


# Dietary yeast influences ethanol sedation in *Drosophila* via serotonergic neuron function

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## Abstract

Abuse of alcohol is a major clinical problem with far-reaching health consequences. Understanding the environmental and genetic factors that contribute to alcohol-related behaviors is a potential gateway for developing novel therapeutic approaches for patients that abuse the drug. To this end, we have used *Drosophila melanogaster* as a model to investigate the effect of diet, an environmental factor, on ethanol sedation. Providing flies with diets high in yeast, a routinely used component of fly media, increased their resistance to ethanol sedation. The yeast-induced resistance to ethanol sedation occurred in several different genetic backgrounds, was observed in males and females, was elicited by yeast from different sources, was readily reversible, and was associated with increased nutrient intake as well as decreased internal ethanol levels. Inhibition of serotonergic neuron function using multiple independent genetic manipulations blocked the effect of yeast supplementation on ethanol sedation, nutrient intake, and internal ethanol levels. Our results demonstrate that yeast is a critical dietary component that influences ethanol sedation in flies and that serotonergic signaling is required for the effect of dietary yeast on nutrient intake, ethanol uptake/elimination, and ethanol sedation. Our studies establish the fly as a model for diet-induced changes in ethanol sedation and raise the possibility that serotonin might mediate the effect of diet on alcohol-related behavior in other species.

## KEYWORDS

ethanol, alcohol, *Drosophila*, behavior, sedation, diet

## 1 | INTRODUCTION

Consumption of alcohol (ethanol) has a wide range of pleasurable effects including psychomotor stimulation,<sup>1,2</sup> general improvement in mood, and relief of stress.<sup>3</sup> Additionally, however, abuse of alcohol has far-reaching, negative health consequences.<sup>4,5</sup> Alcohol abuse contributes to 3% to 4% of all preventable deaths worldwide, increases the risk for specific forms of cancer, and is responsible for hundreds of billions of dollars in costs annually within the United States alone.<sup>4</sup>

Both environmental and heritable genetic factors contribute to the risk for abusing alcohol.<sup>6-9</sup> A better understanding of these environmental and genetic risk factors could ultimately facilitate prevention and treatment of alcohol abuse.

*Drosophila melanogaster* (fruit fly or fly) is a leading invertebrate model for investigating molecular-genetic mechanisms that influence alcohol-related behaviors.<sup>10-13</sup> Behavioral responses of flies to alcohol include locomotor stimulation at low doses,<sup>14,15</sup> sedation at higher doses,<sup>16-22</sup> development of seizures upon withdrawal of alcohol,<sup>23</sup>

and development of tolerance after prior exposure to the drug.<sup>24-26</sup> Additionally, flies will voluntarily consume alcohol,<sup>27</sup> and they develop exposure-dependent alcohol preference.<sup>28-31</sup> All of the behavioral responses to ethanol in flies are also found in other species including humans,<sup>11</sup> strongly suggesting that alcohol likely has conserved effects on nervous system function. Consistent with this possibility, many genes or genetic pathways that influence behavioral responses to alcohol in flies have also been implicated in various aspects of alcohol-related behaviors in other model organisms (eg, *Clic*,<sup>20</sup> GABA signaling,<sup>32,33</sup> *slo* potassium channels,<sup>34</sup> and NPF/NPY signaling<sup>35,36</sup>) as well as various aspects of alcohol consumption and abuse in humans (eg, *Adh*,<sup>31,37-39</sup> *Rsu1*,<sup>40</sup> *AUTS2*,<sup>41</sup> *Ryr1*<sup>16</sup>). Thus, at least some of the mechanisms underlying alcohol-related behavior in model organisms might also impact alcohol abuse in humans.

In addition to genetic factors, approximately 50% of the risk for abusing alcohol is influenced by the environment.<sup>6-9</sup> Diet is possibly one of the key—but largely underappreciated—environmental factors that influences alcohol phenotypes in humans. Supplementation of the diet with tryptophan decreases alcohol craving in human binge drinkers exposed to stress.<sup>42</sup> Additionally, patients with higher body mass indexes (BMIs) are at an increased risk for heavy alcohol intake,<sup>43</sup> development of alcohol dependence,<sup>44</sup> and alcohol abuse.<sup>45</sup> Diet also influences multiple alcohol-related behaviors in rodents<sup>46-49</sup> and *Caenorhabditis elegans*.<sup>50</sup> Furthermore, variants in the genes *FTO* and *CPNE5* are associated with both obesity and multiple alcohol phenotypes in humans,<sup>51-53</sup> and several genes in flies might regulate both food intake and behavioral responses to alcohol.<sup>54</sup> These studies collectively suggest that diet and diet × genotype interactions might play important roles in multiple aspects of alcohol-related behavior in animals and impact risk for alcohol-related phenotypes in humans.

Several studies suggest that the serotonin (5-hydroxytryptophan (5-HT)) system might modulate or mediate the effects of diet on behavioral responses to alcohol. In flies, for example, dietary yeast influences brain 5-HT levels,<sup>55</sup> serotonergic neurons regulate feeding,<sup>56-58</sup> the 5-HT<sub>2A</sub> receptor impacts dietary protein consumption,<sup>55</sup> and 5-HT is implicated in ethanol sedation.<sup>59</sup> Additionally, there is a large literature linking 5-HT to alcohol problems in humans (eg, <sup>60-64</sup>). Despite the insights of the studies summarized here, the possibility that 5-HT signaling underlies diet-induced changes in behavioral responses has not been formally addressed.

In this report, we describe results from our studies on the role of diet in alcohol sedation in *Drosophila*. We chose flies for these studies because of their conserved alcohol-related behaviors,<sup>11,14-31</sup> the powerful tools available for performing genetic analyses in this model,<sup>65</sup> the ability to measure both ethanol sedation (see above) and food intake,<sup>66</sup> the ability to control food composition,<sup>55,66,67</sup> and the known genetic connections between fly alcohol behavior and human alcohol abuse.<sup>16,31,37-41</sup> We report that dietary yeast significantly impacts ethanol sedation in flies, possibly by influencing ethanol uptake/elimination. We also report that the effect of dietary yeast on ethanol sedation and uptake/elimination depends on serotonergic neuron function. Our studies establish flies as a model for exploring diet-induced changes in alcohol sedation and suggest that the

serotonergic system might be a conserved regulator of the underlying processes.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

*Drosophila* agar type II and cotton plugs for vials were from Apex BioResearch Products (Genesee Scientific, San Diego, CA); saf-instant yeast, Lesaffre Yeast Corp. (Milwaukee, WI); yellow corn meal, The Quaker Oats Co. (Chicago, IL); MP Bakers (101400) and MP Brewers (903312) yeast, MP Biomedicals (Solon, OH); table sugar (sucrose), Richmond Restaurant Service (Richmond, VA); methyl 4-hydroxybenzoate (Tegosept), chloramphenicol, tetracycline, and ampicillin, Sigma-Aldrich (St. Louis, MO); FD&C Blue No. 1, Spectrum Chemical Manufacturing Corp. (Gardena, CA); polypropylene culture bottles (AS-355) and cotton plugs, Fisher Scientific; polystyrene narrow vials (89092-722), VWR International; gas drying tube caps (199610000), Bel-Art Products (Wanye, NJ); feeder caps for Con-Ex studies (FCS13/16NA1), MOCAP (Park Hills, MO); 200 (41-6304) and 400 (41-6140)  $\mu\text{m}$  mesh, Ted Pella, Inc. (Redding, CA); Alcohol Reagent Set (A7504), Pointe Scientific, Inc. (Canton, MI).

### 2.2 | Fly stocks and husbandry

The *w*[A], Lausanne-S (LS), Oregon-R (OR), and Samarkand (Sam) strains (stock numbers 5905, 4268, 25211, and 4270, respectively), UAS-Tetanus Toxin Light Chain<sup>68</sup> (stock number 28837), and two *Trh*-Gal4<sup>69</sup> drivers (stock numbers 38388 and 38389) were obtained from the Bloomington *Drosophila* stock center (Bloomington, IN). The *r*[A] strain was generated by backcrossing the *w*<sup>+</sup> allele in Canton-S (supplied by Ron Davis, Scripts, Florida) into *w*[A] for seven generations. Flies containing the UAS-Kir2.1<sup>70</sup> transgene were generated in a *w*<sup>1118</sup> genetic background (supplied by Scott Pletcher, University of Michigan).

