregulation of expression of the critical enzymes in trehalose synthesis and the transport protein required for the movement of trehalose from the fat body to the hemolymph to increase both whole-organism and hemolymph trehalose levels. Furthermore, we found that these modulations of metabolic physiology occurred independently of potential internal cues pertaining to actual physiological levels of the molecule in question. For instance, increasing hemolymph trehalose levels via a high trehalose diet did not affect the upregulation of the expression of trehalose synthesis genes. Though more than two molecules need to be tested [e.g. determining whether loss of the ability to sense lipid or protein, or a different type of carbohydrate, such as fructose, leads to increased levels of these nutrients internally], the novelty of these results implies that we may have uncovered a general paradigm concerning nutrient-sensing and internal metabolism. Additionally, these data help to inform our original question of how loss of sensory function modulates lifespan, as severe effects on physiology due to loss of solely sensory perception are in accordance with the likewise substantial changes in longevity we and others have found in sensory mutants.

An obvious progression of this work proved similarly illustrative, that was, the determination of how specific gustatory inputs impinged upon neuroendocrine signaling pathways to exert their effects. Though we tested the potential requirement of many types of neuroendocrine signaling networks, our data showing that nutrient synthesis and metabolism were significantly altered in both *ppk28* and *Gr5a* mutants steadied our focus on signaling mechanisms coordinating control of nutrient homeostasis. Indeed, the *Drosophila* homologs of the glucagon and insulin signaling pathways – the major regulators of carbohydrate and lipid metabolism in both flies and mammals – proved to be significantly involved in the circuitry governing lifespan alteration in trehalose and water-sensing mutants. Importantly, we found that the glucagon-like adipokinetic hormone [AKH] signaling pathway was required for lifespan extension of *ppk28* mutants and was significantly upregulated due to loss of sensory function pertaining to external water. On the other hand, we propose that the insulin-like peptide [ILP] signaling pathway is likely an important effector of shortevity seen in *Gr5a* mutants, as expression of *dILP2* was upregulated due to loss of function of *Gr5a*. We did not, however, have the time to directly test its requirement for this response, nor, furthermore, whether actual circulating levels of dILP2 protein were increased. The ILP signaling pathway may also be playing a role in the longevity of *ppk28* mutants, in this case through its downregulation, as expression levels of *dILP2* are decreased in these flies and the longevity extension is dependent on the function of dFoxO, the main downstream transcription factor of ILP signaling, which is active under hypoinsulinemic states. These findings were important on two counts.

First, we were able to gain novel insight into neuroendocrine control of lifespan. It was not, in truth, surprising that long-lived mutants might have decreased levels of ILP signaling whereas short-lived mutants had increased levels, as the inverse correlation between longevity and insulin/ILP signaling has been demonstrated across species. Our work simply adds more data to support this paradigm. It was much more novel to find that upregulation of glucagon/AKH signaling increased lifespan in flies, both in itself, as well as in the context of control via *ppk28*-expressing neural input. These data were key to understanding a previous finding that ubiquitous, ectopic overexpression of the AKH peptide extended lifespan (Katewa et al., 2012), as it offered the first evidence of the positive correlation between AKH signaling and longevity in a physiologically relevant framework. The hypothesis burgeoning from the finding that these pathways may have antagonistic effects on not only metabolism but also longevity is a fascinating one that requires further experimentation.

Second, these data also enabled us to characterize whether or not at least some shared signaling mechanisms are utilized by discrete gustatory inputs to alter lifespan. As discussed previously, one primary goal of using a circuit-mapping approach to understanding regulation of longevity is to determine potential points of convergence through which separate circuits may share similar regulatory mechanics. In this regard, we found that ILP-ergic cells may represent one such node, and that positive and negatively-regulating circuits may oppositely perturb these cells ultimately to exert their effects. So, too, may AKH-ergic cells be a convergence point. Furthermore, by extension from these studies, it could be posited generally that diverse inputs overlap

downstream at the point of signaling pathways to enact behavioral and physiological change.

Taking these findings pertaining to the effects of modulation of *Drosophila* gustatory perception in the context of our initial research question – that is, how sensory input modulates organismal aging – we have utilized this well-characterized and genetically malleable model to provide the clearest detail yet attained pertaining to the underlying cellular and metabolic circuitry responsible for sensory-mediated regulation of aging. Namely, we have shown that differing taste inputs impinge [almost certainly indirectly] upon overlapping and discrete neuroendocrine signaling pathways to enact physiological modulation of the fly, including to aid in the internal production of missing sensory information about a given taste molecule. These neuroendocrine alterations turn out to be powerful in their ability to increase or decrease overall lifespan, especially under longitudinal and consistent activation or downregulation. These results have the possibility to shed light on chemosensory control of longevity in other organisms, including humans. Furthermore, they may provide a stepping off point for delving into lifespan modulation by other sensory modalities, notably by providing further credence for a model of neurosensory regulation of aging [i.e. neurosensory cell to signaling cell to physiological change to lifespan effect] that could be exploited in other sensory contexts mediated by different types of sensory cells.

Chapter 5.B. Toward a More Precise Mapping of Regulatory Neural Circuits

Before exploring the possible future paths of this research as it applies to targeting constituents capable of increasing healthy lifespan, it is important to understand how a

greater resolution of the responsible neural circuitry of this control can be achieved. This will afford the ability to maximize the number of points in the circuit with the potential to be targeted as well as to more precisely determine the contribution of each to eventual physiological and lifespan outcomes. In this way, the most effectual parts can become the source of primary focus. While our studies presented here as well as others have provided evidence for the breadth of aging regulation via gustatory information specifically – and sensory information in general – they quite obviously demonstrate that much additional work is necessary in defining both the precise cellular relationships responsible for transducing peripheral stimulation into alterations of signaling networks, as well as how, where, and when these signals exert their effects.

Much work has certainly been performed characterizing target transcriptional networks and peripheral tissues of endocrine signaling [e.g., the *Drosophila* insulin-like peptides (Kannan and Fridell, 2013)] yet less information has been uncovered regarding the neural regulation of endocrine signaling pathways. A focus on the neurobiological bases of sensory-regulated circuits, and especially how constituent neural connections alter signaling cascades, arguably represents the greatest ability to advance our understanding. Do different sensory cues utilize the same or different neurons to transduce their signal? Do discrete inputs regulate signaling networks similarly in a highly comparable or dissimilar way? Many insights can plausibly be gleaned through this emphasis.

