PHYSIOLOGY

Yeast volatiles double starvation survival in *Drosophila*

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Organisms make decisions based on the information they gather from their environment, the effects of which affect their fitness. Understanding how these interactions affect physiology may generate interventions that improve the length and quality of life. Here, we provide evidence that exposure to live yeast volatiles during starvation significantly extends survival, increases activity, and slows the rate of triacylglyceride (TAG) decline independent of canonical sensory perception. We demonstrate that ethanol (EtOH) is one of the active components in yeast volatiles that influences these phenotypes and that EtOH metabolites mediate dynamic mechanisms to promote *Drosophila* survival. Silencing R4d neurons reverses the ability of high EtOH concentrations to promote starvation survival, and their activation promotes EtOH metabolism. The transcription factor *foxo* promotes EtOH resistance, likely by protection from EtOH toxicity. Our results suggest that food-related cues recruit neural circuits and modulate stress signaling pathways to promote survival during starvation.

INTRODUCTION

In nature, the unpredictability of food in both time and space means that nearly all organisms will experience repeated periods of nutritional stress during their lifetime, with prolonged deprivation leading to debilitation and, eventually, death. Animals confront this challenge by altering behavior and physiology in an effort to increase their survival until new food resources are found. Strategies include modulating activity to balance foraging effort with energy utilization, reducing reproduction to conserve stored resources, and strengthening sensory perception to better detect new nutrient sources (*1*–*6*). Starvation resistance can, therefore, be considered an extreme form of adaptive physiology that is modulated continuously in response to external and internal cues that convey the expectation of finding food and the remaining energy available to do so.

While much has been written about physiological correlates of starvation resistance in many species, our understanding of the molecular mechanisms that coordinate the dynamic, integrated response during this challenge remains limited. For example, a causal relationship between starvation resistance and body lipid reserves is well documented in *Drosophila* (*7*), leading to the notion that starvation survival is primarily a function of the amount of stored fat [triacylglyceride (TAG)] at the time of nutrient deprivation (*8*). However, these conclusions are based on experiments conducted in constant laboratory conditions in which all indications of food are absent, thus overlooking many factors that may alter the response dynamics. Notably, even in these simplified conditions, genetic deficiencies in TAG mobilization can, in some cases, increase starvation resistance, which suggests that starving flies actively regulate energy mobilization and utilization during starvation in a way that does not simply maximize survival (*9*).

Sensory perception of food cues during starvation may be one important factor influencing survival. The smell of live yeast modulated activity and life span in *Drosophila*, and loss of the olfactory coreceptor *Orco*/*Or83b*, which renders flies broadly anosmic, increased life span and starvation resistance (*10*). Similar effects were observed in the nematode worm, *Caenorhabditis elegans* (*11*, *12*). The effects of live yeast on *Drosophila* are likely mediated, at least in part, by the perception of CO_2 released by active respiration, as loss of the CO_2 coreceptor *Gr63a* abrogated yeast effects and extended starvation resistance (*13*). Taste inputs are influential as well. The loss of water taste perception through deletion of the receptor *ppk28*, for example, resulted in enhanced starvation resistance (*14*). While most of these manipulations likely influenced starvation resistance by altering physiology before starvation itself (e.g., some of these manipulations also affected the amount of stored TAG), there is evidence that sensory perception during nutrient deprivation also has notable physiological effects. Sweet taste perception through neurons expressing gustatory receptors *Gr5a* and *Gr64* was necessary and sufficient for sleep fragmentation in low-nutrient environments, and this response was suppressed by activation of *Gr66a*-expressing bitter-sensing neurons (*15*). Last, gustatory perception of sweetness is both necessary and sufficient to suppress starvation-induced sleep loss, and gustatory perception during starvation influences survival (*15*). These data suggest that sensory manipulations during starvation may reveal previously unidentified behavioral and physiological mechanisms that influence starvation resistance.

The molecular mechanisms by which food cues influence physiology in peripheral tissues are varied, as are the functional neural networks required to transduce their effects. For example, the effects of gustatory perception of sweetness on starvation-induced behavioral changes required signal transduction in octopaminergic and dopaminergic neurons (*16*). Octopaminergic neural activity also correlated with fly starvation sensitivity through the regulation of the adipokinetic hormone receptor (*AkhR*) (*17*). Starvation-induced sleep loss in *Drosophila* required octopaminergic neurons, adipokinetic hormone, and the insulin-responsive transcription factor *foxo*, which influenced the remodeling of small ventral lateral neuron dorsal projections upon nutrient deprivation (*18*). Other neuronal populations are known to be activated in response to starvation, including *corazonin*-expressing neurons, serotonergic neurons, and ellipsoid body R4 neurons (*19*). However, whether these neurons are involved in the sensory perception of food cues or in general behavioral or physiological responses to starvation remains unknown.

In this study, we sought to identify neural and peripheral mechanisms through which survival is acutely modulated during nutrient deprivation in response to perceived information about the environment.

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We found that indirect exposure to live yeast volatiles during nutrient deprivation doubled the starvation survival of *Drosophila*, independent of changes in activity. This effect did not involve sensory perception through canonical sensory systems. A similar, but smaller effect was observed when flies were indirectly exposed to low concentrations of ethanol (EtOH) vapor, which is one volatile produced by live yeast. Increased starvation resistance required enzymes that degrade EtOH as well as the transcription factor *foxo* and the activity of a small subset of ellipsoid body neurons (the R4d subset), which are likely involved in EtOH detoxification and metabolism, respectively. Our data suggest that metabolites from internalized, food-based volatiles may be used both as energy sources and as neuronal signaling molecules to modulate survival and metabolism while an organism is under extreme nutrient stress. A deeper understanding of the ecological factors and biological processes that influence starvation resistance is broadly relevant in *Drosophila* and other species, where it is often associated with increased body size, extended longevity, reduced reproduction, and delayed larval growth, suggesting the possibility that these traits share underlying mechanisms (*20*, *21*).

RESULTS

Exposure to volatiles from live yeast significantly increases starvation survival

To investigate the effects of food-related odorants on *Drosophila* physiology, we developed a simple and robust indirect exposure paradigm to semiautonomously monitor fly starvation resistance and activity. We placed the TriKinetics DAM2 platform in a closed environment that contained environmental compounds from which the flies were physically separated but were putatively able to see and smell (fig. S1A). We found that indirect yeast exposure of either female or male Canton-S flies more than doubled the mean starvation survival time compared with unexposed flies (Fig. 1, A and B). Indirect exposure to killed yeast had no effect on starvation survival (fig. S1B), and yeast colonies were never observed on standard yeast extract, peptone, and dextrose (YPD) plates placed outside of the culture flask, ruling out the possibility that yeast cells were traveling into the activity tubes where the flies could ingest them to obtain nutrients. These patterns of survivorship were also observed in replicate experiments involving both male and female flies from another laboratory stock, demonstrating that the phenotype was robust (fig. S2, A and B).

One plausible hypothesis to explain our results posits that indirect live yeast exposure triggers a reduction in activity to slow the rate at which stored energy is depleted during the starvation period. To explore this hypothesis, we examined the activity of individual flies throughout their survival. Visual examination of event-history plots, which display the relationship between cohort survival and individual-level activity for each treatment, indicated that activity was not decreased and was instead perhaps increased when flies were indirectly exposed to live yeast (fig. S2, C to E). It was also apparent that the longest-lived flies did not exhibit activity patterns that were qualitatively different from short-lived flies in either cohort. Detailed follow-up experiments revealed that indirect yeast exposure significantly increased activity during the first 24 to 36 hours after exposure, which is before the beginning of starvation-induced death (Fig. 1C and fig. S2F). In addition, starvation-induced sleep loss was not different in the first 36 hours of starvation and was modestly reduced in exposed flies during the subsequent 12 hours (Fig. 1D). Last, we examined the effect of sweet taste on these phenotypes because it has been shown to reverse starvation-induced behavioral changes in the absence of nutrient consumption (*16*). We found that the addition of sweet but noncaloric arabinose to the agar during starvation eliminated the acute burst of activity but had no impact on the increased starvation resistance following indirect live yeast exposure (Fig. 1, E and F). We concluded from these data that indirect exposure to live yeast produces a transient burst of activity that is not required for long-term improvements in starvation resistance, the latter of which likely results from physiological changes induced by one or more yeast volatiles.