Flies were grown to adulthood at 25°C/65% relative humidity with a 12-hour light/dark cycle on standard food medium (2Y10S3C: 2% saf-instant yeast, 10% sugar, 3.3% cornmeal, 1% agar, 2 g/L Tegosept, 0.125 g/L chloramphenicol, 0.02 g/L tetracycline, and 0.1 g/L ampicillin) supplemented with live yeast. Flies (3 to 5 days old) were collected under light CO<sub>2</sub> anesthesia, sex separated, and placed in fresh food vials containing the media indicated in the main text prior to the described studies.

In studies using yeast paste (live or heat-killed (autoclaved at 122°C for 1 h using the dry cycle of a Hirayama HV-50 autoclave) saf-instant yeast (35% w/v) in water), flies were collected and placed into fresh food vials (containing 2Y10S3C as described above) and provided yeast paste (1 g/vial) via inverted caps from 50-mL conical tubes placed in the open ends of the vials. For studies using nylon mesh barriers, caps from gas drying tubes were bored out, circular pieces of nylon mesh were melted into the caps, and the cap-nylon

mesh inserts were placed in the vials to provide an approximately 2-cm gap between the flies and the yeast paste.

The media in vials were 2Y10S3C (described above); 2Y10S3C missing antimicrobials, missing one or two nutrient components, or with all components diluted as described in the main text; 2Y10S3C supplemented with additional yeast, sugar, or cornmeal as described in the main text; or 2Y, 10Y, 20Y or 30Y (2%, 10%, 20%, or 30% yeast w/v in 1% agar). Unless otherwise noted, yeast indicates saf-instant bakers yeast.

### 2.3 | Ethanol sedation, ethanol rapid tolerance, and internal ethanol

Ethanol sedation (determined as sedation time 50 (ST50), the time required for 50% of flies to become sedated) and rapid tolerance (the ratio of a second ST50 to a first ST50) were measured as previously described<sup>17,19</sup> using vapor from 85% ethanol. For analysis of internal ethanol, flies were exposed to ethanol vapor for the times indicated in the figure legends and then homogenized in 200  $\mu$ L of distilled water. Homogenates were centrifuged to pellet debris, and ethanol content in the supernatants was determined as previously described via a spectrophotometric method.<sup>17,19</sup>

### 2.4 | Media and nutrient consumption

Intake of food medium was measured using consumption excretion of 1% FD&C Blue 1 in the indicated media using the sum of the dye excreted in the vial (ExVial) and the internal dye (INT) to reflect the volume of media consumption as described.<sup>66</sup> Flies were reared and collected as described above and placed on the indicated food medium containing 1% FD&C Blue 1 for 24 hours, and then, ExVial and INT were determined. Nutrient consumption (fold of 2Y) was estimated as  $([\text{ExVial} + \text{INT}] \times [\text{yeast concentration}]) \div ([\text{mean 2Y ExVial} + \text{INT}] \times [\text{yeast concentration}])$ .

### 2.5 | Total, dry, and water weight

Adult flies were reared and collected as above and weighed to determine total weight in groups of 11 while anesthetized in tared 1.5-mL snap-cap tubes with perforated lids. Tubes of flies were incubated at 50°C (ambient humidity) for 24 or more hours to volatilize water content and weighed to obtain dry weight. Water weight was determined as the difference between total and dry weight. Total, dry, and water weights for each tube were expressed as milligrams per fly. Each tube of 11 flies generated a single datum.

### 2.6 | Brain 5-HT levels

r[A] females, reared and collected as described above, were fed 2Y or 30Y media for 2 days. Brains were dissected from flies, and 5-HT was measured essentially as previously described.<sup>71-73</sup> In brief, single fly brains were dissected, homogenized, diluted with 10  $\mu$ L 20  $\mu$ M

perchloric acid (to prevent transmitter degradation), and then tissue content determined with capillary electrophoresis with fast scan cyclic voltammetry detection.

### 2.7 | Statistical analyses

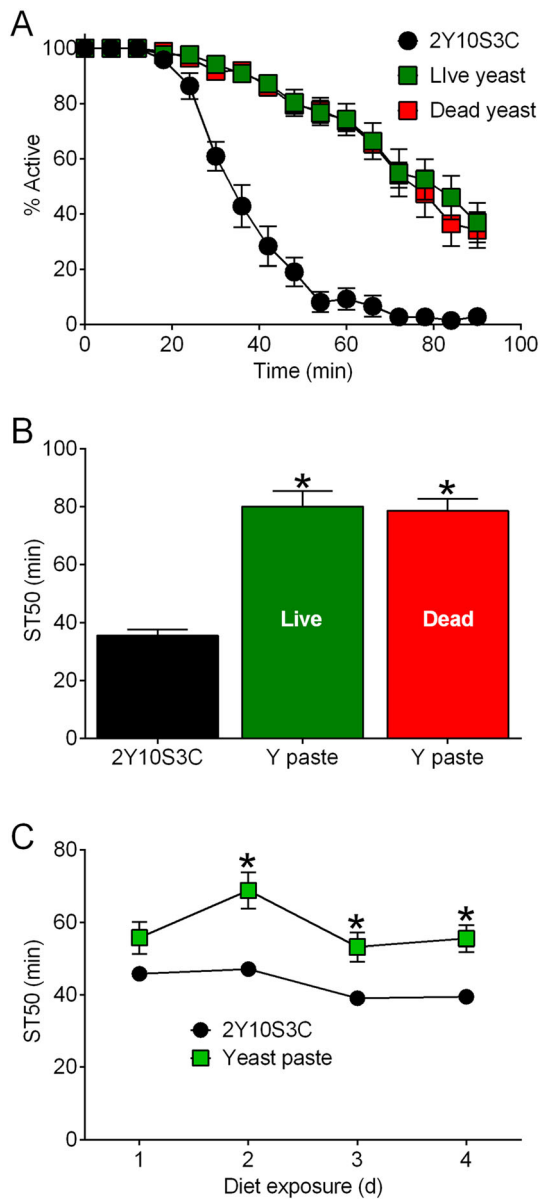
All data were normally distributed (Prism 6.07, GraphPad Software Inc., San Diego, CA) and were therefore analyzed with standard parametric tests (two-tailed *t* tests, one- and two-way ANOVAs, Bonferroni's multiple comparisons (BMCs)) using Prism 6.07 (GraphPad Software Inc., San Diego, CA). *P* values less than .05 were considered to represent statistically discernable differences. All *P* values are reported in the figure legends, and all data are reported as mean  $\pm$  SEM.

## 3 | RESULTS

Drugs, enzyme inhibitors, and other molecules can be administered (ie, fed) to flies via a simple paste made of yeast (*Saccharomyces cerevisiae*) and water (eg, <sup>74-76</sup>). While establishing this drug treatment regimen for investigating alcohol behavior in flies, we found that flies exposed to a standard food medium supplemented with a paste made from live yeast and water were substantially resistant to ethanol sedation (Figure 1A (time courses), B (sedation time 50 (ST50) values)) compared with flies provided only a standard medium containing 2% yeast, 10% sucrose, and 3.3% cornmeal (hereafter 2Y10S3C). The resistance to ethanol sedation was evident by 2 days of exposure to yeast paste and persisted during at least 4 days of continuous exposure (Figure 1C).