As this work stands, the connections between GRN and neuroendocrine cell are not well defined. Of note, GRNs map to the SOG region of the fly brain, an area not innervated by the neuroendocrine cells implicated in gustatory control of lifespan. dILP-

producing median neurosecretory cells (Brogiolo et al., 2001) are located more dorsally in the brain than the SOG whereas AKH-producing *corpora cardiaca* cells are located ventral to the brain itself (Lee and Park, 2004). This geographic separation suggests that at least one and possibly several pairs of interneurons serve to relay a signal from a gustatory cell to a hormone-producing cell. The identity of these interneurons, however, remains unknown. The initial section of this thesis introduced several strategies available in the *Drosophila* system for establishing neural connections, as well as for testing the requirement of candidate neural populations for a given phenotype, such as augmented nutrient levels or lengthened lifespan [see Chapter 1.E.]. These genetic tools will certainly be necessary for further refining the neurobiological control of neuroendocrine signaling necessary for mediating gustatory effects on aging. Indeed, we have begun to utilize these techniques for this very purpose, though no neuronal populations have yet been implicated. As an added benefit, this work will provide insight into the regulation of endocrine signaling in general – of high relevance as the ability of hormonal pathways to enact potent effects on organismal physiology has been well characterized, including in this work.

Additionally, knowledge of how downstream targets are modulated due to varying environmental input will also be critical in determining the full scope – including timing and spatial resolution – of sensory-mediated lifespan control. Again, though our work has not fleshed out these realities for water and trehalose-sensing inputs, the *Drosophila* system is well suited for this analysis. As noted in the introductory chapter, inducible manipulations – via both temperature and drugs – are now commonplace in the fly geneticist's toolbox. These afford the ability to avoid developmental complications

and to manipulate cell populations and signaling pathways for short periods, or only at a specific time of an organism's life. This will prove useful in determining, for example, if a given physiological modulation early in life is sufficient to enact long-term changes in longevity, or whether longitudinal alteration is required. So too, are reagents with high utility for parsing cell and tissue-specific contributions to a given phenotype. These include restricted driver lines which can narrow the expression of the downstream effector to activate or inhibit a candidate cell population – in the case of neurons – or to knock down expression of a gene in question in these cells or to ablate them altogether. Each strategy provides potential insight into the circuitry responsible for transducing sensory information into changes in longevity. Specifically for our work, it would be helpful to understand the tissues which neuroendocrine molecules are perturbing. Knockdown of the receptor for these molecules in separate tissues [i.e. the AKH receptor in the fat body, or in neurons] may shed light on this transaction.

It is thus apparent that characterizing the cell types and molecules required in gustatory-mediated regulatory circuits, while critical, is not sufficient to fully discern, and, in turn, target, them. In addition, a knowledge of when, where, and with what potency affected downstream events are occurring will be necessary to accomplish this goal. It is important to note once again that the system we have utilized for our studies is as capable as any in making this refinement a reality, yet the experiments required were beyond the time scale of this thesis project. Nonetheless, work on these circuits, as well as those mediated by other varieties of sensory modalities, should move forward toward this end.

Chapter 5.C. Targeting Neurosensory Circuitry to Achieve Healthy Aging

This work was performed to gain understanding on multiple fronts, including of both basic science and translationally-relevant questions, and was, arguably, successful in obtaining information in this regard. The implications of our studies for understanding basic principles of taste perception and signaling pathway regulation have been previously discussed in this chapter. As a final synthesis of the results from our work, then, it is necessary to note on what accounts there has been novel insight gained and how this knowledge might be utilized to benefit the general health of an organism. On the other hand, it is also important to assess where this work has been incomplete and what future experimentation might aid in clarifying the exact workings of these circuits.

It is clear that we have been able to pinpoint specific lifespan effects to discrete gustatory inputs in the fly. In general, sweet and pheromone-sensitive inputs promote a longer lifespan whereas bitter and water-sensitive inputs promote a shorter one. The principles of taste perception are broadly conserved between the fly and mammals, including a similar range of taste modalities and classes of genes required for recognition of tastants (Yarmolinsky et al., 2009). Though no specific work has been done to link human gustation and aging, there is some data, as noted in the introductory chapter, suggesting a substantial link between taste perception and physiological alteration. Though these were short-term studies focused on an endurance task, longitudinal manipulation of taste receptors may have significant effects on long-term health and lifespan. It may also turn out to be the case, of course, that similar manipulation of sensory perception has a much greater effect on longevity of

invertebrate model systems which possess a considerably shorter and more malleable average lifespan than a human. In humans, the effect of even severe alterations in taste [i.e. a compound which causes complete inhibition of a given taste receptor] may lead to much less substantial lifespan modulation. As an additional consideration, too, tasting – especially high-quality food – is generally viewed as a positive human experience, and the possibility of potential health benefits may not be enough to overcome the loss of this ability. Thus, targeting taste inputs themselves may have some utility [e.g. pharmaceuticals which selectively alter taste input function or specialized diets removing certain tastants], but may not represent the most enjoyable strategy for promoting organismal healthspan.