Effects of indirect exposure to live yeast are independent of canonical sensory perception

We next sought to determine whether the observed effects of indirect, live yeast exposure were mediated by sight, smell, or taste. Each of these sensory modalities is known to influence aging, physiology, and/or starvation resistance (*10*, *11*, *17*, *18*). Smell-blind flies that lack *Orco*, *Ir25a*, *Ir8a*, and *Gr63a* receptors exhibited a similar increase in starvation resistance when exposed to yeast volatiles compared with the control animals (Fig. 2A), as did flies with their antenna and maxillary palps removed (Fig. 2B). Visually blind flies, created by expressing the proapoptotic gene *hid* in glass multimer reporter (GMR)-expressing cells, exhibited a normal increase in starvation resistance upon indirect exposure to live yeast, establishing that sight is not required for the survivorship effect (Fig. 2C). Taste-blind *Pox-neuro* (*Poxn*) mutant flies, in which most chemosensory bristles were transformed into mechanosensory bristles, also showed significantly increased starvation resistance when indirectly exposed to live yeast (Fig. 2D). Furthermore, neither the water sensor, *ppk28*, nor the CO_2 coreceptor, *Gr63a*, was required for improvements in starvation survival upon yeast volatile exposure (Fig. 2, E and F). Visual inspection of these plots suggested that some of these sensory manipulations may reduce the magnitude of the response, but this was not supported by statistical analyses, which reveal no significant genotype × treatment interactions. This may be due to reduced power for this test. In contrast to control animals, loss of olfaction significantly reduced activity during the first 24 hours after yeast exposure, while manipulations of taste and sight did not significantly affect the activity response (table S1). These data reinforced the notion that live yeast volatiles affect starvation survival and activity independently, and they suggested that smell is required for the burst of activity following exposure to live yeast volatiles but that none of the canonical sensory perception modalities are involved in mediating their effect on starvation resistance.

EtOH is an active component of yeast volatiles that increases starvation resistance

Yeast fermentation produces at least 270 volatiles, including a variety of alcohols, acids, and esters, as well as water and CO_2 (*22*). We determined that the live yeast solution to which flies were indirectly exposed increased humidity and CO_2 in the exposure chamber over a 24-hour period (table S2). Although sensory perception of water and CO_2 was ruled out, we nevertheless wondered whether they could be acting more directly on organismal physiology. To explore this idea, we first removed both water and CO_2 from yeast volatiles by passing exposed air through a column containing both the CO_2 absorbent, Ascarite (II), and the desiccant, Drierite, before delivery into the starvation chamber. Yeast volatiles treated in this manner

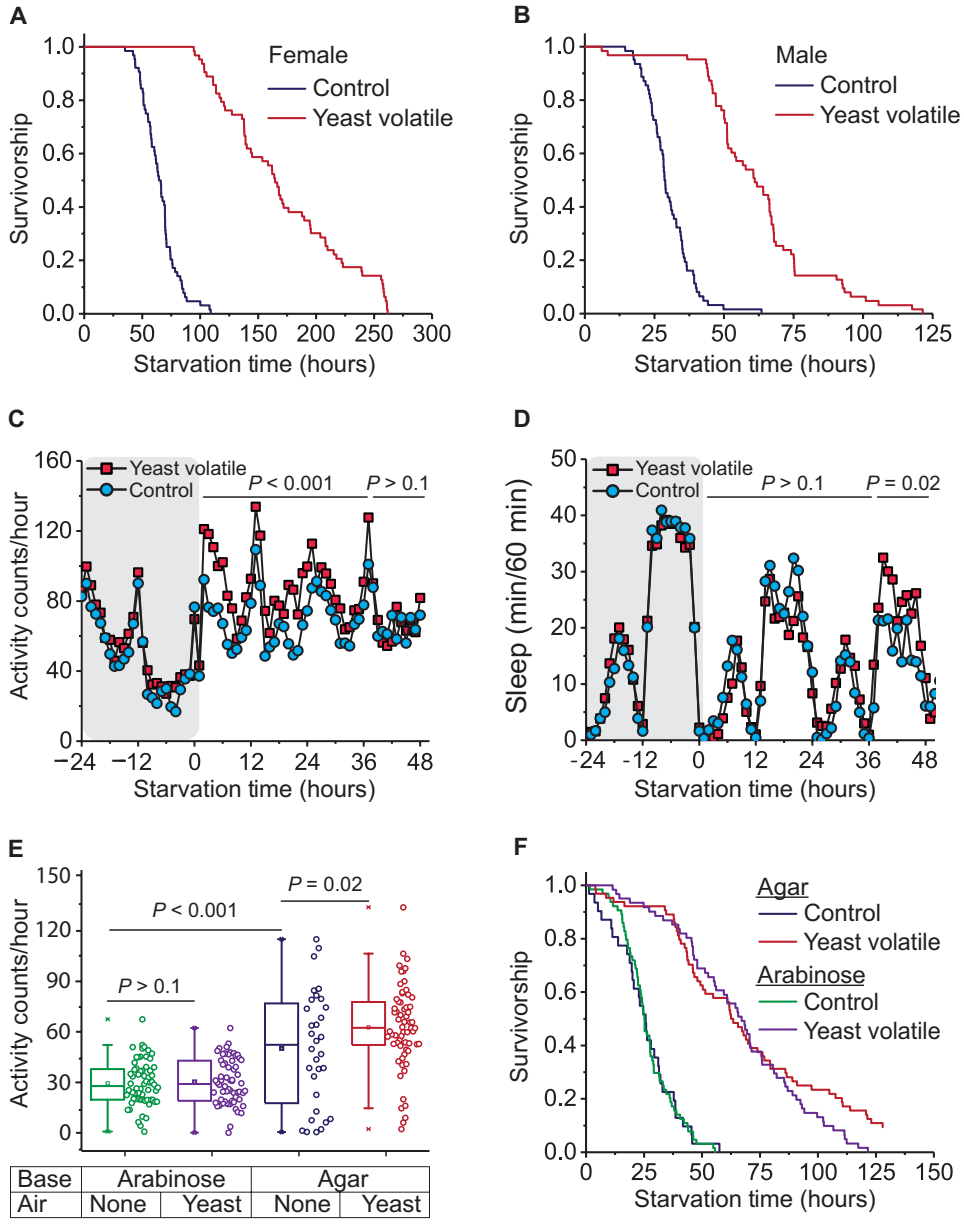

Fig. 1. Indirect exposure to live yeast volatiles doubled starvation survival. (**A** and **B**) Indirect exposure to live yeast volatiles significantly increased starvation survival of Canton-S female (A) and male (B) flies; $n = 64$ flies for each treatment and $P < 0.001$ using a log-rank test. (**C**) Indirect exposure to live yeast volatiles induced an acute and transient increase in activity over a 48-hour exposure period; $n = 32$ female flies for each treatment and $P < 0.001$ or $P > 0.1$ using a t test to compare the total activity between 0 and 36 hours and between 36 and 48 hours, respectively. (**D**) Exposure to yeast volatiles reduced sleep loss later in the starvation period; $n = 32$ female flies for each treatment and $P > 0.1$ or $P = 0.02$ using a t test to compare between 0 and 36 hours and between 36 and 48 hours, respectively. The shaded region in (C) and (D) is fly activity before starvation and exposure to the yeast volatiles. (**E** and **F**) Taste of 100 mM sweet, noncaloric arabinose in agar-suppressed starvation-induced hyperactivity over a 36-hour period (E) but did not affect starvation resistance (F). (E) $n = 64$ female flies for each treatment, and a t test was used for analysis. (F) $n = 64$ female flies for each treatment, and $P > 0.1$ when comparing the yeast volatile plus or minus arabinose using log-rank analysis.

failed to prolong starvation survival (Fig. 3A), suggesting that CO_2, water, and/or a water-soluble compound(s) are required for enhanced starvation resistance. Removing humidity alone significantly reduced, but did not eliminate, the effects of indirect yeast exposure on starvation resistance (Fig. 3B). Last, changes in humidity alone or CO_2 alone were not sufficient to modulate starvation resistance (Fig. 3, C and D). We concluded that while changes in relative humidity and CO_2 were not directly causing changes in starvation survival, one or both may potentiate the effect, likely by influencing the availability of active compounds (*23*).

We next examined other candidate molecules emitted by live yeast. We found that indirect exposure of flies to 2.5% acetic acid, 5% 1-butanol, 5% isopropanol, or 0.3% propionic acid had no effect on starvation-induced survivorship (Fig. 4A and fig. S3A). Indirect exposure to 2.5% EtOH, however, significantly increased starvation survival (Fig. 4A). Follow-up experiments revealed a concentration-dependent