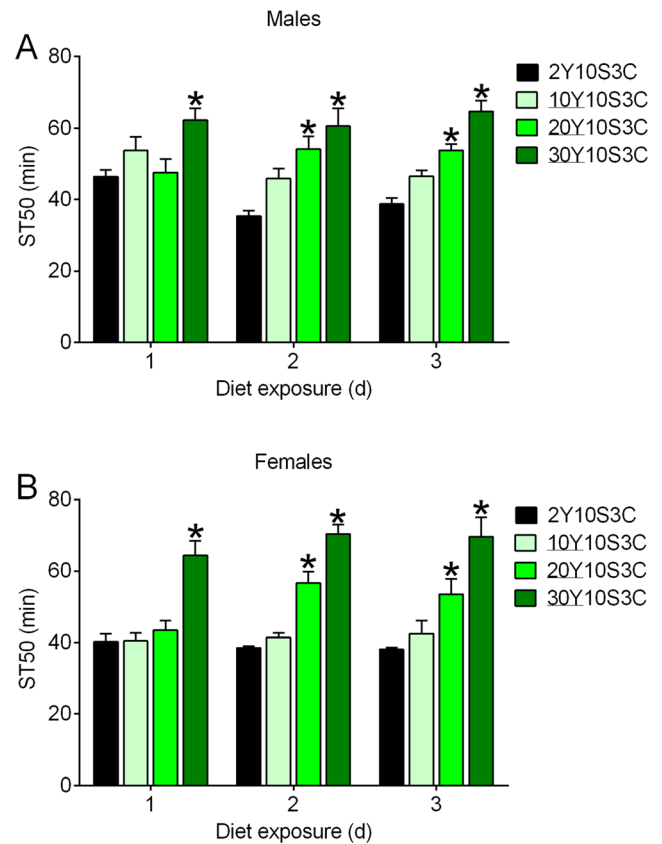
Yeast produce ethanol via fermentation,<sup>77-80</sup> including under conditions used to rear *Drosophila*.<sup>17</sup> To address the possibility that the ethanol resistance in flies fed yeast paste reflected tolerance in response to ethanol produced by the supplemented yeast, we fed flies paste made of heat-killed yeast (which would be incapable of fermentation) and then assessed ethanol sedation. Flies fed heat-killed yeast paste were resistant to ethanol sedation compared with flies fed standard food, and ethanol sedation in flies fed heat-killed and live yeast paste was indistinguishable (Figure 1A (time courses), B (ST50 values)). Therefore, fermentation and ethanol production by supplemented yeast is not required for the yeast-induced change in resistance to ethanol sedation.

Flies were provided with supplemental yeast paste in the studies reported in Figure 1. To address the possibility that increasing the concentration of yeast incorporated into agar-based fly media (versus supplementation with yeast paste) was capable of altering ethanol sedation, we assessed ST50 values in flies fed our standard fly medium (2% yeast, 2Y10S3C) and in media containing 10% (10Y10S3C), 20% (20Y10S3C), and 30% (30Y10S3C) yeast. Increasing the yeast concentration increased ST50 values in males (Figure 2A) and females (Figure 2B). Flies fed 20% yeast medium had increased ST50s after exposure to the diet for two or more days, whereas flies fed medium with 30% yeast had greater ST50 values after one or more days on the



**FIGURE 1** Exposure to dietary yeast paste alters ethanol sedation sensitivity. A, w[A] females fed a paste of live or dead yeast (35% w/v) for 2 days took longer to become sedated compared with flies fed 2Y10S3C (standard) medium (two-way ANOVA; time,  $P < .0001$ ; yeast,  $P < .0001$ ; interaction,  $P < .0001$ ; \*Bonferroni's multiple comparisons (BMCs) versus 2Y10S3C;  $P < .0001$ ;  $n = 7-8$  per data point). B, ST50 values derived from (A). Yeast (Y) paste had a significant overall effect on ST50s (one-way ANOVA,  $P < .0001$ ,  $n = 7-8$ ). ST50s were greater in flies fed live or dead yeast versus 2Y10S3C medium (\*BMC,  $P < .0001$ ). ST50s were not distinguishable between flies fed live or dead yeast paste (BMC, .9682). C, Dietary yeast paste increased ST50 values in r[A] females (two-way ANOVA; yeast,  $P < .0001$ ; time, .0029; interaction, .3486; \*BMC, .0136 to  $<.0001$ ;  $n = 8$ )

diet (Figure 2). Increasing the yeast concentration in agar-based medium, like supplemental yeast paste, is therefore capable of eliciting resistance to ethanol sedation. Rearing flies on 2Y10S3C and 30Y10S3C promoted comparable patterns of adult emergence over time (Figure S1A) and comparable total numbers of progeny (Figure S1B), suggesting that our standard 2Y01S3C medium is not nutrient



**FIGURE 2** Supplementation of dietary media with yeast alters ethanol sedation. Flies were fed the indicated media for 1 to 3 days. Concentrations (w/v) of yeast (Y) used are underlined. ST50s in r[A] males (A) and females (B) were influenced by supplementing the diet with yeast (two-way ANOVA; yeast,  $P < .0001$ ; diet exposure time in males, .2665; diet exposure time in females, .0852; interaction in males, .0681; interaction in females, .2749;  $n = 6$ ). Compared with flies fed 2Y10S3C medium, ST50s were increased in flies fed media supplemented with yeast (\*BMCs, .068 to  $<.0001$ )

deficient, and therefore, the yeast-driven changes in ST50 (Figure 2) are likely to be related to nutrient supplementation versus restoration of sufficient nutrients. The data in Figure 2 also suggest that dietary yeast did not need to be alive to elicit resistance to ethanol sedation since the agar-based media were generated by boiling.

It seemed possible that increasing nutrient components other than yeast in dietary media might also influence ethanol sedation. We therefore fed flies standard agar-based media supplemented with sucrose or cornmeal and then measured their ST50s. We found that increasing these other nutrient components of dietary media for 1 to 3 days of feeding did not systematically or substantially alter ST50 values in males (Figures S2A and S2C) or females (Figures S2B and S2D). Although these experiments do not formally rule out a potential role for dietary sucrose or cornmeal in fly ethanol sedation resistance, they do indicate that altering these two components of the diet likely has a much more modest (if any) effect on ethanol sedation compared with yeast.

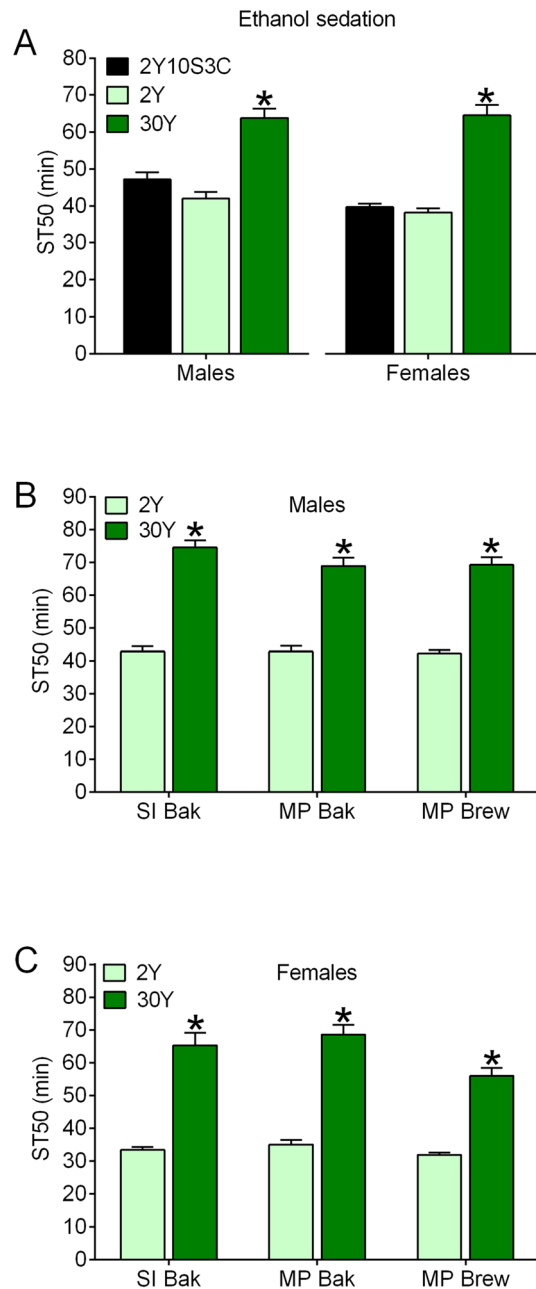
It also seemed possible that omitting other components of the fly media could affect ethanol sedation. We therefore measured ST50

values in male and female flies fed 2Y10S3C media with (+ATC) or without (-ATC) the antibiotics ampicillin, chloramphenicol, and tetracycline (Figure S3A) and with (+TEG) and without (-TEG) the antifungal Tegosept (Figure S3B). Additionally, to test whether omission of one or more of the nutrient components of 2Y10S3C medium could alter ethanol sedation, we assessed ST50 values in flies fed media that did not contain yeast, sucrose, or cornmeal individually (Figure S4A), lacked combinations of yeast and sucrose, sucrose and cornmeal, or yeast and cornmeal (Figure S4B), contained diluted media components (0.5× and 0.25×, Figure S4C), or contained no yeast, sucrose, or cornmeal (0×, Figure S4C). Ethanol sedation was not significantly affected by the omission of antibiotics from the media (Figure S3A), consistent with a previous report from our group,<sup>17</sup> nor by omitting or reducing Tegosept (Figure S3B), yeast, sugar, or cornmeal (Figures S4A and S4B), or all nutrient components (Figure S4C).