Moving downstream in our neural circuits of interest, the details we uncovered pertaining to gustatory regulation of signaling pathways have had high utility in a number of ways. First, they have led to a new clarity concerning which taste inputs affect which endocrine signaling mechanisms. We have shown that information from *ppk28*-expressing neurons eventually leads to the modulation of glucagon-like AKH signaling whereas information from *Gr5a*-expressing neurons likely eventually impinges upon ILP signaling. Thus, we have learned that gustatory inputs do not alter longevity simply by activating or inhibiting a singular signaling pathway. It is important to note, however, that this does not necessarily imply that there are no points of convergence within these separate circuits, a major goal, as previously explained, in mapping multiple regulatory networks. We have not characterized the likely multiple connections between GRNs and endocrine cells, a route which at least passes through several steps in the CNS, but also potentially to a peripheral tissue before coming back to the brain

[e.g. the release of a humoral factor from the fat body]. Until this is more clearly understood, it remains a formal possibility that similar cell populations and signaling molecules – which might induce or inhibit neuroendocrine signaling – may be utilized by circuits that begin with separate gustatory inputs. Second, our studies have aided in the understanding of regulation of lifespan by neuroendocrine signaling itself. Though it has long been known and extensively shown that an increase in ILP signaling reduces lifespan across species, we were able to show that this holds true in sweet-tasting gustatory mutants, further adding to the large body of evidence in support of this paradigm. Of perhaps greater significance, though, was the finding that activation of AKH-ergic cells, both in the context of lack of water gustatory information as well as in otherwise control flies, extended lifespan. Though there was some preliminary evidence suggesting this may be the case, our work was the first to show this was true when activation was limited to solely AKH-ergic cells in a physiological context. The possibility that ILP and AKH signaling have somewhat opposing biological effects as well as modulate lifespan in an opposing manner has not escaped our attention. Whether or not there is communication between these two signaling mechanisms to regulate longevity was not part of our work and remains a ripe area of further investigation. Of these two sets of data, the latter is likely the most useful for our ability to intervene normal physiology to increase the length of wellness experienced in humans, especially as these pathways are highly conserved. Therapeutics which target signaling pathways do not come with the caveat of altering sensory experience, and, as such, represent a potentially fruitful set of constituents to modulate through dietary or pharmaceutical measures. Particularly in the context of our work, interventions which might selectively

augment glucagon signaling may prove beneficial to human healthspan. Again, though, our lack of complete understanding of these mechanisms from our studies must caution any certain conclusion. A greater knowledge of how much activation of this pathway, at what time, and in which tissues it is the most useful – and conversely, in which contexts it may actually be harmful – is certainly required, and yet another subsequent line of work derived from our initial findings.

Finally, our work understanding how taste inputs might work to regulate internal nutrient levels may also prove of benefit, not only in their long-term effects on longevity, but also as an acute measure of inducing rapid physiological change. Of particular note, our work showing that loss of at least the two inputs we studied shifted internal organismal physiology toward the production of the metabolite recognized by these sensors, represented a quite unexpected – though not completely unintuitive – and intriguing arc in our investigation. Currently, dietary and environmental regimes [e.g. exercise] are recommended to control nutrient storage and metabolism. Our studies suggest the plausibility of a new avenue of control. For instance, could blood sugar be reduced simply by activating carbohydrate-sensing receptors in the absence of actual sugar? Could lipid or protein levels be modulated without austere changes in diet? The potential advantageousness of these manipulations is certainly tantalizing. As has become a common theme, though, this must be tempered with the reality of what remains to be determined. First, it must be understood whether this is a general rule of thumb or holds true only for the two contexts which we have analyzed. This requires not only the study of other known chemosensory receptors which recognize metabolically important tastants, but also the discovery and characterization of receptors for nutrients

– such as protein and lipid – which are not currently known. If the link between external sensing and internal metabolism of a given metabolite does turn out to occur as a general standard, then further work in other organisms, including mammals, needs to be performed to determine if this regulation is conserved across species. Only then should a major effort be placed into targeting sensory receptors as a means to enact specific physiological changes.

Uniting these multiple sets of observations as a whole, we have unequivocally pushed the fields of sensory biology, neuroendocrine regulation, and aging forward by providing novel insight and potential therapeutic targets as a result of our experimentation. Though there is much left to be uncovered, the *Drosophila* system provides the attributes and genetic tools to expand the knowledge for which our studies set the foundation. The genetic and functional conservation between the fly and mammalian systems, including humans, lends the possibility of transducing this knowledge into beneficial physiological interventions for our own species.