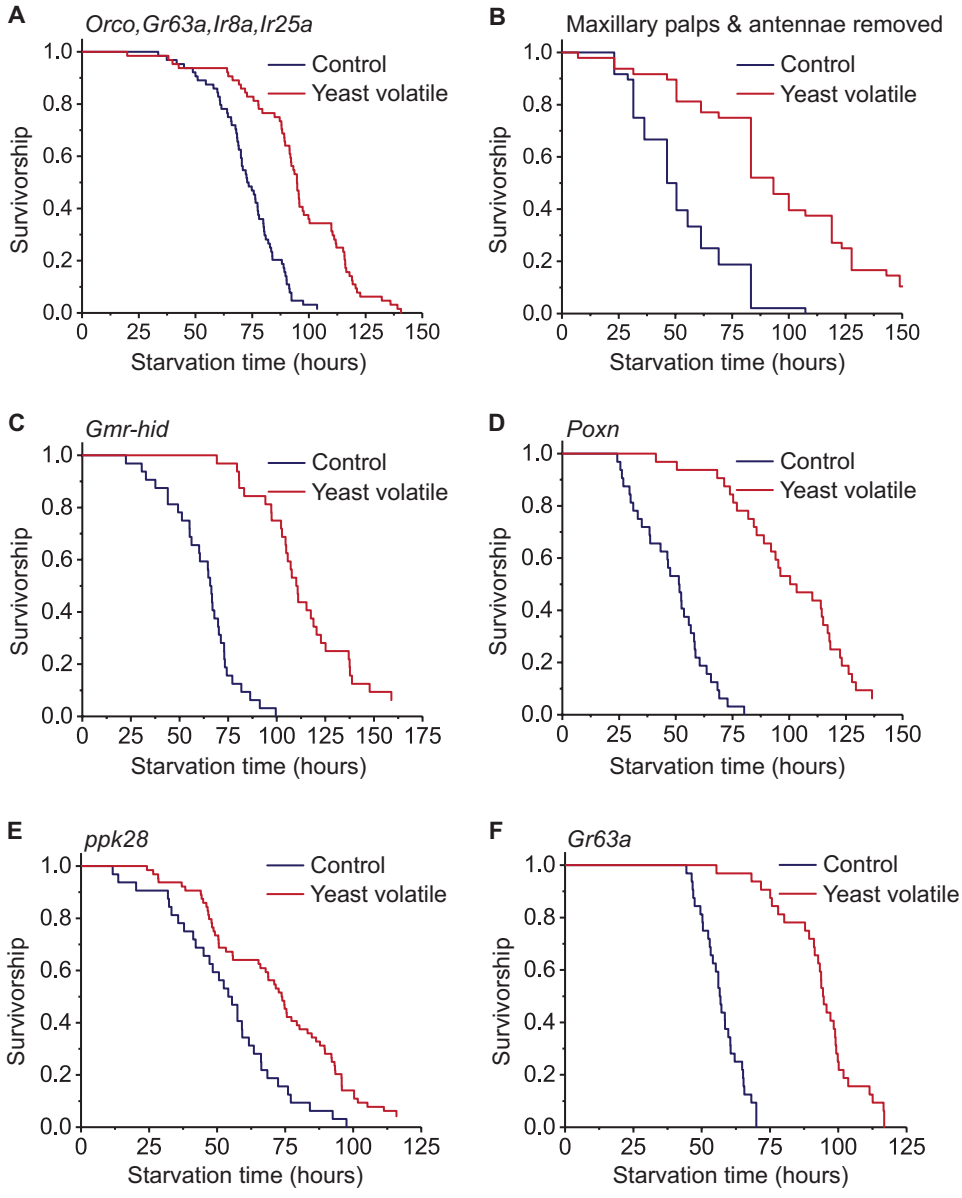

Fig. 2. Canonical sensory perception pathways were not required for the effect of yeast volatiles on starvation resistance. (**A** to **D**) Smell-blind (A), flies with antenna and maxillary palps removed (B), vision-blind (C), or taste-blind flies (D) showed significantly increased starvation resistance upon yeast volatile exposure. (**E** and **F**) Flies lacking the *ppk28* water receptor (E) or the CO_2 coreceptor, *Gr63a* (F), also displayed significantly increased starvation resistance upon yeast volatile exposure. In each experiment, $n = 64$ female flies for each treatment, and $P < 0.001$ when comparing control and yeast volatile treatments via log-rank analysis.

effect of indirect EtOH exposure where starvation survival was maximized at a concentration near 10% and not further increased at 20% (Fig. 4B). After reflecting on previous survival data (e.g., Fig. 3, A and B), we established that our CO_2 removal column also eliminated the measurable EtOH produced by live yeast and that our process of removing humidity alone significantly reduced it (Fig. 4C), which is consistent with EtOH as an active component of yeast volatiles with respect to starvation survival. Indirect exposure to live yeast extended starvation survival to roughly the same magnitude as did exposure to 10% EtOH, and simultaneous exposure to both did not further extend survival (fig. S3B), which is also consistent with this idea. Notably, exposure to live yeast decreased the starvation survival of flies exposed to 20% EtOH (fig. S3B), indicating that higher levels of EtOH exposure may be deleterious, an observation that was confirmed in subsequent experiments (fig. S3C).

We found that the magnitude of induced starvation resistance among treatments did not reflect a simple relationship with the amount of EtOH vapor in the air, suggesting some complexity in the response. Relative to untreated air in the chamber, there was ~1.8-fold more EtOH found in the air from chambers with yeast volatiles, while there was 2.2-, 4.3-, and 6.8-fold more EtOH in the air of 5, 10, and 20% EtOH solutions, respectively (Fig. 4D). Thus, yeast volatiles alone increased starvation resistance significantly more than 5% EtOH exposure and to the same extent as both 10 and 20% despite producing less than one-third the amount of airborne

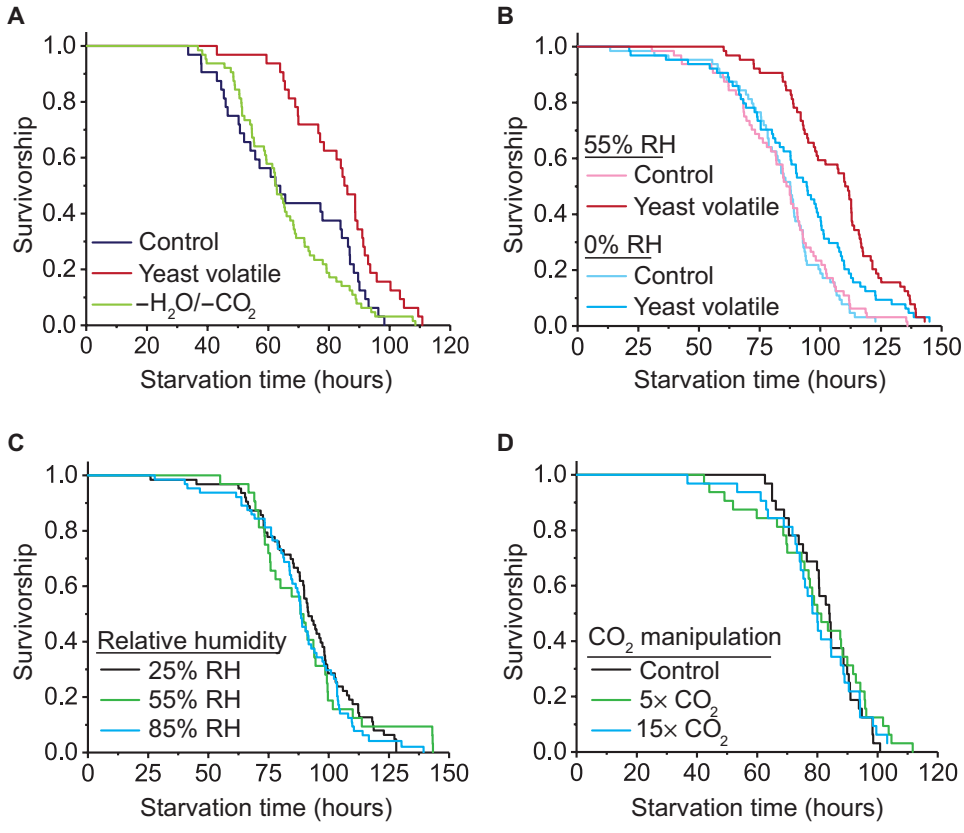

Fig. 3. Humidity and CO_2 may be required but were not sufficient to alter starvation resistance when exposed to yeast volatiles. (**A**) Yeast volatiles with water and CO_2 removed failed to prolong starvation resistance. Using log-rank analysis, $P > 0.1$ when comparing the control to $–H_2O/–CO_2$, and $P < 0.001$ when comparing the control to yeast volatile or $–H_2O/–CO_2$ to yeast volatile exposure. (**B**) Removing humidity alone reduced the effects of yeast volatiles on starvation resistance. Using log-rank analysis, $P = 0.01$ when comparing the control to yeast volatiles at 0% relative humidity (RH), and $P < 0.001$ comparing the control to yeast volatiles at 55% RH. (**C** and **D**) Manipulating humidity (C) or CO_2 levels (D) alone in the experimental chamber was not sufficient to alter starvation resistance. Using log-rank analysis, $P > 0.1$ when comparing between treatments. In each experiment shown, $n = 64$ female flies per treatment type.

EtOH. The EtOH concentration in the fly exhibited a similar relationship: Flies indirectly exposed to 5 and 20% EtOH had significantly more internal EtOH compared with flies exposed to yeast volatiles (Fig. 4E). Together these data suggest that EtOH is a key component of live yeast volatiles that increases starvation resistance of *Drosophila* upon indirect exposure. The amount of EtOH found inside and outside the flies does not associate with the magnitude of starvation resistance, suggesting that its effectiveness is potentiated by unknown factors in yeast volatiles and may be inhibited at higher concentrations.

It is known that fed flies exhibit a mild preference for low concentrations of EtOH when mixed with food odors (*24*). We therefore asked whether flies in our experimental conditions found higher concentrations of pure EtOH vapor attractive. Using a binary T-maze apparatus (*25*), we found that starved flies exhibited a modest positive preference index (PI) that was significantly greater than 0 ($P = 0.04$; fig. S3D), while fed flies did not (consistent with previously published data for this concentration; $P = 0.30$). Second, we asked whether starved flies exhibited different activity levels upon exposure to EtOH vapor compared with fed control animals and found that they were more active for up to 12 hours immediately following EtOH exposure ($P = 0.028$; fig. S3E). Together, these data suggest that starvation may sensitize flies to interpret EtOH vapor as indicating nearby food sources, which may lead to physiological changes that increase activity and enhance survival in the hope of locating such resources.