The results in Figures 1 and 2 and S2 to S4 collectively indicate that increasing dietary yeast is capable of increasing resistance to ethanol sedation. To more directly test this possibility, we assessed ST50 values in males and females fed our standard 2Y10S3C medium, a medium with 2% yeast as the only nutrient (2Y), or a medium with 30% yeast as the only nutrient (30Y). ST50 values were indistinguishable in flies fed 2Y10S3C and 2Y media (Figure 3A; left, males; right, females), consistent with our previous studies using media lacking sucrose or cornmeal (Figure S4). As expected, ST50 values were significantly greater in male and female flies fed a 30Y diet compared with both 2Y10S3C and 2Y (Figure 3A). These results confirm that manipulating the concentration of dietary yeast in the absence of other nutrients is sufficient for altering ethanol sedation.

The studies reported in all figures discussed thus far used saf-instant bakers (SI Bak) yeast. To address whether SI Bak yeast was unique in its ability to elicit resistance to ethanol sedation, we tested whether yeast from other sources could alter ST50 values (Figure 3 B,C). Males (Figure 3B) and females (Figure 3C) fed media containing 30% (30Y) SI Bak, MP bakers (MP Bak), or MP brewers (MP Brew) yeast were resistant to ethanol sedation compared with their sex-matched counterparts fed media with 2% yeast (2Y) from each source. Media with 30% of all three yeast sources had comparable effects on resistance to ethanol sedation in males (Figure 3B), whereas 30% MP Brew yeast had a smaller effect than the other two yeast sources in females (Figure 3C). The ability to induce resistance to ethanol sedation appears to be a common property of yeast. Additionally, our studies suggest that there could be subtle yeast × sex effects on ethanol sedation.

Like mammals, flies develop rapid ethanol tolerance, quantified as the change in resistance to ethanol during a second ethanol challenge after recovery from an initial exposure to the drug.<sup>24</sup> To determine whether a high yeast diet altered rapid tolerance in flies, we fed flies 2Y or 30Y media, measured their ethanol-naive ST50 values, allowed them to recover for 4 hour, and then measured their ST50 values during a second ethanol exposure. Males (Figure S5A) and females (Figure S5B) fed 2Y and 30Y media developed robust rapid tolerance, but the development of rapid tolerance to ethanol was not altered by diet in either sex (Figures S5A and S5B). This suggests that high



**FIGURE 3** Effects of multiple types of dietary yeast influences ethanol sedation. A, Flies were fed the indicated media for 2 days. ST50s were greater in r[A] males and females fed 30% yeast (30Y) compared with 2Y10S3C or 2% yeast (2Y) media (one-way ANOVAs; males,  $P < .0001$ ; females,  $P < .0001$ ; \*BMC versus other groups,  $P < .0001$ ;  $n = 8$ ). ST50s were increased in male (B) and female (C) flies fed 30Y versus 2Y media for 2 days (individual two-way ANOVAs: males—yeast concentration,  $P < .0001$ ; yeast source, .2509; interaction, .3232; females—yeast concentration,  $P < .0001$ ; yeast source, .0048; interaction, .1087; \*BMC versus 2Y,  $P < .0001$ ;  $n = 8$  for all groups). ST50s in females were lower on 30Y MP Brew than in 30Y SI Bak and MP Bak (BMC, .0202 and .0012, respectively)

concentrations of dietary yeast influence initial ethanol sedation, but not the development of rapid tolerance.

Flies from different genetic backgrounds can vary substantially in their feeding,<sup>66,81</sup> alcohol,<sup>22,82</sup> and other behaviors.<sup>83,84</sup> To determine

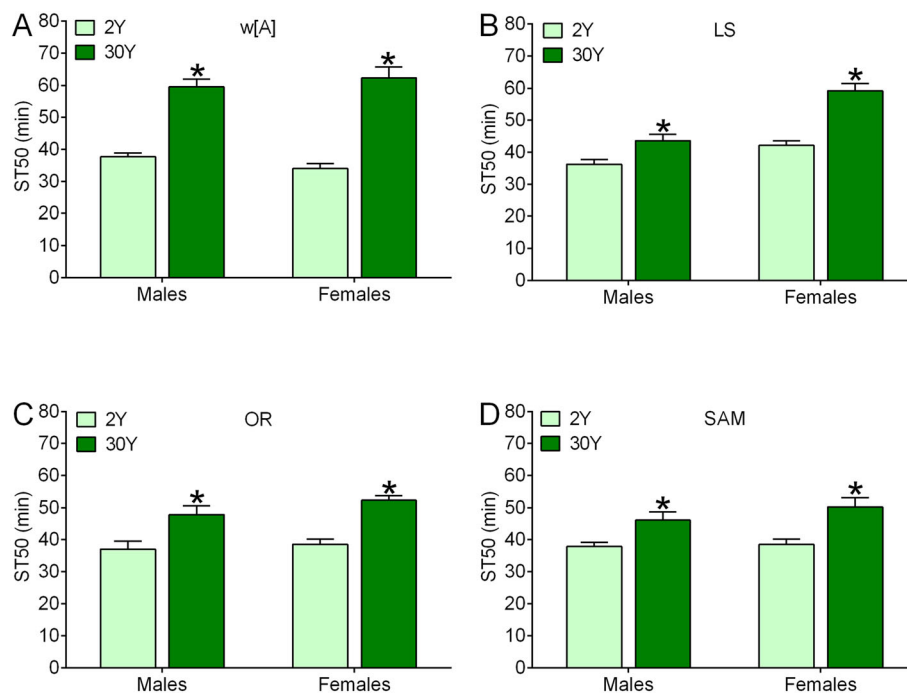
whether the effect of dietary yeast supplementation on resistance to ethanol sedation was a common property of flies, we measured ST50 values in four additional control strains (*w[A]*, Lausanne-S (LS), Oregon-R (OR), and Samarkand (SAM)) after feeding them 2Y or 30Y media for 2 days (Figure 4) or 1 day (Figure S6). Males and females fed 30Y medium had elevated ST50 values compared with flies fed 2Y medium in all cases. The magnitude of the supplemental yeast effect on ST50 values varied across the additional control strains tested (eg, compare *w[A]* and SAM in Figure 4A,D), consistent with widely appreciated genetic background effects on behavior. Although the effect of dietary yeast on ST50 values varied across the control strains tested, these data indicate that the increased resistance to ethanol sedation in response to supplemental dietary yeast is a common feature of flies. Additionally, these data confirm that providing flies with an elevated yeast diet for one or more days is sufficient to increase their resistance to ethanol sedation.