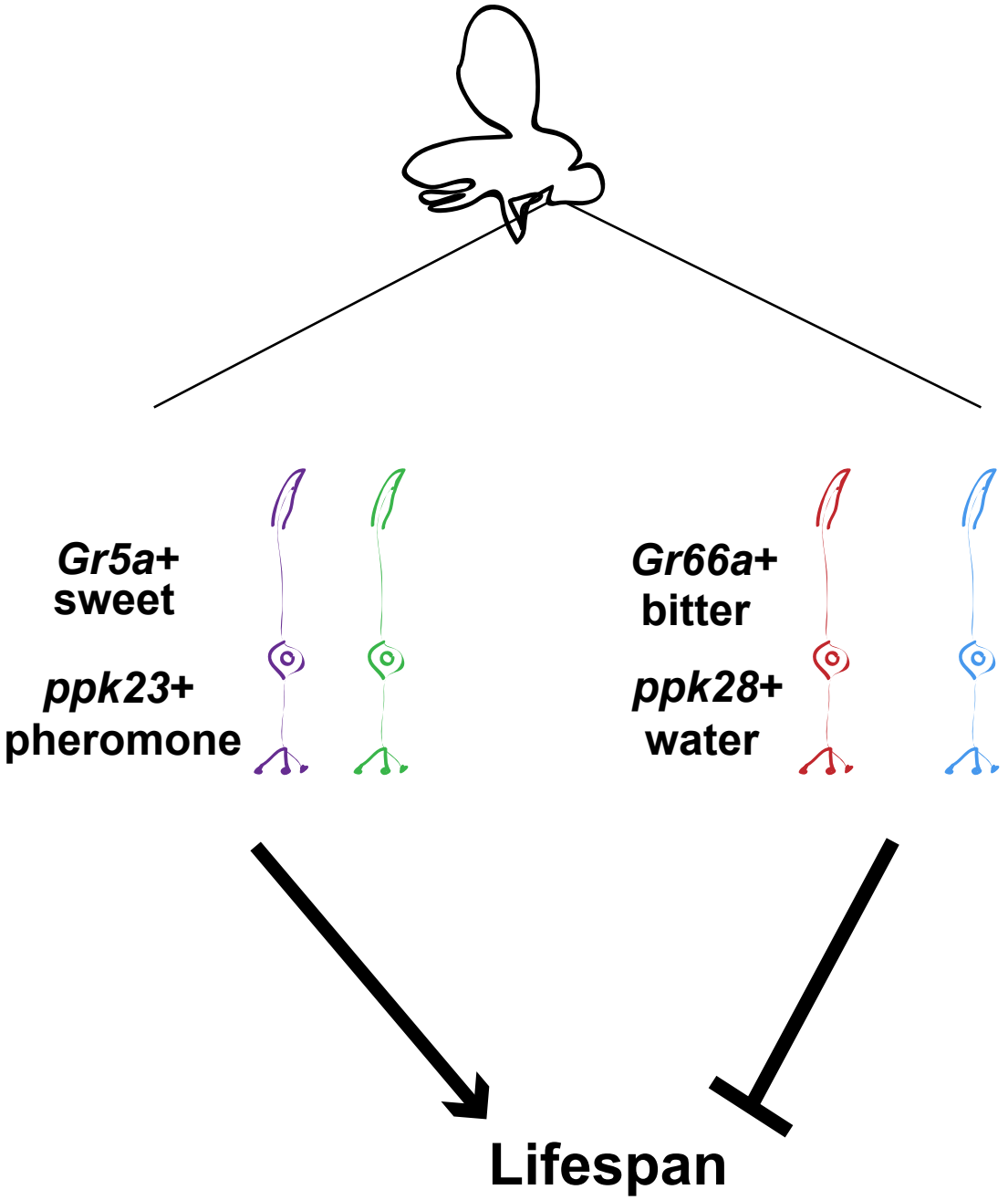


Figure 5.1. Longevity Regulation by Discrete Gustatory Inputs in *Drosophila*. The control of lifespan by distinct gustatory inputs recognizing stereotyped classes of tastants.

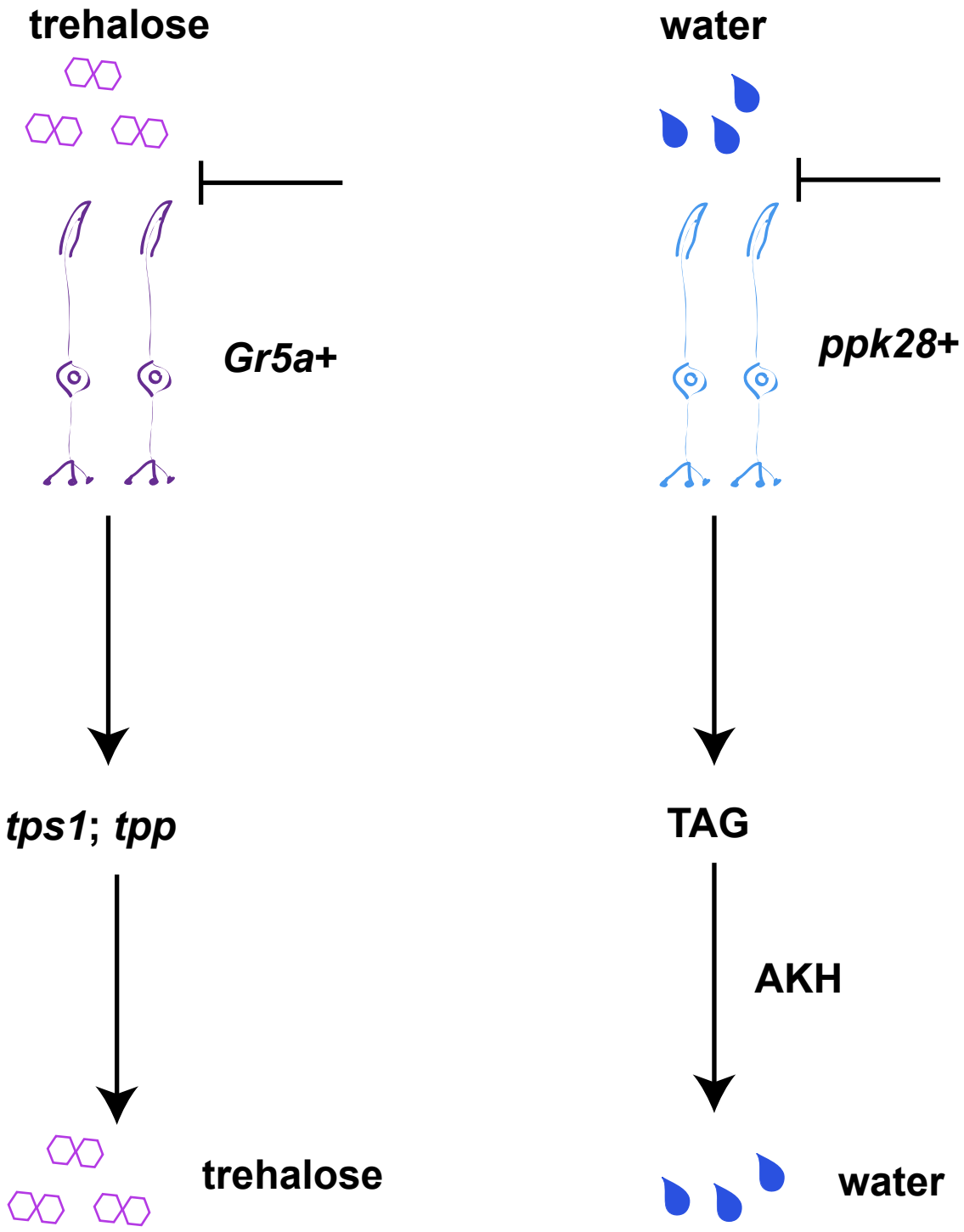


Figure 5.2. Internal Response to Loss of Gustatory Perception. Loss of the ability to externally sense both water and trehalose enacts a metabolic response which increases internal levels of these molecules.

References

Alcedo, J., and Kenyon, C. (2004). Regulation of *C. elegans* longevity by specific gustatory and olfactory neurons. Neuron *41*, 45-55.

Apfeld, J., and Kenyon, C. (1999). Regulation of lifespan by sensory perception in *Caenorhabditis elegans*. Nature *402*, 804-809.

Ash, C.E., and Merry, B.J. (2011). The molecular basis by which dietary restricted feeding reduces mitochondrial reactive oxygen species generation. Mech Ageing Dev *132*, 43-54.