EtOH metabolism is required for the effects of yeast volatiles on survivorship

To better characterize the molecular mechanisms by which yeast volatiles and EtOH increase starvation survivorship, we performed a targeted screen of genes known to be involved in putatively relevant physiological processes, including fat metabolism, EtOH metabolism, and stress resistance. Genes involved in fat metabolism were of interest because our initial characterization suggested that TAG levels were significantly higher in starved flies exposed to live yeast volatiles for 48 hours or longer relative to unexposed control animals (fig. S4A). With this in mind, we tested whether genes involved in TAG catabolism were required, including adipokinetic hormone (*Akh*), its receptor (*AkhR*), and triacylglycerol lipase (*bmm*), and none were (fig. S4, B to D, and table S3). We next tested whether genes involved in EtOH metabolism were involved. Loss of either gene that encodes the essential alcohol metabolism enzymes, alcohol dehydrogenases (*Adh*) or aldehyde dehydrogenase (*Aldh*), eliminated the effect of live yeast volatiles and 10% EtOH exposure on starvation survival (Fig. 5, A and B, and table S3) and sensitized

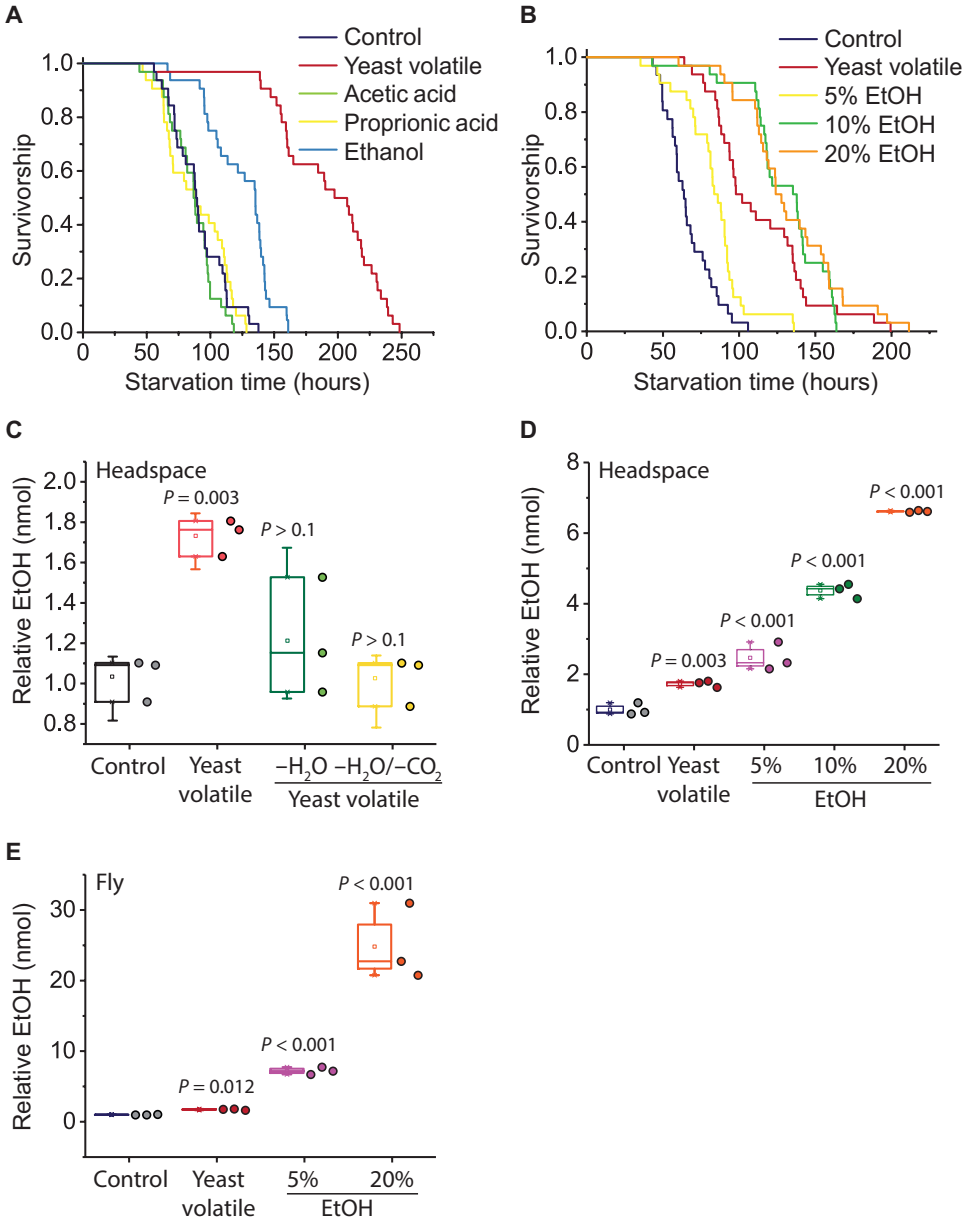

Fig. 4. EtOH is an active component found in yeast volatiles that increased starvation resistance. (**A**) Indirect exposure to 2.5% EtOH significantly increased starvation survival, but exposure to 5% acetic acid or 0.3% propionic acid did not. In this experiment, $n = 64$ female flies for each treatment. Using a log-rank analysis, $P = 0.003$ when comparing the control to 2.5% EtOH, and $P < 0.001$ when comparing the control to yeast volatiles, or $P > 0.1$ when comparing the control to 5% acetic acid or 0.3% propionic acid. (**B**) The effects of EtOH exposure are concentration dependent ($n = 64$ female flies per treatment). Using a log-rank analysis, $P < 0.001$ when comparing the control to each treatment, and $P > 0.1$ when comparing the 10% EtOH to the 20% EtOH exposure groups. (**C**) Yeast volatiles with water removed or both water and CO_2 removed contain less EtOH. In this experiment, $n = 3$ biological replicates, and a *t* test was used to compare the control to each treatment. (**D**) Relative EtOH levels in the air of live yeast or EtOH solutions. In this experiment, $n = 3$ biological replicates, and a *t* test was used to compare the control to each treatment. (**E**) Relative EtOH levels inside flies that were exposed to yeast volatiles or EtOH solution for 24 hours. Here, $N = 3$ biological replicates of 20 female flies in each treatment, and a t test was used to compare the control to each treatment.

flies to 20% EtOH exposure, to which they were significantly shorter-lived. We also tested genes that have been implicated in general stress resistance and aging, including silent information regulators (Sirtuins), target of rapamycin(TOR), insulin/insulin growth-like factor (IGF) signaling, and forkhead box transcription factors (FOXOs). While insulin-like peptides 2, 3, and 5 were not required for EtOH or yeast volatiles to increase starvation survival (table S3; see $dIlp^{2,3,5}$ mutant phenotype), we found that flies carrying a putative null mutation of the insulin-responsive transcription factor *foxo* ($foxo^{\Delta 94}$) exhibited a significantly reduced effect of indirect 10% EtOH exposure and were sensitized to 20% EtOH, similar to loss of *Adh/Aldh*. Unexpectedly, however, live yeast volatiles effectively increased