Altering the diet can lead to changes in the body mass of flies.<sup>85,86</sup> To determine if yeast supplementation altered body mass in our experiments, we measured total, dry, and water weight in several different control flies fed 2Y and 30Y media for 1 day. Compared with flies fed 2Y medium, flies on 30Y had increased total body mass in 9 of 10 cases, increased dry mass in 7 of 10 cases, and increased water weight in 8 of 10 cases (Table S1). To address if body mass might impact ethanol sedation, we explored whether total, dry, or water weight correlated with ST50 values in flies on 30Y vs 2Y media. Total, dry, and water weight did not correlate with ST50s in males or females

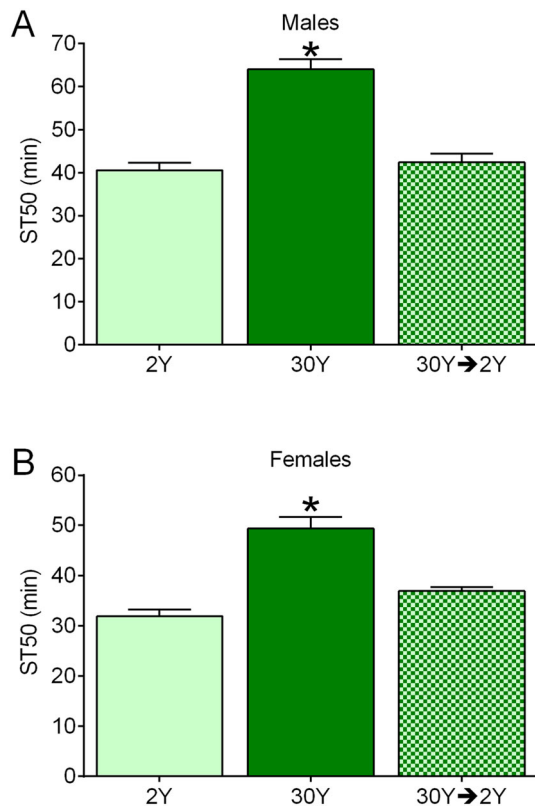
(Table S2). Additionally, feeding 30Y medium for 1 day increased ST50 values in males and females of all genotypes tested (Figures 2 and S6), even though some groups of animals did not have changes in total, dry, or water weight (Table S1). Thus, flies fed 30Y medium had increased total, dry, and water weight in most cases, but these changes were not required for altered ethanol sedation and body mass did not predict ST50 values.

To determine whether the effect of a high yeast diet on resistance to ethanol sedation was reversible, we fed flies 30Y medium for 2 days, switched them to 2Y for 2 days, then assessed their ST50 values. Flies fed 30Y food for 2 days were resistant to ethanol sedation compared with flies fed 2Y for 2 days as expected (Figure 5A, males; Figure 5B, females). In contrast, flies fed 30Y medium for 2 days and then switched to 2Y food for 2 days had ST50 values that were indistinguishable from flies fed 2Y medium only (Figure 5A (males), B (females)). The resistance to ethanol sedation driven by supplemental dietary yeast is therefore readily reversible in both males and females.

Flies are well known to adjust the volume of media they consume in response to changes in nutrient concentration in their diet.<sup>66,81,87</sup> This compensatory feeding is thought to help maintain steady total nutrient intake,<sup>81,87</sup> although this phenomenon does not always occur.<sup>66</sup> To address whether flies provided with 30Y medium consumed more nutrients than flies fed 2Y medium, we performed consumption-excretion (Con-Ex) studies using FD&C Blue 1 as a food tracer.<sup>66</sup> Males and females both consumed decreased volumes of



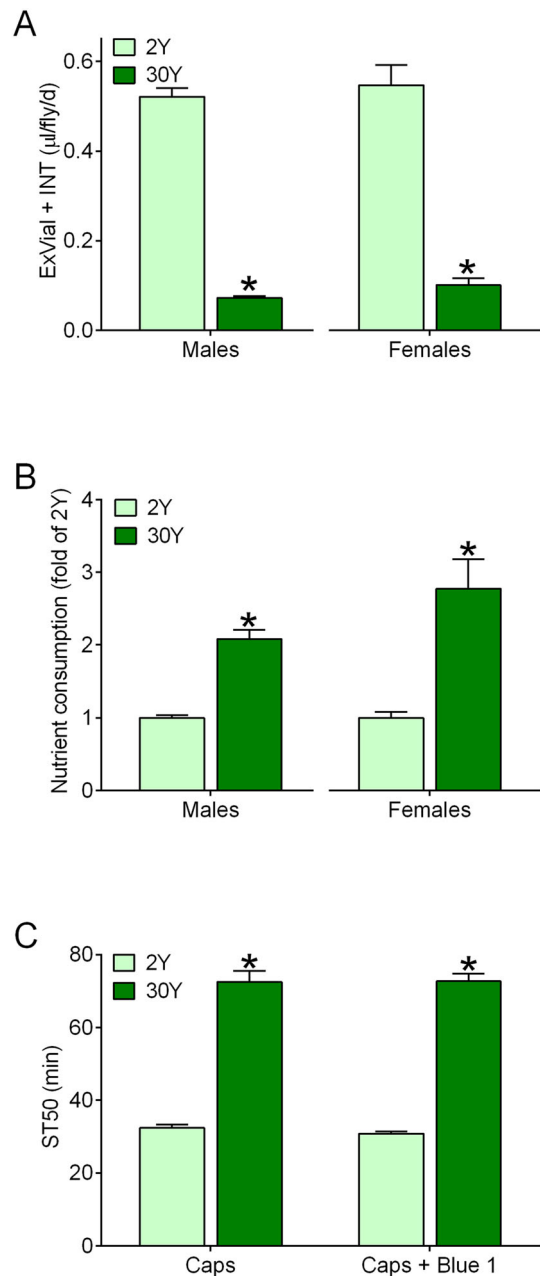
**FIGURE 4** Dietary yeast impacts ethanol sedation in flies from several different genetic backgrounds. Compared with flies fed 2Y medium, ST50s were increased in male and female *w[A]* (A), LS (B), OR (C), and Sam (D) after 2 days of feeding on 30Y medium (individual two-way ANOVAs; *w[A]*—yeast concentration,  $P < .0001$ ; sex, .8266; interaction, .1857; LS— yeast concentration,  $P < .0001$ ; sex,  $P < .0001$ ; interaction, .0137; OR—yeast concentration,  $P < .0001$ ; sex, .1756; interaction, .4990; Sam—yeast concentration, .0002; sex, .2905; interaction, .4390; \*BMC versus 2Y, .0299 to  $<.0001$ ;  $n = 6$  for all groups in all panels)



**FIGURE 5** Reversible effects of dietary yeast on ethanol sedation. Dietary regimen impacted ST50 values in males (A) and females (B) (individual one-way ANOVAs for effect of diet; males,  $P < .0001$ ; females,  $P < .0001$ ;  $n = 8$ ). Compared with flies fed only 2Y medium, ST50 values were increased in males and females fed 30Y medium for 2 days (\*BMC,  $P < .0001$ ;  $n = 8$ ), but not in flies fed 30Y for 2 days and then switched to 2Y for an additional 2 days (BMC; males,  $P > .9999$ ; females,  $p = 0.1097$ )

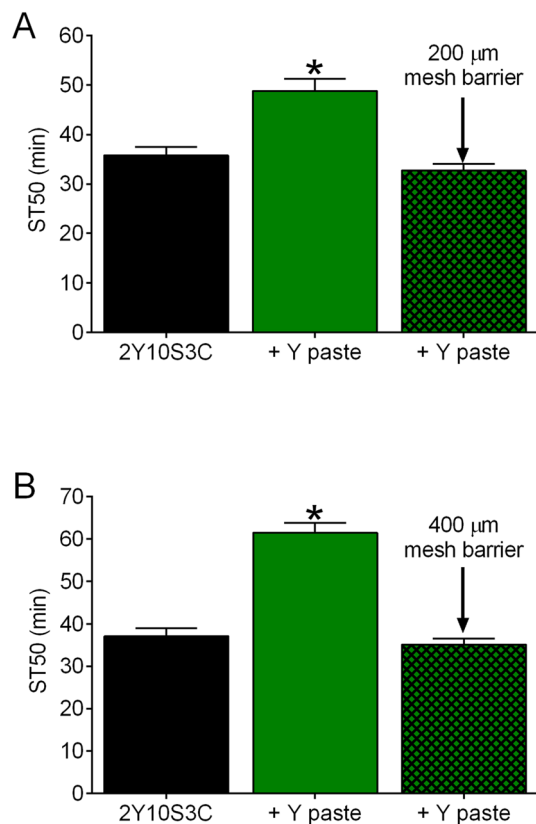
30Y versus 2Y media as anticipated (Figure 6A). Given that 30Y medium has 15-fold the yeast concentration of 2Y medium, the level of consumption represented in Figure 6A results in 30Y-fed flies ingesting at least 2-fold the total nutrients as flies fed 2Y (Figure 6B; males, left; females; right). Importantly, consumption of media from the feeder caps in Con-Ex experiments and the presence of FD&C Blue 1 in the media did not have discernable effects on yeast-induced resistance to ethanol sedation (Figure 6C). These data show that increased yeast nutrient consumption accompanies the increase in resistance to ethanol sedation, suggesting that they are causally linked.