Atasoy, D., Betley, J.N., Su, H.H., and Sternson, S.M. (2012). Deconstruction of a neural circuit for hunger. Nature *488*, 172-177.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in *Drosophila* neurons developing without synaptic transmission. J Neurosci *21*, 1523-1531.

Barrios, A., Ghosh, R., Fang, C., Emmons, S.W., and Barr, M.M. (2012). PDF-1 neuropeptide signaling modulates a neural circuit for mate-searching behavior in *C. elegans*. Nat Neurosci *15*, 1675-1682.

Barzilai, N., Huffman, D.M., Muzumdar, R.H., and Bartke, A. (2012). The critical role of metabolic pathways in aging. Diabetes *61*, 1315-1322.

Becker, A., Schloder, P., Steele, J.E., and Wegener, G. (1996). The regulation of trehalose metabolism in insects. Experientia *52*, 433-439.

Belgacem, Y.H., and Martin, J.R. (2006). Disruption of insulin pathways alters trehalose level and abolishes sexual dimorphism in locomotor activity in *Drosophila*. J Neurobiol *66*, 19-32.

Bellen, H.J., Levis, R.W., Liao, G., He, Y., Carlson, J.W., Tsang, G., Evans-Holm, M., Hiesinger, P.R., Schulze, K.L., Rubin, G.M*., et al.* (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. Genetics *167*, 761-781.

Benton, R. (2008). Chemical sensing in *Drosophila*. Curr Opin Neurobiol *18*, 357-363.

Bharucha, K.N., Tarr, P., and Zipursky, S.L. (2008). A glucagon-like endocrine pathway in *Drosophila* modulates both lipid and carbohydrate homeostasis. J Exp Biol *211*, 3103-3110.

Bishop, N.A., and Guarente, L. (2007). Two neurons mediate diet-restriction-induced longevity in *C. elegans*. Nature *447*, 545-549.

Boll, W., and Noll, M. (2002). The *Drosophila Pox neuro* gene: control of male courtship behavior and fertility as revealed by a complete dissection of all enhancers. Development *129*, 5667-5681.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. (2001). An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. Curr Biol *11*, 213-221.

Buck, L., and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell *65*, 175-187.

Burnett, C., Valentini, S., Cabreiro, F., Goss, M., Somogyvari, M., Piper, M.D., Hoddinott, M., Sutphin, G.L., Leko, V., McElwee, J.J.*, et al.* (2011). Absence of effects of *Sir2* overexpression on lifespan in *C. elegans* and *Drosophila*. Nature *477*, 482-485.

Cameron, P., Hiroi, M., Ngai, J., and Scott, K. (2010). The molecular basis for water taste in *Drosophila*. Nature *465*, 91-95.

Carter, J.M., Jeukendrup, A.E., and Jones, D.A. (2004). The effect of carbohydrate mouth rinse on 1-h cycle time trial performance. Med Sci Sports Exerc *36*, 2107-2111.

Carter, M.E., Soden, M.E., Zweifel, L.S., and Palmiter, R.D. (2013). Genetic identification of a neural circuit that suppresses appetite. Nature.

Cava, E., and Fontana, L. (2013). Will calorie restriction work in humans? Aging (Albany NY) *5*, 507-514.

Christensen, A.P., and Corey, D.P. (2007). TRP channels in mechanosensation: direct or indirect activation? Nat Rev Neurosci *8*, 510-521.

Chyb, S., Dahanukar, A., Wickens, A., and Carlson, J.R. (2003). *Drosophila Gr5a* encodes a taste receptor tuned to trehalose. Proc Natl Acad Sci U S A *100 Suppl 2*, 14526-14530.

Colombani, J., Andersen, D.S., and Leopold, P. (2012). Secreted peptide *Dilp8* coordinates *Drosophila* tissue growth with developmental timing. Science *336*, 582-585.

Conti, B. (2008). Considerations on temperature, longevity and aging. Cell Mol Life Sci *65*, 1626-1630.

Conti, B., Sanchez-Alavez, M., Winsky-Sommerer, R., Morale, M.C., Lucero, J., Brownell, S., Fabre, V., Huitron-Resendiz, S., Henriksen, S., Zorrilla, E.P.*, et al.* (2006). Transgenic mice with a reduced core body temperature have an increased life span. Science *314*, 825-828.

Couto, A., Alenius, M., and Dickson, B.J. (2005). Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. Curr Biol *15*, 1535-1547.

Dahanukar, A., Lei, Y.T., Kwon, J.Y., and Carlson, J.R. (2007). Two Gr genes underlie sugar reception in *Drosophila*. Neuron *56*, 503-516.

Davis, R.L. (2011). Traces of *Drosophila* memory. Neuron *70*, 8-19.

Dethier, V.G. (1976). The hungry fly: a physiological study of the behavior associated with feeding (Cambridge, Mass., Harvard University Press).

Diederen, J.H., Oudejans, R.C., Harthoorn, L.F., and Van der Horst, D.J. (2002). Cell biology of the adipokinetic hormone-producing neurosecretory cells in the locust corpus cardiacum. Microsc Res Tech *56*, 227-236.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S.*, et al.* (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature *448*, 151-156.

Dommer, D.H., Gazzolo, P.J., Painter, M.S., and Phillips, J.B. (2008). Magnetic compass orientation by larval *Drosophila melanogaster*. J Insect Physiol *54*, 719-726.

Evans, D.R., and Mellon, D., Jr. (1962). Electrophysiological studies of a water receptor associated with the taste sensilla of the blow-fly. J Gen Physiol *45*, 487-500.

Feinberg, E.H., Vanhoven, M.K., Bendesky, A., Wang, G., Fetter, R.D., Shen, K., and Bargmann, C.I. (2008). GFP Reconstitution Across Synaptic Partners (GRASP) defines cell contacts and synapses in living nervous systems. Neuron *57*, 353-363.

Folk, D.G., Han, C., and Bradley, T.J. (2001). Water acquisition and partitioning in *Drosophila melanogaster*: effects of selection for desiccation-resistance. J Exp Biol *204*, 3323-3331.

Fontana, L., Partridge, L., and Longo, V.D. (2010). Extending healthy life span--from yeast to humans. Science *328*, 321-326.