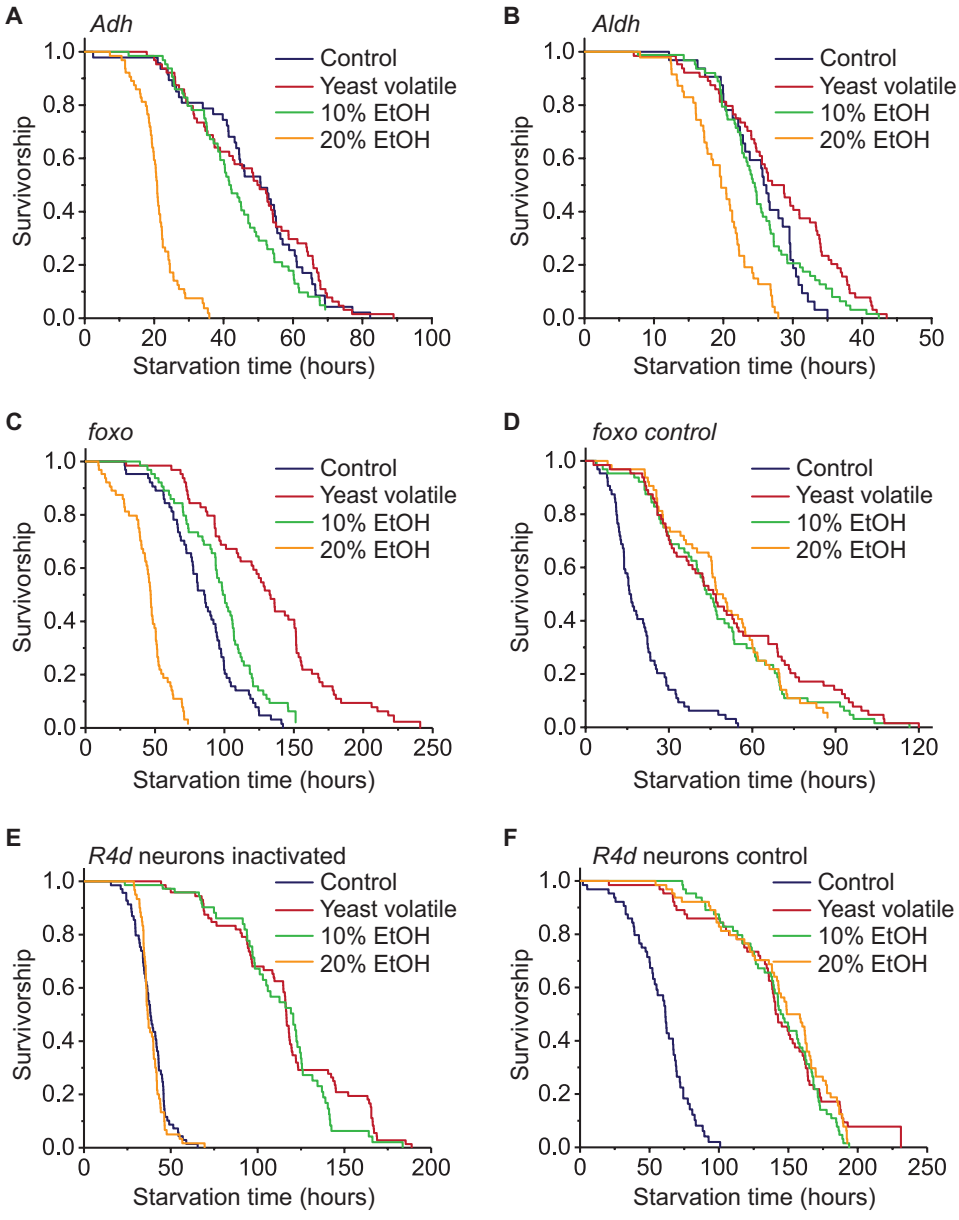

Fig. 5. EtOH-metabolizing genes, the transcription factor *foxo*, and R4d ring neurons were required for the ability of yeast and/or EtOH to affect starvation resistance. (**A** and **B**) Starvation survival of flies lacking alcohol dehydrogenase, *Adh* (A), or aldehyde dehydrogenase, *Aldh* (B), was unaffected by yeast volatiles and indirect exposure to 10% EtOH and was reduced by indirect exposure to 20% EtOH. Using log-rank analysis, $P > 0.1$ when comparing the control to yeast volatiles or 10% EtOH, and $P < 0.001$ when comparing the control to 20% EtOH. (**C**) *foxo* mutant flies exhibited an altered starvation response upon indirect exposure to EtOH but an extension of survival upon exposure to yeast volatiles that was similar to control flies. Using log-rank analysis, $P = 0.004$ when comparing the control to 10% EtOH, and $P < 0.001$ when comparing the control to 20% EtOH or yeast volatiles. (**D**) The genetic control of *foxo* mutant flies displayed significantly increased starvation resistance when exposed to yeast volatiles or EtOH. Using a log-rank analysis, $P < 0.001$ when comparing the control to yeast or EtOH groups. (**E**) Inactivation of R4d ring neurons eliminated the effect of 20% EtOH exposure but had no effect on enhanced starvation survivorship upon indirect exposure to 10% EtOH or to yeast volatiles. Using a log-rank analysis, $P > 0.1$ when comparing the control to 20% EtOH, and $P < 0.001$ when comparing the control to yeast volatiles or 10% EtOH. (**F**) Control flies that contain the R4d-Gal4 construct alone displayed significantly increased starvation resistance upon indirect yeast or EtOH exposure. Using a log-rank analysis, $P < 0.001$ comparing the control to yeast volatile or EtOH groups. In all panels, $n = 64$ female flies for each treatment.

starvation survival in *foxo* mutant flies, suggesting that yeast and EtOH effects are, at least in part, mediated by distinct molecular mechanisms (Fig. 5, C and D).

We next focused on neural regulators that have been reported to regulate starvation resistance or other stress responses. We found that loss of neuropeptide F (NPF) (NPF^{sk2} mutants), ablation of corazonin-expression cells (*Crz*-Gal4 × UAS-*Rpr*), loss of octopamine biosynthesis (Tbh^{nM18} mutants), loss of serotonin biosynthesis (Trh^{C01440} mutants or serotonin receptor mutants), and loss of specific dopaminergic receptors (*DopEcR* and *Dop1R1* mutants) all

failed to abrogate the ability of yeast odorants or EtOH to increase starvation survival (table S3). We then concentrated on neuronal function in the central complex of the brain because it has been reported to be involved in mediating behavioral and physiological responses to starvation or EtOH (*26*–*28*). Inactivating broader subsets of ring neurons in the ellipsoid body or selectively inhibiting three of the six total ring neural subsets did not eliminate the effects of EtOH vapor or yeast volatile exposure on starvation survivorship (fig. S5 and table S3). Targeted inhibition of one specific subset of ring neurons, termed R4d neurons (*29*), reversed the effects of 20% EtOH on starvation survival but had no effect on the extension induced by 10% EtOH or by live yeast volatiles (Fig. 5, E and F).

R4d ring neurons regulate the rate of EtOH metabolism, while *foxo* regulates stress responses to high EtOH concentrations

Given the results thus far, it seemed reasonable to consider the possibility that flies were directly receiving energy from the EtOH vapor. In other words, perhaps the flies were simply "eating the air" and were deriving sufficient calories from ingestion to prolong starvation survival. The observation that EtOH metabolism through *Adh* and *Aldh* was required for the beneficial effects was consistent with this idea. However, we also noted a marked lack of correspondence between the amount of EtOH in the surrounding air and the magnitude of starvation life-span extension. This was perplexing because a simple prediction of a consumption-only model is that the higher the EtOH in the air, the more potential food and, thus, the greater the starvation survival. Yet, live yeast volatiles were far more effective than this model would predict based on EtOH concentration alone (which equated to indirect exposure to a 2% EtOH solution; Fig. 4E), and indirect exposure to 20% EtOH did not further prolong starvation in wild-type flies compared with 10% EtOH. To accommodate these observations, we further posited that EtOH-derived metabolites at low concentrations may also serve as signaling molecules to promote physiological changes that extend survival and that non-EtOH components of live yeast volatiles might serve to modulate EtOH effects, either through metabolism or signaling. On the other hand, high concentrations of unmetabolized EtOH may be sufficiently toxic to kill flies before starvation, as evidenced by the sensitivity of *Adh*/*Aldh* mutants to indirect 20% EtOH exposure and the higher internal EtOH concentrations in these mutant flies (fig. S6A).

To explore the likelihood that EtOH and yeast volatiles also act to modulate metabolic pathways, we asked whether inactivation of R4d neurons or loss of *foxo* affected the ability of flies to metabolize EtOH. By measuring the internal EtOH concentrations in the fly at various times following exposure to a high-concentration vapor, we found that inactivation of R4d neurons significantly slowed EtOH clearance, while loss of *foxo* and exposure to yeast volatiles had no effect (Fig. 6, A to C). As expected, this resulted in higher steady-state EtOH levels and enhanced expression of *Adh* in flies with inactivated R4d neurons but not in *foxo* mutant flies (Fig. 6D and fig. S6, B and C). Activation of R4d ring neurons using transgenic expression of a temperature-sensitive, transient receptor potential cation channel (TRPA1) was not sufficient to prolong starvation survival when EtOH was absent but was sufficient to further enhance starvation survival following indirect exposure to 20% EtOH (Fig. 6, E and F). Together, these data suggested that R4d ring neuron activation potentiated the rate of EtOH clearance and, thus, increased the effects of EtOH vapor exposure, either through signaling or by harvesting energy from the air. On the other hand, loss of R4d neuron function significantly reduced EtOH clearance, thereby promoting accumulation of internal EtOH concentrations whose toxicity at high concentrations might effectively reverse the beneficial effects of EtOH on starvation survival.

Given that loss of *foxo* had no effect on EtOH clearance (Fig. 6B) and that *foxo* mutant flies exhibited sensitivity to EtOH exposure (Fig. 5C) without changes in internal EtOH levels (fig. S6B), we postulated that this transcription factor might instead mediate important detoxification processes or resistance to high internal EtOH, independent of R4d function. Consistent with this interpretation, we found that RNA interference (RNAi)–mediated knockdown of *foxo* in R4d neurons had no effect on the ability of yeast volatiles, 10% EtOH, or 20% EtOH to increase starvation survival (fig. S7). We also quantified the expression of several *foxo* target genes, including the oxidative stress response gene *Catalase* (*Cat*), antimicrobial peptides *Drosmycin* (*Dros*) and *Diptericin* (*Dipt*), and autophagy-regulation genes *Atg7* and *Atg16*. Abundances of *Cat*, *Dros*, *Atg7*, and *Atg16* mRNA were unchanged by indirect exposure to EtOH or live yeast volatiles, while expression of *Dipt* trended higher in flies from both treatments (Fig. 7A). Notably, up-regulation of *Dipt* was completely *foxo* dependent (Fig. 7B), suggesting that immune response genes may contribute to EtOH resistance (*30*) and/or that they represent markers of *foxo* activity (*31*). It should be noted that loss of canonical immune response mediators, including the predominant nuclear factor κB transcription factors in adult flies (*Rel* and *Dif*), had no effect on starvation survival following indirect exposure to EtOH or live yeast volatiles (fig. S8), suggesting that the latter interpretation is likely correct, as has been shown in other contexts (*31*).