Olfactory cues from yeast influence life span in flies.<sup>88</sup> To determine if olfactory cues from supplemental yeast are sufficient to elicit resistance to ethanol sedation, we assessed whether mesh barriers that prevented flies from directly contacting the yeast paste blocked the change in ST50 values. We used barriers with two different mesh sizes to test this possibility because (1) we reasoned that barriers of both sizes would eliminate the ability of flies to contact the food surface and (2) the larger mesh size would be more porous to olfactory cues from the yeast paste. Compared with flies fed standard medium, flies that physically contacted supplemental yeast paste were resistant



**FIGURE 6** Control *r[A]* flies consume more nutrients from 30Y versus 2Y media. A, Flies consumed-excreted lower volumes (ExVial +INT) of 30Y medium compared with 2Y medium during 24-hour Con-Ex studies (\*two-tailed  $t$  tests,  $P < .0001$ ; males,  $n = 8$ ; females,  $n = 6$ ). B, Flies consumed more nutrients (relative to 2Y, calculated from (A)) from 30Y medium compared with 2Y (\*two-tailed  $t$  tests; males,  $P < .0001$ ,  $n = 8$ ; females,  $.0016$ ;  $n = 6$ ). C, Flies fed 30Y medium had increased ST50 values compared with flies fed 2Y medium when all media were provided in feeder caps (Caps) for 2 days. Including Blue 1 in the media had no effect on ST50 values (two-way ANOVA; yeast concentration,  $P < .0001$ ; Blue 1,  $.7200$ ; interaction,  $.6652$ ; \*BMCs versus 2Y,  $P < .0001$ ;  $n = 12$ )

to ethanol sedation (Figure 7A,B) as expected (Figure 1). In contrast, ST50 values in flies that could not contact the supplemental yeast due to mesh barriers were indistinguishable from flies fed a standard



**FIGURE 7** Effect of dietary yeast paste on ST50 values requires physical contact. Flies had access to the indicated media for 2 days. Compared with flies fed 2Y10S3C medium, ST50 values were increased in flies that had access to yeast paste (green bars), but not in flies that were prevented from physically contacting the yeast paste by a mesh barrier (green hatched bars). There was an overall effect of treatment group in *w[A]* (A) and *r[A]* (B) females (one-way ANOVAs,  $P < .0001$ ,  $n = 8$  in A and B). ST50s were greater in flies with access to yeast paste compared with the other groups (\*BMC, .0003 to  $<.0001$ ). ST50s were indistinguishable in flies fed 2Y10S3C and in flies prevented from physically contacting the yeast paste (BMC (A) .8415; (B)  $P > .9999$ )

diet only (Figure 7A,B). The yeast-induced resistance to ethanol sedation therefore requires physical contact with, and presumably consumption of, the supplemental yeast to produce resistance to ethanol sedation.

The mechanism by which dietary yeast influences ethanol sedation in flies is of obvious interest. Intriguingly, a high yeast diet increases brain 5-HT levels in flies<sup>55</sup> [confirmed here (30Y:  $439.8 \pm 89.0$  fmol/brain,  $n = 11$ ; 2Y:  $231.7 \pm 36.9$  fmol/brain,  $n = 14$ ; *t* test,  $p = 0.0282$ )]. Additionally, serotonergic neuron function is important for regulating food consumption in larval and adult flies<sup>56–58</sup>; the 5-HT<sub>2A</sub> receptor plays a role in preference for dietary protein consumption in flies,<sup>55</sup> and 5-HT has been implicated in fly ethanol sedation.<sup>59</sup> Furthermore, there is a large literature linking 5-HT to alcohol problems in humans (eg,<sup>60–64</sup>). These findings collectively suggested that there could be mechanistic connections between serotonergic neurons and the effect of dietary yeast on ethanol sedation. To address

this possibility, we determined if suppression of serotonergic neurons influenced the effect of dietary yeast on ST50 or the consumption of high yeast medium.

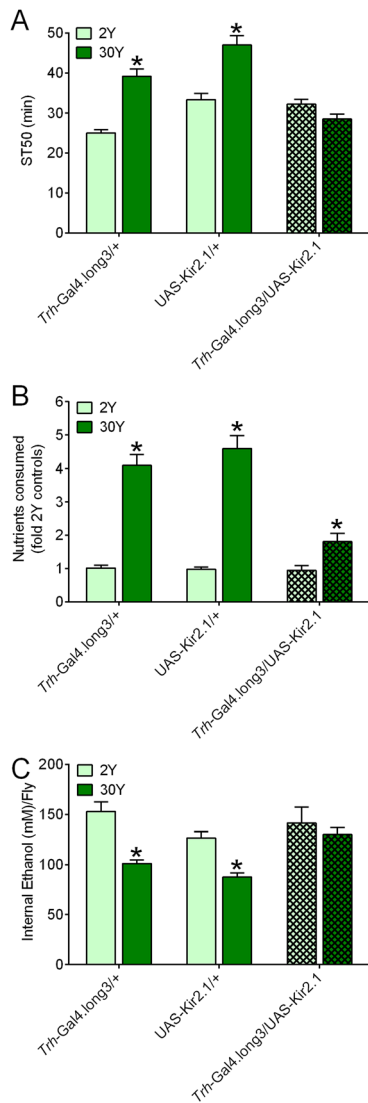
Compared with 2Y medium, ST50 values were increased by 30Y diet in control flies with the *Trh-Gal4.long3* or the *Trh-Gal4.long2* driver alone, a UAS-Kir2.1 transgene alone, or a UAS-TeTxLC(E2) transgene alone (first four bars, Figures 8A, 9A, S7A, S8A). These control flies also consumed more nutrients when fed 30Y medium (first four bars, Figures 8B, 9B, S7B, S8B). Inhibition of serotonergic neurons by expression of UAS-Kir2.1 (which hyperpolarizes neurons<sup>69</sup>) via *Trh-Gal4.long3* (Figure 8A, hatched bars) or via *Trh-Gal4.long2* (Figure S7A, hatched bars) blocked the effect of 30Y medium on ST50 values. Similarly, *Trh-Gal4*-driven expression of tetanus toxin light chain (UAS-TeTxLC(E2), which inhibits vesicle release<sup>68</sup>) in serotonergic neurons blocked the effect of yeast supplementation on ST50 values (Figures 9A and S8A, hatched bars). The effect of a high yeast diet on ethanol sedation therefore requires functional serotonergic neurons.

Regarding media consumption, flies expressing Kir2.1 via *Trh-Gal4.long3* had greater intake of nutrients when fed 30Y vs 2Y media (Figure 8B), but not when Kir2.1 was expressed by *Trh-Gal4.long2* (Figure S7B). Flies expressing tetanus toxin via both *Trh-Gal4* drivers consumed significantly more nutrients from 30Y versus 2Y media (Figures 9B and S8B). Thus, inhibition of serotonergic neurons did not consistently block the increase in nutrient intake on 30Y medium, but these flies appeared to consume fewer nutrients than control genotypes when on 30Y.