Frank, C.L. (1988). Diet Selection by a Heteromyid Rodent: Role of Net Metabolic Water Production. Ecology *69*, 1943-1951.

Friedman, D.B., and Johnson, T.E. (1988). A mutation in the *age-1* gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility. Genetics *118*, 75-86.

Fujishiro, N., Kijima, H., and Morita, H. (1984). Impulse frequency and action-potential amplitude in labellar chemosensory neurons of *Drosophila melanogaster*. Journal of Insect Physiology *30*, 317-325.

Gaillard, I., Rouquier, S., and Giorgi, D. (2004). Olfactory receptors. Cell Mol Life Sci *61*, 456-469.

Galindo, M.M., Schneider, N.Y., Stahler, F., Tole, J., and Meyerhof, W. (2012). Taste preferences. Prog Mol Biol Transl Sci *108*, 383-426.

Garelli, A., Gontijo, A.M., Miguela, V., Caparros, E., and Dominguez, M. (2012). Imaginal discs secrete insulin-like peptide 8 to mediate plasticity of growth and maturation. Science *336*, 579-582.

Gendron, C.M., Kuo, T.H., Harvanek, Z.M., Chung, B.Y., Yew, J.Y., Dierick, H.A., and Pletcher, S.D. (2013). *Drosophila* Life Span and Physiology Are Modulated by Sexual Perception and Reward. Science *343*, 544-548.

Gilbertson, T.A. (2002). Hypoosmotic stimuli activate a chloride conductance in rat taste cells. Chem Senses *27*, 383-394.

Gordon, M.D., and Scott, K. (2009). Motor control in a Drosophila taste circuit. Neuron *61*, 373-384.

Gray, E.M., and Bradley, T.J. (2005). Physiology of desiccation resistance in *Anopheles gambiae* and *Anopheles arabiensis*. Am J Trop Med Hyg *73*, 553-559.

Greer, E.R., Perez, C.L., Van Gilst, M.R., Lee, B.H., and Ashrafi, K. (2008). Neural and molecular dissection of a *C. elegans* sensory circuit that regulates fat and feeding. Cell Metab *8*, 118-131.

Gronke, S., Muller, G., Hirsch, J., Fellert, S., Andreou, A., Haase, T., Jackle, H., and Kuhnlein, R.P. (2007). Dual lipolytic control of body fat storage and mobilization in *Drosophila*. PLoS Biol *5*, e137.

Guevara-Aguirre, J., Balasubramanian, P., Guevara-Aguirre, M., Wei, M., Madia, F., Cheng, C.W., Hwang, D., Martin-Montalvo, A., Saavedra, J., Ingles, S.*, et al.* (2011). Growth hormone receptor deficiency is associated with a major reduction in pro-aging signaling, cancer, and diabetes in humans. Sci Transl Med *3*, 70ra13.

Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., and Garrity, P.A. (2008). An internal thermal sensor controlling temperature preference in *Drosophila*. Nature *454*, 217-220.

Ja, W.W., Carvalho, G.B., Zid, B.M., Mak, E.M., Brummel, T., and Benzer, S. (2009). Water- and nutrient-dependent effects of dietary restriction on *Drosophila* lifespan. Proc Natl Acad Sci U S A *106*, 18633-18637.

Jennings, J.H., Sparta, D.R., Stamatakis, A.M., Ung, R.L., Pleil, K.E., Kash, T.L., and Stuber, G.D. (2013). Distinct extended amygdala circuits for divergent motivational states. Nature *496*, 224-228.

Jiang, G., and Zhang, B.B. (2003). Glucagon and regulation of glucose metabolism. Am J Physiol Endocrinol Metab *284*, E671-678.

Kannan, K., and Fridell, Y.W. (2013). Functional implications of insulin-like peptides in metabolism, aging, and dietary restriction. Front Physiol *4*, 288.

Kaplan, J.M., and Horvitz, H.R. (1993). A dual mechanosensory and chemosensory neuron in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A *90*, 2227-2231.

Katewa, S.D., Demontis, F., Kolipinski, M., Hubbard, A., Gill, M.S., Perrimon, N., Melov, S., and Kapahi, P. (2012). Intramyocellular Fatty-Acid Metabolism Plays a Critical Role in Mediating Responses to Dietary Restriction in *Drosophila melanogaster*. Cell Metab *16*, 97-103.

Kawano, T., Kataoka, N., Abe, S., Ohtani, M., Honda, Y., Honda, S., and Kimura, Y. (2005). Lifespan extending activity of substances secreted by the nematode *Caenorhabditis elegans* that include the dauer-inducing pheromone. Biosci Biotechnol Biochem *69*, 2479-2481.

Kenyon, C. (2011). The first long-lived mutants: discovery of the insulin/IGF-1 pathway for ageing. Philos Trans R Soc Lond B Biol Sci *366*, 9-16.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993). A *C. elegans* mutant that lives twice as long as wild type. Nature *366*, 461-464.

Khan, S., and Chang, R. (2013). Anatomy of the vestibular system: a review. NeuroRehabilitation *32*, 437-443.

Kim, S.H., Lee, Y., Akitake, B., Woodward, O.M., Guggino, W.B., and Montell, C. (2010). *Drosophila* TRPA1 channel mediates chemical avoidance in gustatory receptor neurons. Proc Natl Acad Sci U S A *107*, 8440-8445.

Kitamoto, T. (2001). Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. J Neurobiol *47*, 81-92.

Kondratov, R.V., Kondratova, A.A., Gorbacheva, V.Y., Vykhovanets, O.V., and Antoch, M.P. (2006). Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. Genes Dev *20*, 1868-1873.

Kwan, K.Y., Allchorne, A.J., Vollrath, M.A., Christensen, A.P., Zhang, D.S., Woolf, C.J., and Corey, D.P. (2006). TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. Neuron *50*, 277-289.

Lai, S.L., and Lee, T. (2006). Genetic mosaic with dual binary transcriptional systems in *Drosophila*. Nat Neurosci *9*, 703-709.

Lee, G., and Park, J.H. (2004). Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster*. Genetics *167*, 311-323.

