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 CO₂ released by active respiration, as loss of the CO₂ 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.
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 semiautomatically 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*, *Irba*, 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 CO₂ 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 CO₂ (22). We determined that the live yeast solution to which flies were indirectly exposed increased humidity and CO₂ in the exposure chamber over a 24-hour period (table S2). Although sensory perception of water and CO₂ 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 CO₂ from yeast volatiles by passing exposed air through a column containing both the CO₂ absorbent, Ascarite (II), and the desiccant, Dririte, before delivery into the starvation chamber. Yeast volatiles treated in this manner...
failed to prolong starvation survival (Fig. 3A), suggesting that CO₂,
water, and/or a water-soluble compound(s) are required for en-
hanced 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
CO₂ alone were not sufficient to modulate starvation resistance
(Fig. 3, C and D). We concluded that while changes in relative hu-
midity and CO₂ 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 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 expo-
sure to 2.5% EtOH, however, significantly increased starvation survival
(Fig. 4A). Follow-up experiments revealed a concentration-dependent
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 CO2 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

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**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 CO2 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.

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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

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**Fig. 3. Humidity and CO₂ may be required but were not sufficient to alter starvation resistance when exposed to yeast volatiles.** (A) Yeast volatiles with water and CO₂ removed failed to prolong starvation resistance. Using log-rank analysis, P > 0.1 when comparing the control to −H₂O/−CO₂, and P < 0.001 when comparing the control to yeast volatile or −H₂O/−CO₂ 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 CO₂ 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.
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 *dlip<sup>2,3,5</sup>* mutant phenotype), we found that flies carrying a putative null mutation of the insulin-responsive transcription factor *foxo* (*foxo<sup>∆94</sup>) 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 starvation survival (fig. 4A). 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* = 0.003 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<sub>2</sub> 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.

**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* = 0.003 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<sub>2</sub> 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.
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<sup>sk2</sup> mutants), ablation of corazonin-expression cells (Crz-Gal4 × UAS-Rpr), loss of octopamine biosynthesis (Tbh<sup>n318</sup> mutants), loss of serotonin biosynthesis (Trh<sup>CO144</sup> mutants or serotonin receptor mutants), and loss of specific dopaminergic receptors (DopEcR and Dop1R1 mutants) all
failed to abrogate the ability of yeast odors 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 survival (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/Adhd 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 Drosomycin (Dros) and Dipterica (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 diptera (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.
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

![Graphs showing data](image-url)
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 serotoninergic 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 (13). 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.

**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.
MATERIALS AND METHODS

*Drosophila* hubandry

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 CO2 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 blooming tent drosophila stock center (BDSC): Canton-S, w^{1118} Sirtuin1^{2A-7.11} (BDSC, #8838), Sirtuin2^{28-2.35} (BDSC, #8839), Stariun4 (BDSC, #8840), DppEcr (BDSC, #10847), and Foxo RNAi (BDSC, #32427). DopIR1 (BDSC, #43667), UAS-Kir2.1 (BDSC, #6596), UAS-rosaer (BDSC, #5823), nSyb-GAL4 (BDSC, #51635), Ppl-GAL4 (BDSC, #58768), Esg-GAL4 (BDSC, #26816), Acdh (BDSC, #3976), Tbr^{3144} (BDSC, #10531), Tkd2-GAL4 (BDSC, #9313), GAL4 covering a broader subset of ring neurons (BDSC, #39805), R2d4 r-mGAL4 (BDSC, #80279), R1-GAL4 (BDSC, #49661), R2-GAL4 (BDSC, #48343), R4d-GAL4 (BDSC, #40074), R4d-GAL4 (BDSC, #39847), R4m-GAL4 (BDSC, #39209), R5-GAL4 (BDSC, #39198), Ref^{z}, and Dff mutant flies weregifts from M. Hedengren. NPF^{87z} was provided by S. Kondo, and ppk28 was a gift from K. Scott. E. Johnson provided the Crz-GAL4 flies. Dllp2 and foxo^{804} 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. Alidh^{A24} and Czr^{R1} mutant flies were provided by F. James and J. Park, respectively. Tbr^{M18} flies were provided by K. Illadi, and Poxn^{AM22–BS2ebR} mutants were provided by J. Alcedo. Gr63a mutant flies were a gift from A. Ray. Serotonin receptor mutant flies were a gift from Y. Yao. Gr63a/orco/Ir8a/Ir25a mutant flies were provided by R. Bento.

**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 3570 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.).

**CO2 measurement**

For quantifying CO2 production by live yeast, 10 g of dry Brewer’s yeast was dissolved in 20 ml of distilled H2O 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 CO2 level. CO2 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 CO2 level in the experimental box, we used CO2-release tablets to slowly release CO2 (ISTA CO2 Tablet Carbon Dioxide 100 Tab Carbon Dioxide–Planted Diffuser Tablets by Aquarium Equip, Amazon). Five CO2 tablets released about 30% of the CO2 released by live yeast over a 24-hour period (table S2); we, therefore, used 5 tablets (for a low CO2 level exposure) or 15 tablets (for a CO2 level exposure that is close to that released by live yeast) in our experiments to achieve different CO2 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 H2O 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 were 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 H2O. 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 Transcript 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−ΔΔCt method normalized to the housekeeping gene Rpl32. The primers used for qPCR are as follows: Atg16_Forward (AGCAGAGGAGGCTCACAAGAG), Atg16_Reverse (TTGTCG- GTTTGCGTTGAC); Atg7_Forward (GCCAAATGTTGTGCATGA), Atg7_Reverse (CAAAATGCTCCTTCGTGGG); Cat_Forward (TCTTGGTTATCCGGTACGAC), Cat_Reverse (GTGAATTGCAAGGAGAA); Dros_Forward (ACCAAGTCTGCTGAGACCTT), Dros_Reverse (TGTATCTCCGGACAGG-CAG); Dipt_Forward (ATTGGACTGAAAGGAGATAGG), Dipt_Reverse (CGGAAATCCTTGGTGTAGT); Adh_Forward (CGTTCAACTCTGTTGGGAT), Adh_Reverse (AGCTCGA- TACGCTTGACGAA); Adh disob (GCCAAATGTCGCA- CATTAT), Adh_Reverse (AGACTGGGCCCAGTTAAG); Rpl32_Foward (ATCGGTTACGGATCAACAA), Rpl32_Reverse (GCAATCTCCTTGCCCTTCT).

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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Yeast volatiles double starvation survival in *Drosophila*
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