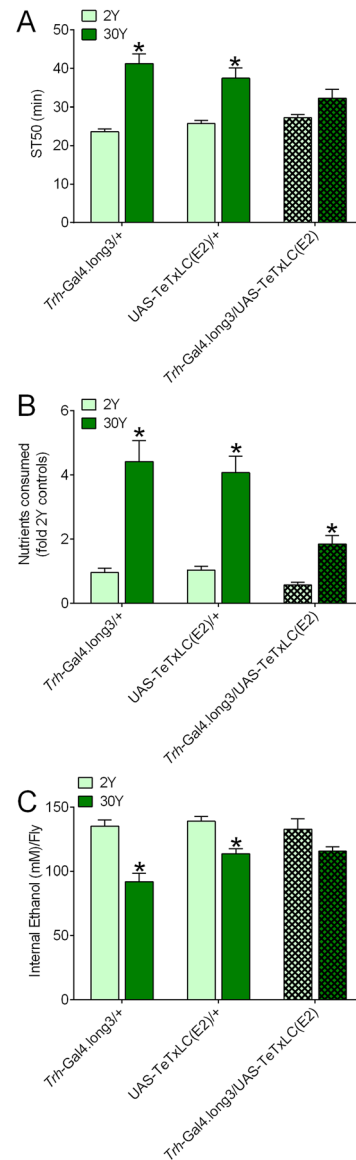
We postulated that a high yeast diet might impact net uptake/elimination of ethanol, and if true, that suppression of serotonergic neurons might influence internal ethanol levels in flies on a high yeast diet. We therefore measured internal ethanol in control flies and in flies expressing either UAS-Kir2.1 or UAS-TeTxLC(E2) in serotonergic neurons when fed 2Y or 30Y media. Internal ethanol concentrations during sedation from exogenous ethanol were decreased in control flies on 30Y vs 2Y media (Figures 8C and 9C, first four bars), indicating that a high yeast diet influences ethanol uptake and/or elimination. Interestingly, the effect of 30Y diet on internal ethanol levels was blocked by inhibition of serotonergic neurons via expression of UAS-Kir2.1 (Figure 8C, hatched bars) or UAS-TeTxLC(E2) (Figure 9C, hatched bars).

The data in Figures 8, 9, S7, and S8 raised the possibility that serotonergic neurons drive yeast consumption which in turn drives internal ethanol levels and ethanol sedation. To further explore this possibility, we determined whether there were correlations between nutrient consumption, internal ethanol levels, and ST50 values using data from Figures 8, 9, S7, and S8. We found strong, significant correlations between all pairs of measures (Figure 10). ST50 and nutrient intake exhibited a positive correlation (Figure 10A), while ST50 and internal ethanol (Figure 10B) as well as nutrient intake and internal ethanol (Figure 10C) exhibited negative correlations. These results support a model in which a high yeast diet leads to serotonergic neuron-dependent increases in nutrient intake, and that increased nutrient intake subsequently alters the uptake or elimination of ethanol resulting in lower internal ethanol levels, ultimately leading to increased ST50 values (Figure 11).

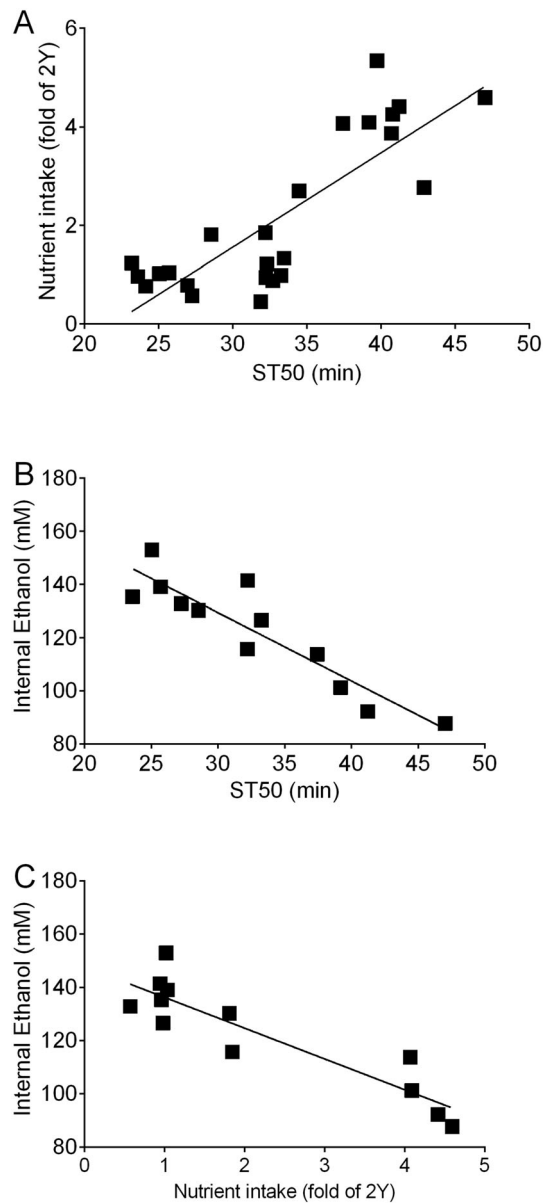




**FIGURE 8** Inhibition of serotonergic neurons with Kir2.1 blunts the effect of a high yeast diet on ethanol sedation, nutrient consumption, and internal ethanol levels. Male flies of the indicated genotypes consumed 2Y or 30Y media for 1 day prior to determination of ST50s, nutrient consumption, and internal ethanol. A, There were overall effects of yeast concentration and genotype on ST50s and an interaction between the two factors (two-way ANOVA; yeast,  $P < .0001$ ; genotype,  $P < .0001$ ; interaction,  $P < .0001$ ;  $n = 8$ ). Compared with flies fed 2Y medium, ST50s were greater in control flies (*Trh-Gal4.long3/+* and *UAS-Kir2.1/+*) on 30Y (\*BMC,  $P < .0001$ ), but not in flies with inhibition of serotonergic neurons (*Trh-Gal4.long3/+*; *UAS-Kir2.1/+*; hatched bars; BMC, .3174). B, Overall, yeast concentration and genotype influenced nutrient consumption, and there was an interaction between yeast and genotype (two-way ANOVA; yeast,  $P < .0001$ ; genotype,  $P < .0001$ ; interaction,  $P < .0001$ ;  $n = 8$ ). All genotypes consumed more nutrients from 30Y than 2Y (\*BMC,  $P < .001$ ). C, Overall, the concentration of dietary yeast and genotype influenced internal ethanol levels after exposure to vapor from 85% ethanol for 36 minutes (two-way ANOVA; yeast,  $P < .0001$ ; genotype, .0072; interaction, .0733;  $n = 8$ ). Internal ethanol was decreased in control flies (*Trh-Gal4.long3/+* and *UAS-Kir2.1/+*) fed 30Y versus 2Y media (\*BMC,  $P \leq .0094$ ), but yeast concentration had no effect on internal ethanol in *Trh-Gal4.long3/+*; *UAS-Kir2.1/+* flies (hatched bars; BMC,  $P > .9999$ )



**FIGURE 9** Expression of tetanus toxin in serotonergic neurons dampens the effect of dietary yeast on ethanol sedation, nutrient intake, and internal ethanol levels. Male flies were fed 2Y or 30Y media for 1 day. A, Overall, yeast concentration, but not genotype, influenced ST50s, and there was an interaction between yeast and genotype (two-way ANOVA; yeast,  $P < .0001$ ; genotype, .3451; interaction, .0058;  $n = 8$ ). Compared with flies fed 2Y medium, control *Trh-Gal4.long3/+* and *UAS-TeTxLC(E2)/+* flies fed 30Y had greater ST50s (\*BMCs,  $P \leq .0002$ ), but dietary yeast had no discernable effect on ST50s in flies expressing tetanus toxin light chain in serotonergic neurons (*Trh-Gal4.long3/+*; *UAS-TeTxLC(E2)/+*; hatched bars; BMC, .1996). B, Yeast and genotype had significant overall effects on nutrient consumption, and there was an interaction between the factors (two-way ANOVA; yeast,  $P < .0001$ ; genotype,  $P < .0001$ ; interaction, .0053;  $n = 6-8$ ). All genotypes consumed more nutrients on 30Y versus 2Y (\*BMCs,  $P \leq .0257$ ). C, Overall, internal ethanol was affected by yeast concentration and genotype, but there was no interaction between the factors (two-way ANOVA; yeast,  $P < .0001$ ; genotype, .0472; interaction, .0524;  $n = 8$ ). Compared with flies fed 2Y, internal ethanol was decreased in control *Trh-Gal4.long3/+* and *UAS-TeTxLC(E2)/+* flies fed 30Y (\*BMCs,  $P \leq .0045$ ), but not in *Trh-Gal4.long3/+*; *UAS-TeTxLC(E2)/+* flies (BMC, .0807)