Lee, S.J., and Kenyon, C. (2009). Regulation of the longevity response to temperature by thermosensory neurons in *Caenorhabditis elegans*. Curr Biol *19*, 715-722.

Lee, Y., Moon, S.J., and Montell, C. (2009). Multiple gustatory receptors required for the caffeine response in *Drosophila*. Proc Natl Acad Sci U S A *106*, 4495-4500.

Libert, S., Bonkowski, M.S., Pointer, K., Pletcher, S.D., and Guarente, L. (2012). Deviation of innate circadian period from 24 h reduces longevity in mice. Aging Cell *11*, 794-800.

Libert, S., Zwiener, J., Chu, X., Vanvoorhies, W., Roman, G., and Pletcher, S.D. (2007). Regulation of *Drosophila* life span by olfaction and food-derived odors. Science *315*, 1133-1137.

Lindemann, B. (1996). Taste reception. Physiol Rev *76*, 718-766.

Linford, N.J., Bilgir, C., Ro, J., and Pletcher, S.D. (2013). Measurement of Lifespan in *Drosophila melanogaster*. J Vis Exp *71*.

Liu, L., Leonard, A.S., Motto, D.G., Feller, M.A., Price, M.P., Johnson, W.A., and Welsh, M.J. (2003). Contribution of *Drosophila* DEG/ENaC genes to salt taste. Neuron *39*, 133-146.

Ludewig, A.H., Izrayelit, Y., Park, D., Malik, R.U., Zimmermann, A., Mahanti, P., Fox, B.W., Bethke, A., Doering, F., Riddle, D.L.*, et al.* (2013). Pheromone sensing regulates *Caenorhabditis elegans* lifespan and stress resistance via the deacetylase SIR-2.1. Proc Natl Acad Sci U S A *110*, 5522-5527.

Marella, S., Fischler, W., Kong, P., Asgarian, S., Rueckert, E., and Scott, K. (2006). Imaging taste responses in the fly brain reveals a functional map of taste category and behavior. Neuron *49*, 285-295.

Marella, S., Mann, K., and Scott, K. (2012). Dopaminergic modulation of sucrose acceptance behavior in *Drosophila*. Neuron *73*, 941-950.

Maruyama, H., Hisatomi, A., Orci, L., Grodsky, G.M., and Unger, R.H. (1984). Insulin within islets is a physiologic glucagon release inhibitor. J Clin Invest *74*, 2296-2299.

Maures, T.J., Booth, L.N., Benayoun, B.A., Izrayelit, Y., Schroeder, F.C., and Brunet, A. (2013). Males Shorten the Life Span of *C. elegans* Hermaphrodites via Secreted Compounds. Science *343*, 541-544 .

Montell, C. (2009). A taste of the *Drosophila* gustatory receptors. Curr Opin Neurobiol *19*, 345-353.

Montell, C. (2011). The history of TRP channels, a commentary and reflection. Pflugers Arch *461*, 499-506.

Moon, S.J., Kottgen, M., Jiao, Y., Xu, H., and Montell, C. (2006). A taste receptor required for the caffeine response in vivo. Curr Biol *16*, 1812-1817.

Moon, S.J., Lee, Y., Jiao, Y., and Montell, C. (2009). A *Drosophila* gustatory receptor essential for aversive taste and inhibiting male-to-male courtship. Curr Biol *19*, 1623-1627.

Murthy, V.N. (2011). Olfactory maps in the brain. Annu Rev Neurosci *34*, 233-258.

Naidu, S.G. (2008). Why does the Namib Desert tenebrionid *Onymacris unguicularis* (Coleoptera: Tenebrionidae) fog-bask? European Journal of Entomology *105*, 829-838.

Ostojic, I., Boll, W., Waterson, M.J., Chan, T.P., Chandra, R., Pletcher, S.D., and Alcedo, J. (2014). Positive and Negative Gustatory Inputs Affect *Drosophila* Lifespan Partly in Parallel to dFOXO. Proc Natl Acad Sci U S A (in press).

Pfeiffer, B.D., Jenett, A., Hammonds, A.S., Ngo, T.T., Misra, S., Murphy, C., Scully, A., Carlson, J.W., Wan, K.H., Laverty, T.R.*, et al.* (2008). Tools for neuroanatomy and neurogenetics in *Drosophila*. Proc Natl Acad Sci U S A *105*, 9715-9720.

Piggott, B.J., Liu, J., Feng, Z., Wescott, S.A., and Xu, X.Z. (2011). The neural circuits and synaptic mechanisms underlying motor initiation in *C. elegans*. Cell *147*, 922-933.

Piper, M.D., Wong, R., Grandison, R.C., Bass, T.M., Martinez, P.M., and Partridge, L. (2010). Water-independent effects of dietary restriction in *Drosophila*. Proc Natl Acad Sci U S A *107*, E54-56; author reply E57.

Poon, P.C., Kuo, T.H., Linford, N.J., Roman, G., and Pletcher, S.D. (2010). Carbon dioxide sensing modulates lifespan and physiology in *Drosophila*. PLoS Biol *8*, e1000356.

Potter, C.J., Tasic, B., Russler, E.V., Liang, L., and Luo, L. (2010). The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. Cell *141*, 536-548.

Ramaekers, A., Magnenat, E., Marin, E.C., Gendre, N., Jefferis, G.S., Luo, L., and Stocker, R.F. (2005). Glomerular maps without cellular redundancy at successive levels of the *Drosophila* larval olfactory circuit. Curr Biol *15*, 982-992.
Ren, D., Navarro, B., Xu, H., Yue, L., Shi, Q., and Clapham, D.E. (2001). A prokaryotic voltage-gated sodium channel. Science *294*, 2372-2375.

Reyes-DelaTorre, A., Pena-Rangel, M., and Riesgo-Escovar, J. (2012). Carbohydrate metabolism in *Drosophila*: reliance on the disaccharide trehalose. Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology, Prof. Chuan-Fa Chang (Ed.)

Rezaval, C., Pavlou, H.J., Dornan, A.J., Chan, Y.B., Kravitz, E.A., and Goodwin, S.F. (2012). Neural circuitry underlying *Drosophila* female postmating behavioral responses. Curr Biol *22*, 1155-1165.