DISCUSSION

In seeking to identify mechanisms through which *Drosophila* use airborne food cues to stimulate adaptations to food scarcity, we found that exposure to live yeast volatiles significantly extended survival, increased activity, and slowed the rate of TAG decline during prolonged periods of starvation. We have concluded that the effect of yeast volatiles on starvation survival is independent of sight, smell, and taste, indicating that the mechanisms through which food cues modulate starvation survival are distinct from those that direct their effects on life span and physiology in nutrient-replete or modestly restricted conditions (*10*–*13*). It is important to keep in mind, however, that our starvation experiments were designed to detect major effects with high probability at the possible expense of missing manipulations with small effect. It may be that much larger experiments or simultaneous loss of multiple sensory inputs provide additional insight. The acute increase in activity that starved animals exhibited upon indirect exposure to yeast was dependent on olfaction and was reversed by sweet but nonnutritious, taste inputs, while survival was not, indicating that behavioral and physiological responses to starvation use different mechanisms. To our knowledge, little is known about the effects of food cues on starvation survival in other species, but at least some responses are conserved across taxa. For example, the smell of food causes hungry mice to become more active and to slow energy mobilization (*32*, *33*). The ability of food cues to trigger behavioral changes and, independently, to alter energy metabolism under starvation conditions in a manner that promotes directed foraging and survival is likely to favor successful discovery of new nutritional resources.

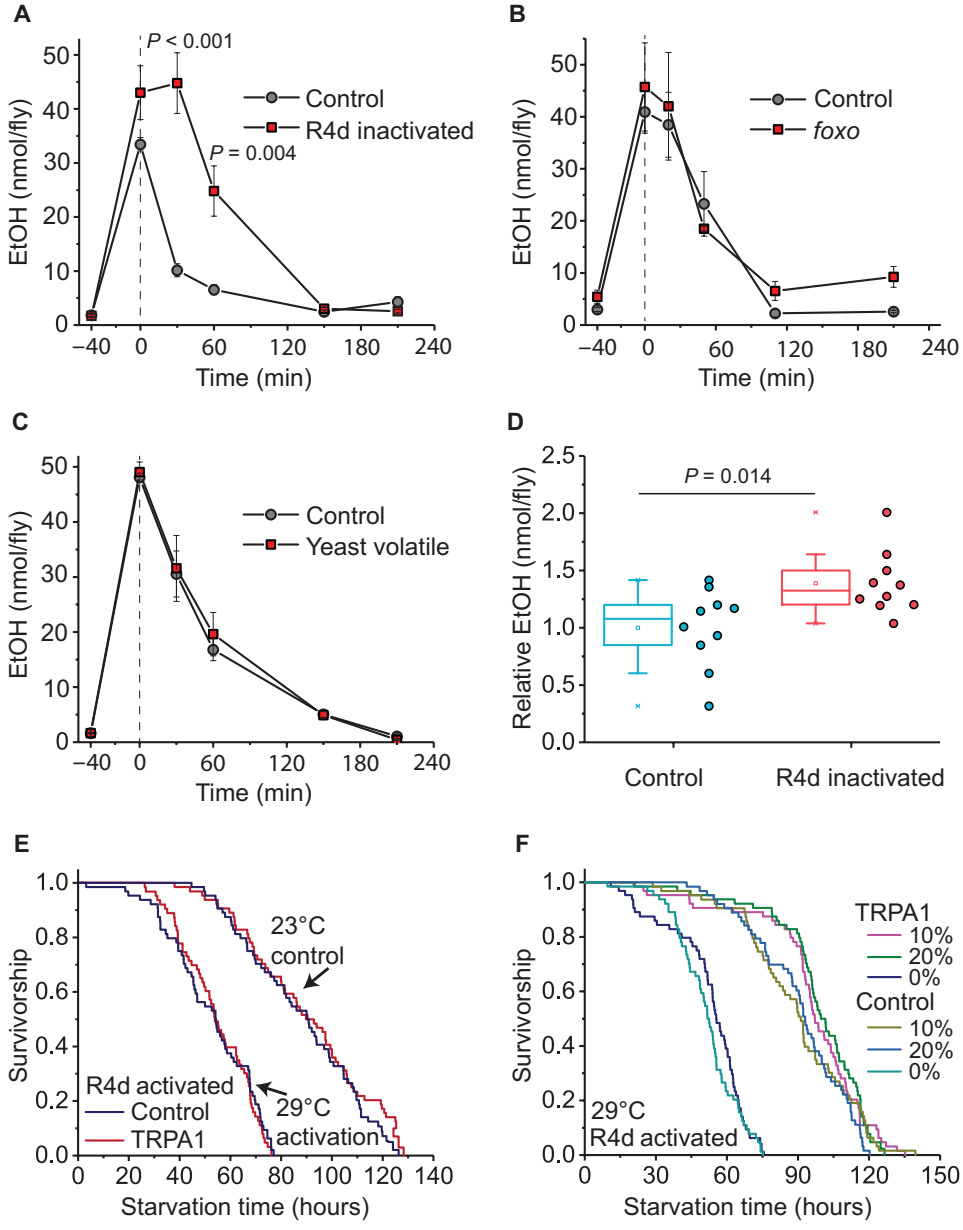

Fig. 6. R4d ring neurons modulated EtOH metabolism and potentiated starvation survival upon indirect EtOH exposure. (**A**) Inactivation of R4d neurons reduced the rate at which internal EtOH was cleared following acute exposure. (**B** and **C**) Neither loss of *foxo* nor yeast volatiles had a significant effect on the rate of EtOH clearance following acute exposure. In (A) to (C), $N = 4$ replicates of 20 female flies in each treatment at each time point, and a *t* test was used to compare EtOH measures between control and R4d inactivated, *foxo*, or yeast volatile at each time point. The dash lines indicate the time to remove EtOH exposure. (**D**) Flies with R4d neurons inactivated exhibited higher internal EtOH abundance after being indirectly exposed to 20% EtOH for 24 hours. In this experiment, $N = 10$ replicates of 20 female flies in each treatment, and a *t* test was used for the analysis. (**E**) Activation of R4d neurons did not prolong starvation resistance in the absence of EtOH; $n = 64$ female flies for each treatment, and a log-rank analysis demonstrated that $P > 0.1$ when comparing the control to TRPA1 at each temperature. (**F**) Activation of R4d neurons enhanced starvation resistance when 20% EtOH was present ($n = 64$ female flies for each treatment). Using a log-rank analysis, $P > 0.1$ when comparing the control flies to TRPA1 flies when EtOH was absent or at 10%, and $P = 0.005$ when comparing the control flies to TRPA1 flies when EtOH was 20%.

Our data suggest that at least one active component of yeast volatiles is EtOH. When flies were indirectly exposed to a dilute EtOH solution that was placed in a beaker alongside starvation chambers, survival was increased significantly. The effect was concentration dependent, with maximum effect achieved when flies were exposed to a 10% EtOH solution; no further increase was observed when a 20% EtOH solution was used. We are currently unable to distinguish whether airborne EtOH is being used directly for energy/adenosine 5′-triphosphate (ATP) production and/or whether certain EtOH metabolites act as signaling molecules to promote broader metabolic changes that enhance starvation resistance per se. In the nematode worm, *C. elegans*, ingestion of low levels of EtOH (1 mM concentration in medium) extended life span in L1 arrested animals (*34*). While formally possible, it does seem remarkable that the amount

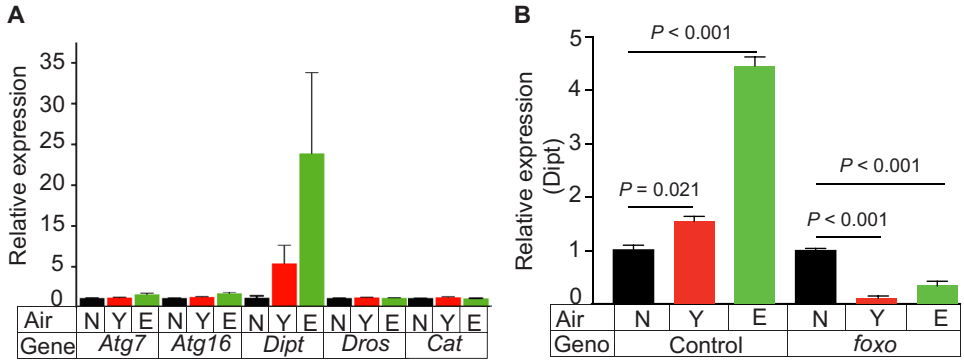

Fig. 7. Expression of the antimicrobial peptide, *Diptericin*, was increased in a *foxo*-dependent manner when flies were indirectly exposed to yeast or EtOH. (**A**) Indirect exposure of flies to 20% EtOH trended toward increased *Diptericin* (*Dipt*) mRNA expression in wild-type flies. (**B**) *Dipt* mRNA was significantly increased in control flies and depended on the presence of *foxo*. (A and B) N = no exposure, Y = yeast exposure, and E = 20% EtOH exposure. In both panels, *N* = 3 or 4 biological replicates of 20 female flies in each treatment. (A) The relative expression of each gene was normalized to its expression in the no exposure flies, and *t* tests were used to compare the no exposure group to the yeast or EtOH exposed groups. (B) The relative expression of *Dipt* was normalized to its expression in the no exposure group, and *t* tests were used to compare the no exposure group to yeast or EtOH exposed groups.

of EtOH flies might absorb from the air would provide sufficient calories to double starvation survival. Even if this were the case, however, it seems likely that additional components of yeast volatiles may also be important because the magnitude of their effect on life span greatly exceeded that predicted based on EtOH levels alone, which was empirically equivalent to indirect exposure to a 2% EtOH solution that only marginally increased starvation survival. EtOH metabolism, however, was required in all cases, suggesting that yeast volatiles somehow gate or potentiate EtOH effects. Similar relationships between metabolism and food cues have been described in other *Drosophila* species. Acetone and methanol, for example, have been shown to extend life span of *Drosophila mojavensis* only when yeast is present (*35*).

An alternative view, which is not mutually exclusive, is that absorbed EtOH vapor exerts direct effects in the brain to regulate metabolic changes that promote starvation survival (*36*). In *Drosophila*, specific neuronal populations have been shown to modulate EtOH sensitivity, tolerance, and behavioral responses, including serotonergic neurons, corazonin neurons, ellipsoid body neurons, and dopaminergic neurons (*27*, *37*–*39*). While none of these previously described groups were influential in modulating starvation survival in response to indirect yeast or EtOH exposure, we found that a few ellipsoid body neurons, the R4d subset, were required specifically for the effects of indirect exposure to high EtOH solutions (e.g., 20%). Broadly inhibiting all ellipsoid body neurons, however, did not affect the response to indirect EtOH exposure, indicating the possibility of a compensatory network among different ellipsoid body subsets (*40*). Experimental activation of R4d neurons promoted EtOH metabolism by increasing catabolism, reducing free EtOH in the fly, and increasing starvation survival in an EtOH-dependent manner. Together, these results imply that R4d neurons may be recruited in specific conditions to orchestrate changes in organism metabolism in a way that is in line with environmental conditions.

Regardless of how EtOH acts to improve starvation resistance, high levels can be toxic, and our data suggest that stress response pathways are required to ensure extended starvation survival. When presented at higher levels, EtOH toxicity has been reported in both *C. elegans* and *Drosophila*, where exposure to high EtOH caused lower antioxidant capacity and decreased life span in the worm (*41*) and increased oxidative stress and death in the fly (*42*). We found evidence that the toxic effect of EtOH exposure at higher concentrations may be mediated by the conserved insulin-responsive forkhead transcription factor, *foxo*, as a foxo target was increased upon indirect exposure to EtOH, consistent with increased *foxo* activation. Furthermore, while flies lacking *foxo* showed a significant increase in survivorship in response to lower EtOH concentrations, they were significantly shorter-lived than control animals upon starvation when indirectly exposed to higher EtOH concentrations. Because loss of *foxo* did not affect EtOH metabolism or internal abundance, we interpret these results as suggesting that *foxo* is involved in promoting EtOH tolerance. It would, therefore, be permissive of extended starvation survival upon higher EtOH exposure.

Previous work has shown that sweet taste perception alone, without nutrient intake, attenuates starvation-induced sleep loss in flies, suggesting an interplay between sensory and metabolic perception of nutrient availability in regulating physiology and other complex behaviors (*15*). While we are unable to formally rule out the possibility that flies derive ATP from food-derived volatiles, airborne calories are unlikely to explain all the patterns that we observed. In mice that have been acutely starved, the sight, smell, and/or taste of food rapidly reversed the activation state of neurons induced by energy deficit, and this depends on nutritional state (*43*, *44*). Similarly in nature, nutrient-derived volatiles would be strongly paired with nearby food availability, and adaptive responses that maximize the likelihood of finding and exploiting these resources would be favored by natural selection. Our data suggest a coordinated response in the brain and in peripheral tissues that couples changes in metabolism to stress responses. This effect does not depend on known pathways involved in canonical sensory perception, aging, fat metabolism, or EtOH tolerance, suggesting the possibility of unknown metabolic nutrient sensors that are tuned to relevant environmental cues. A deeper understanding of how organisms across taxa perceive and respond to environmental stress may offer substantial benefit to humans attempting to maintain maximal health in the face of food shortages, unstable environmental conditions, and food insecurity (*45*, *46*) and in this way represent one path toward understanding global stress response.

MATERIALS AND METHODS

Drosophila husbandry

All fly stocks were maintained on a standard cornmeal-based growth medium at 25°C with 60% humidity under a 12:12 light-dark cycle. Flies carrying temperature-sensitive transgenes were reared in 23°C until they were used in the experiments, which was at 7 to 10 days old unless otherwise stated. Larval density was controlled by aliquoting 32 μl of collected eggs into rearing bottles (www.flystuff.com, #8003) containing 25 ml of standard cornmeal-based growth medium. Following eclosion, adult flies were transferred into rearing bottles containing SY10% medium (10% sucrose and 10% yeast) and allowed to mate for 2 days, after which the sexes were separated under light CO_2 anesthesia. Twenty male or female flies were placed into individual vials (www.flystuff.com, #8002) and allowed to age for 5 to 7 days before use unless otherwise stated.

Fly stocks

The following stocks were obtained from Bloomington Stock Center bloominton drosophila stock center (BDSC): *Canton-S*, w^{1118}, $Sirtuin1^{2A\text{-}7\text{-}11}$ (BDSC, #8838), $Sirtuin2^{5B\text{-}2\text{-}35}$ (BDSC, #8839), *Sirtuin4* (BDSC, #8840), *DopEcR* (BDSC, #10847), and *Foxo_RNAi* (BDSC, #32427). *Dop1R1* (BDSC, #43667), *UAS-Kir2.1* (BDSC, #6596), *UAS-reaper* (BDSC, #5823), *nSyb-GAL4* (BDSC, #51635), *Ppl-GAL4* (BDSC, #58768), *Esg-GAL4* (BDSC, #26816), *Adh* (BDSC, #3976), Trh^{c01440} (BDSC, #10531), *Tdc2-GAL4* (BDSC, #9313), *GAL4* covering a broader subset of ring neurons (BDSC, #39805), R2,3d,4 m-*GAL4* (BDSC, #SS02709), R1-*GAL4* (BDSC, #49661), R2-*GAL4* (BDSC, #48343), R3d-*GAL4* (BDSC, #40074), R4d-*GAL4* (BDSC, #48487), R4m-*GAL4* (BDSC, #39209), R5-*GAL4* (BDSC, #39198), Rel^{E38}, and *Dif* mutants were gifts from M. Hedengren. NPF^{sk2} was provided by S. Kondo, and *ppk28* was a gift from K. Scott. E. Johnson provided the *Crz-GAL4* flies. $DIlp^{2,3,5}$ and $foxo^{\Delta 94}$ were a gift from L. Partridge, and *UAS-Tsc1-Tsc2* were from J. H. Lee. *Akh*, *AkhR*, and *bmm* mutant flies were a gift from M. Dus. $Aldh^{\Delta 24}$ and $CrzR^{01}$ mutant flies were provided by F. James and J. Park, respectively. Tbh^{nM18} flies were provided by K. Iliadi, and $Poxn^{\Delta M22-B5\Delta XB}$ mutants were provided by J. Alcedo. *Gr63a* mutant flies were a gift from A. Ray. Serotonin receptor mutant flies were a gift from Y. Rao. *Gr63a/orco/Ir8a/Ir25a* mutant flies were provided by R. Bento.

Yeast and EtOH exposure

A live yeast solution was prepared by dissolving 10 g of dry Brewer's yeast (Fleischmann's Yeast, #2182) in 20 ml of distilled H_2O. For experiments that involved dead yeast, the live yeast mixture was heated in the microwave for 2 min, after which zero growth was observed on standard YPD media. The EtOH solution was prepared by diluting 100% EtOH (Fisherbrand, #HC800) in distilled H_2O to the required concentrations. Unless otherwise stated, flies were exposed to yeast or EtOH by placing 20 ml of the respective solution in a 200-ml beaker and placing the beaker together with the flies in a plastic container (Snapware Airtight 29-Cup Rectangular Food Storage Container, Amazon), which was then sealed with its respective lid. Air exchange was achieved through two holes on the side of the box. For experiments in which a controlled airflow (1 LPM) was used to expose flies to yeast volatiles (Fig. 3, A and B), an odor delivery system was used (Analytical Research System, model# OLFM-ADS, 4AFM1C), and air was passed through a beaker containing the relevant solution before being directed into the sealed container. In all cases, the flies had no direct physical contact with the yeast or EtOH solutions.