Rhea, J.M., Wegener, C., and Bender, M. (2010). The proprotein convertase encoded by *amontillado* (*amon*) is required in *Drosophila corpora cardiaca* endocrine cells producing the glucose regulatory hormone AKH. PLoS Genet *6*, e1000967.

Rizki, G., Iwata, T.N., Li, J., Riedel, C.G., Picard, C.L., Jan, M., Murphy, C.T., and Lee, S.S. (2011). The evolutionarily conserved longevity determinants HCF-1 and SIR-2.1/SIRT1 collaborate to regulate DAF-16/FOXO. PLoS Genet *7*, e1002235.

Robertson, H.M. (1998). Two large families of chemoreceptor genes in the nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae* reveal extensive gene duplication, diversification, movement, and intron loss. Genome Res *8*, 449-463.

Rollo, I., Williams, C., Gant, N., and Nute, M. (2008). The influence of carbohydrate mouth rinse on self-selected speeds during a 30-min treadmill run. Int J Sport Nutr Exerc Metab *18*, 585-600.

Roman, G., Endo, K., Zong, L., and Davis, R.L. (2001). P[Switch], a system for spatial and temporal control of gene expression in *Drosophila melanogaster*. Proc Natl Acad Sci U S A *98*, 12602-12607.

Rubin, J., Matsushita, K., Ballantyne, C.M., Hoogeveen, R., Coresh, J., and Selvin, E. (2012). Chronic hyperglycemia and subclinical myocardial injury. J Am Coll Cardiol *59*, 484-489.

Rulifson, E.J., Kim, S.K., and Nusse, R. (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science *296*, 1118-1120.

Satirapoj, B. (2012). Nephropathy in diabetes. Adv Exp Med Biol *771*, 107-122.

Sato, K., Pellegrino, M., Nakagawa, T., Vosshall, L.B., and Touhara, K. (2008). Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature *452*, 1002-1006.

Schepers, R.J., and Ringkamp, M. (2009). Thermoreceptors and thermosensitive afferents. Neurosci Biobehav Rev *33*, 205-212.

Shostal, O.A., and Moskalev, A.A. (2012). The genetic mechanisms of the influence of the light regime on the lifespan of *Drosophila melanogaster*. Front Genet *3*, 325.

Slack, C., Giannakou, M.E., Foley, A., Goss, M., and Partridge, L. (2011). dFOXO-independent effects of reduced insulin-like signaling in *Drosophila*. Aging Cell *10*, 735-748.

Slone, J., Daniels, J., and Amrein, H. (2007). Sugar receptors in *Drosophila*. Curr Biol *17*, 1809-1816.

Smith, E.D., Kaeberlein, T.L., Lydum, B.T., Sager, J., Welton, K.L., Kennedy, B.K., and Kaeberlein, M. (2008). Age- and calorie-independent life span extension from dietary restriction by bacterial deprivation in *Caenorhabditis elegans*. BMC Dev Biol *8*, 49.

Stanfel, M.N., Shamieh, L.S., Kaeberlein, M., and Kennedy, B.K. (2009). The TOR pathway comes of age. Biochim Biophys Acta *1790*, 1067-1074.

Steuerman, R., Shevah, O., and Laron, Z. (2011). Congenital IGF1 deficiency tends to confer protection against post-natal development of malignancies. Eur J Endocrinol *164*, 485-489.

Sukul, N.C., and Croll, N.A. (1978). Influence of potential difference and current on the electrotaxis of *Caenorhaditis elegans*. J Nematol *10*, 314-317.

Tatar, M., Bartke, A., and Antebi, A. (2003). The endocrine regulation of aging by insulin-like signals. Science *299*, 1346-1351.

Thistle, R., Cameron, P., Ghorayshi, A., Dennison, L., and Scott, K. (2012). Contact chemoreceptors mediate male-male repulsion and male-female attraction during *Drosophila* courtship. Cell *149*, 1140-1151.

Tissenbaum, H.A., and Guarente, L. (2001). Increased dosage of a *sir-2* gene extends lifespan in *Caenorhabditis elegans*. Nature *410*, 227-230.

Venken, K.J., Popodi, E., Holtzman, S.L., Schulze, K.L., Park, S., Carlson, J.W., Hoskins, R.A., Bellen, H.J., and Kaufman, T.C. (2010). A molecularly defined duplication set for the X chromosome of *Drosophila melanogaster*. Genetics *186*, 1111-1125.

Waterson, M.J., Chung, B.Y., Harvanek, Z.M., Ostojic, I., Alcedo, J., and Pletcher, S.D. (2014). The water sensor *ppk28* modulates *Drosophila* lifespan and physiology through AKH signaling. Proc Natl Acad Sci U S A (in press).

Watson, K.J., Kim, I., Baquero, A.F., Burks, C.A., Liu, L., and Gilbertson, T.A. (2007). Expression of aquaporin water channels in rat taste buds. Chem Senses *32*, 411-421.

White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. Philos Trans R Soc Lond B Biol Sci *314*, 1-340.

Whittaker, A.J., and Sternberg, P.W. (2004). Sensory processing by neural circuits in *Caenorhabditis elegans*. Curr Opin Neurobiol *14*, 450-456.

Williams, R.W. (2000). Mapping genes that modulate mouse brain development: a quantitative genetic approach. Results Probl Cell Differ *30*, 21-49.

Wu, Q., Clark, M.S., and Palmiter, R.D. (2012). Deciphering a neuronal circuit that mediates appetite. Nature *483*, 594-597.

Xiao, R., Zhang, B., Dong, Y., Gong, J., Xu, T., Liu, J., and Xu, X.Z. (2013). A genetic program promotes *C. elegans* longevity at cold temperatures via a thermosensitive TRP channel. Cell *152*, 806-817.

Yarmolinsky, D.A., Zuker, C.S., and Ryba, N.J. (2009). Common sense about taste: from mammals to insects. Cell *139*, 234-244.

Yates, S.K., and Brown, W.F. (1981). Light-stimulus-evoked blink reflex: methods, normal values, relation to other blink reflexes, and observations in multiple sclerosis. Neurology *31*, 272-281.