Starvation resistance and activity measurement

Female flies were sorted on the third day after eclosion and maintained on SY10 media for 5 to 7 days before being used for experiments. Starvation resistance was measured using a high-throughput starvation survival assay that was developed in our laboratory and that takes advantage of the real-time activity recording by TriKinetics *Drosophila* activity monitors. Briefly, individual flies were placed into activity tubes with 2% agar and placed in DAM2 monitors (www.trikinetics.com). Activity counts were collected every 30 s; time of death was considered to be the last census time in which the activity count was greater than zero and was accompanied by at least two other periods of nonzero activity within a 6-hour window. This algorithm allowed an objective determination of death time and avoided artifacts caused by bumping or occasional false-positive measures from the DAM2. This assay was validated with visual observation. Analysis software written in the R programming language is freely available at https://github.com/PletcherLab/DAMSurvival.

Triglyceride measurement

Measures of lipid storage were obtained using Infinity Triglyceride reagent (Thermo Fisher Scientific, # TR22421). Eight replicates of 10 flies were placed in standard vials containing 2% agar. Each vial was covered by a fine steel mesh to allow ready air exchange with the surrounding environment. As described above, these vials were cohoused with the yeast solution in a tightly sealed plastic container. At the desired time point, the flies were collected and homogenized in 300 μl of phosphate-buffered saline + 0.1% Triton X-100 (IBI Scientific, #18D0151) on ice. Homogenates were then centrifuged at 3750*g* for 1 min to settle debris, after which 5 μl of supernatant was withdrawn and mixed with 150 μl of Infinity Triglyceride reagent warmed to 37°C. The solution was incubated at 37°C for 10 min, and TAG levels were estimated from absorbance at 520 nm as per the manufacturer's instructions. Absorbance readings were collected using a Synergy2 plate reader (BioTek Inc.).

CO_2 measurement

For quantifying CO_2 production by live yeast, 10 g of dry Brewer's yeast was dissolved in 20 ml of distilled H_2O and kept in a tightly sealed plastic box (the same equipment as that used for starvation survival experiments, see above) for 24 hours at 25°C. Yeast headspace air was removed from the box using a 25-ml syringe and immediately injected into respirometry chambers. A Sable Systems Respirometry System, including a Li-COR LI-7000 carbon dioxide analyzer, mass flow controllers (MFC2), and a UI-2 analog signal unit, was used to determine CO_2 level. CO_2 measures for each sample were obtained using the EXPDATA software from Sable Systems, following adjustment using a proportional baseline. Eight replicate samples were analyzed from each container, and three containers each of yeast augmented and control were sampled.

To manipulate the CO_2 level in the experimental box, we used CO_2-release tablets to slowly release CO_2 (ISTA CO_2 Tablet Carbon Dioxide 100 Tab Carbon Dioxide–Planted Diffuser Tablets by Aquarium Equip, Amazon). Five CO_2 tablets released about 30% of the CO_2 released by live yeast over a 24-hour period (table S2); we, therefore, used 5 tablets (for a low CO_2 level exposure) or 15 tablets (for a CO_2 level exposure that is close to that released by live yeast) in our experiments to achieve different CO_2 concentrations.

Behavioral preference assays

Choice was measured using binary traps made from commercially available T connectors (McMaster-Carr part number 5372K615) with 200-μl pipette tips, which were trimmed, attached to opposite ends of the T connectors to form one-way doors that end in small collection chambers. Twenty female Canton-S flies were introduced into the central arm of the maze, with 10 μl of H_2O in one arm of the T-connector and 10 μl of 10% EtOH in the other arm. Behavioral preference was measured in a dark room under dim 660-nm red light at 24°C, and behavior was observed at 3, 6, 9, and 12 min. A PI at each time point was computed as follows: [number of flies in EtOH (NE) − number of flies in unexposed arms (NC)]/(NC + NE). The fraction of flies that participated in the experiments was calculated as: (NC + NE)/20. Average PI values are weighted mean values among replicates with weights proportional to the number of animals that made a choice. Participation rates for all of the T-maze assays were >50%.

EtOH measurement

EtOH levels were determined using an EtOH assay kit (Megazyme, K-ETOH). To estimate the amount of EtOH in yeast volatiles, yeast volatiles were bubbled in 3 ml of water for 24 hours. Ten microliters of the resulted water was used to determine EtOH level by measuring the absorbance at 340 nm as per the manufacturer's instructions. To determine the amount of EtOH in the fly, 20 female flies were ground in 200 μl of tris-HCl (pH 7.5) and then centrifuged at 10,000 rpm for 15 min at 4°C. Ten microliters of supernatant was used for the EtOH level determination. A standard curve was generated using the EtOH standard in the kit following the instructions to estimate EtOH levels in each sample.

To evaluate the EtOH metabolism rate, flies were exposed to 3 ml of 50% EtOH added to the cotton plug in the fly vial. A fine mesh was used to separate flies from the plug. After 40 min of exposure, the flies were flipped into new vials and collected in liquid nitrogen at different time points for EtOH measurement. To investigate whether yeast volatiles influence EtOH metabolism, flies were exposed to EtOH as above. After 40-min exposure, they were flipped into new vials and divided into two groups. One group of flies were cohoused with 20 ml of live yeast solution, and the other group of flies were cohoused with 20 ml of water. Flies were sampled, and the EtOH level was determined using the same method.

RNA extraction and reverse transcription quantitative polymerase chain reaction

Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, #15596026) following the manufacturer's instructions. In general, for each treatment and control group, four replicates of 20 female flies were collected into nuclease-free lysing tubes with matrix D beads (MPbio, #6913-500-129984) and 500 μl of TRIzol. Flies were homogenized for two 30-s pulses at 5 M/s. Lysates sat at room temperature for 5 min, and then 120 μl of chloroform (Sigma-Aldrich, #c2432) was added for phase separation. About 250 μl of RNA containing supernatant was transferred to a new nuclease-free tube and mixed with an equal volume of isopropanol (Sigma-Aldrich, #c2432) to precipitate the RNA. The RNA was then washed twice with 70% cold EtOH (Sigma-Aldrich, #E7023), air dried, and dissolved in 50 μl of nuclease-free H_2O. RNA concentration and quality were checked using a NanoDrop One machine (Thermo Fisher Scientific). Complementary DNA (cDNA) was prepared from 1 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, #4368814), and the resulted cDNA were diluted five times before use. Individual Quantitative polymerase chain reactions (qPCRs) were composed of the following: 10 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, #4367659), 7 μl of water, 1 μl of diluted cDNA, and 1 μl each of the forward and reverse primers at 10 mM. The settings for qPCR amplification consisted of 95°C for 5 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 30 s. The melt curve was used to confirm the specificity of primers. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to the housekeeping gene *Rpl32*. The primers used for qPCR are as follows: *Atg16*_Forward (AGCAGGAGGAGCTCACAGAG), *Atg16*_Reverse (TTGTTCGTTTGCTGTTCGAC); *Atg7*_Forward (GCCAAGAATGGTGTGCATGA), *Atg7*_Reverse (CAAATGCTCCTTCGTCTGGG); *Cat*_Forward (TTCTGGTTATCCCGTTGAGC), *Cat*_Reverse (GGTAATGGCACCAGGAGAAA); *Dros*_Forward (ACCAAGCTCCGTGAGAACCTT), *Dros*_Reverse (TTGTATCTTCCGGACAGGCAG); *Dipt*_Forward (ATTGGACTGAATGGAGGATATGG), *Dipt*_Reverse (CGGAAATCTGTAGGTGTAGGT); *Adh*_Forward (CGTTCAACTCCTGGTTGGAT), *Adh*_Reverse (AGCTCGATAGCCTTGACGAA); *Aldh*_Forward (GGCAAGTCGCCAAACATTAT), *Aldh*_Reverse (AGCACTGGCCCATGTTAAAG); *Rpl32*_Forward (ATCGGT TACGGATCGAACAA), *Rpl32*_Reverse (GACAATCTCCTTGCGCTTCT).

Statistical analysis

For starvation assays, groupwise and pairwise comparisons among the survivorship curves were performed using the DLife computer software (*47*), and survivorship curves generated from the *Drosophila* Activity Monitor System were performed using an R package developed by the Pletcher laboratory (https://github.com/PletcherLab/DAMSurvival). *P* values were obtained using the log-rank analysis (select pairwise comparisons and group comparisons or interaction studies) as noted. Interaction *P* values were calculated using Cox regression when the survival data satisfied the assumption of proportional hazards. For all box plots, the box represents SEM (centered on the mean), and the whiskers represent 10/90%. For TAG, activity, and EtOH measures, *P* values were obtained by using a standard two-sided *t* test or analysis of variance (ANOVA) after verifying normality and equality of variances. Details of the statistical analysis are presented in the figure legends.

For preference assays, *P* values comparing the PI between starved and fully fed flies were obtained using a randomization procedure and the statistical software R. Briefly, the null distribution of no difference among treatments was obtained by randomizing individual PIs obtained from groups of 20 flies among all measures (maintaining block structure when appropriate) and 100,000 *t* statistics. *P* values were determined by computing the fraction of null values that were equal or more extreme to the observed *t* statistic. Mean preference values were plotted and weighted by the number of choosing flies in each trial, with the error bars representing the SEM.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/20/eabf8896/DC1

View/request a protocol for this paper from *Bio-protocol*.

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