**FIGURE 10** Correlations between ST50, nutrient intake, and internal ethanol levels. Data from Figures 8, 9, S7, and S8 were combined to assess correlations between ST50, nutrient intake, and internal ethanol levels. A, There was a positive correlation between ST50 and nutrient intake (Pearson  $r = 0.827$ ,  $P < .0001$ ,  $n = 24$ ). B, ST50 values inversely correlated with internal ethanol levels (Pearson  $r = -0.913$ ,  $P < .0001$ ,  $n = 12$ ). C, Nutrient intake negatively correlated with internal ethanol levels (Pearson  $r = -0.903$ ,  $P < .0001$ ,  $n = 12$ ). Lines are best fit linear regressions

## 4 | DISCUSSION

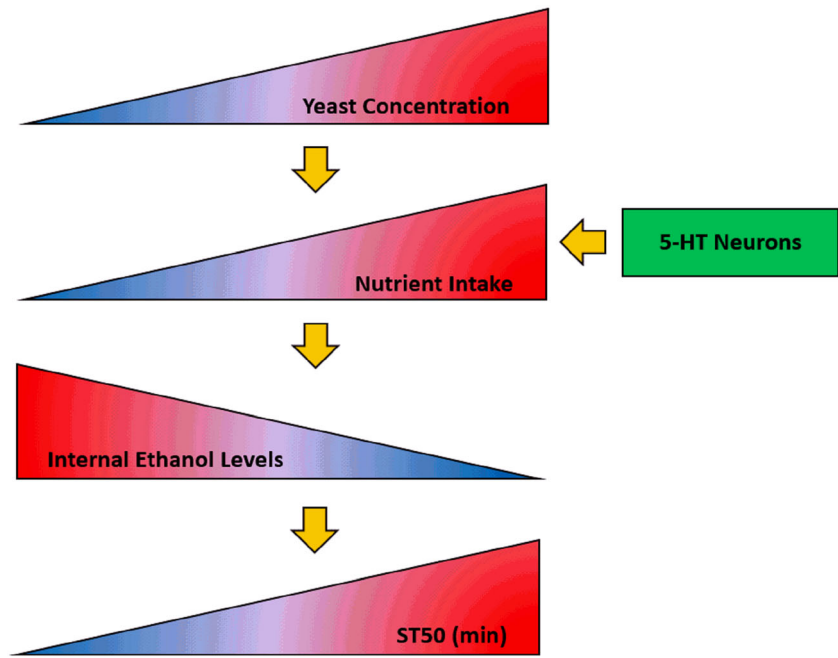
Fruit flies are an important genetic model organism for investigating the molecular basis of a plethora of physiological outputs including alcohol-related behaviors,<sup>11,14–31</sup> food consumption,<sup>66,81</sup> and responses to diet.<sup>67,85,89–97</sup> To the best of our knowledge, our studies are the first to integrate these three areas of biology in the fly. We find that increasing the concentration of yeast in the diet, but not increasing other dietary components or decreasing all components of our standard

medium, makes flies resistant to ethanol sedation. The resistance to ethanol sedation requires physical access to dietary yeast, is a common property of yeast, is seen in both males and females of multiple control strains, is reversible, appears to be caused by a mechanism independent of rapid tolerance, and is associated with increased yeast nutrient consumption as well as decreased internal ethanol levels. Importantly, the effect of a high yeast diet on ethanol sedation and internal ethanol levels is blunted by inhibition of serotonergic neurons.

In principle, our data on yeast supplementation and ethanol sedation could be interpreted in two ways. One interpretation is that yeast supplementation of a diet otherwise capable of supporting growth and normal behavior causes resistance to ethanol sedation. A second, alternative interpretation is that decreasing the concentration of dietary yeast below that required for normal growth and behavior leads to ethanol sedation sensitivity. We favor the former interpretation for several reasons. In previous studies, adult flies reared on our standard medium weigh approximately the same (eg, approximately 1 mg for females<sup>19,20,22</sup>) as flies grown under routine conditions used in other labs (eg,<sup>98</sup>). In the studies reported here, flies reared on our standard 2Y10S3C and supplemented 30Y10S3C media emerged with similar time courses and in the same numbers. These results suggest that flies grown on 2Y10S3C are not nutrient-deprived. Additionally, the increased resistance to alcohol sedation in our studies requires yeast concentrations in excess of 10%, which is higher than yeast concentrations used in routine fly culture. Our interpretation of these observations is that yeast supplementation of a diet otherwise sufficient in nutrients is capable of increasing resistance to ethanol sedation. It is extremely challenging, however, to formally rule out the possibility that flies fed our standard medium are not at least somewhat nutrient-deprived. Thus, it is a matter of perspective whether our data are interpreted to mean that yeast supplementation increases resistance to ethanol sedation or that yeast restriction decreases resistance to ethanol sedation. Importantly, either interpretation wholly supports the hypothesis that the concentration of yeast in the fly diet influences ethanol sedation.

Each *Drosophila* laboratory can and often does use a unique recipe for fly media. Differences in fly media composition could lead to variability in baseline ethanol sedation or potentially a lack of reproducibility of results across laboratories. We suggest that it become standard practice in the field to report all components and the concentrations used for fly media for studies on alcohol sedation as has been suggested previously for studies in other areas.<sup>67</sup>

The ability to manipulate ethanol sedation by changing the yeast concentration in the fly diet expands the utility of the *Drosophila* model for investigating genes and genetic pathways that underlie alcohol-related behaviors. With our data as a backdrop, the fly model should be suitable for pursuing at least three major areas of research: molecular and cellular mechanisms like serotonergic signaling that drive nutrient consumption as it relates to ethanol sedation, nutrient-driven changes in ethanol uptake and/or elimination, and pathways downstream of nutrient intake that change behavioral responses to alcohol. It is interesting to speculate that at least some genetic manipulations known to influence resistance to ethanol



**FIGURE 11** Model for effect of dietary yeast on ethanol sedation. As the yeast concentration in the diet increases, nutrient intake increases, internal ethanol levels decrease, and the time to sedation (ST50) is extended. 5-HT neurons positively regulate nutrient intake and thereby influence the effect of dietary yeast on internal ethanol and ST50

sedation in flies or other species might relate to one or more of these three areas.

Dietary yeast influences brain 5-HT content in flies<sup>55</sup>; 5-HT likely plays a role in fly ethanol sedation,<sup>59</sup> and 5-HT is connected to problematic alcohol consumption in humans (eg,<sup>60-64</sup>). Additionally, serotonergic neurons and serotonin signaling are important for hunger/satiety and feeding behavior in both larval and adult flies.<sup>56-58,99</sup> Our studies in flies suggest that serotonergic neurons might influence ethanol sedation via effects on nutrient consumption and ethanol uptake/elimination, raising the possibility that there could be a link between 5-HT, diet, and alcohol-related behavior in other species.

The effect of diet on alcohol-related behavior is not unique to flies. In *C. elegans*, mutations that disrupt synthesis of eicosapentaenoic acid (EPA, an omega-3 polyunsaturated fatty acid) blunt the development of acute functional tolerance to alcohol, and dietary supplementation with this fatty acid facilitates acute functional tolerance.<sup>50</sup> Reduced caloric intake in rats enhances the alcohol deprivation effect and reinstatement of ethanol-seeking behavior,<sup>46</sup> and food deprivation decreases alcohol drinking in mice.<sup>47</sup> Furthermore, providing mice with different, but otherwise routinely used, laboratory diets influences ethanol drinking, ethanol consumption, and ethanol-induced locomotion,<sup>48</sup> and altering EPA in the diet of mice influences both ethanol sensitivity and consumption.<sup>49</sup> These results indicate that diet-induced changes in alcohol-related behavior are a common feature of metazoans. Therefore, identification of the underlying mechanisms via studies like those described here has the potential to be valuable for both prevention and treatment of AUD.

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#### AUTHOR CONTRIBUTIONS

M.G. initially conceived the project. R.E.S., M.R.M., B.C.S., E.K.D., H.-F. F., B.J.V., K.L.S., S.D.P., and M.G. designed the experiments. R.E.S., M. R.M., B.C.S., E.K.D., K.L.S., and H.-F.F. performed the studies. R.E.S., B. C.S., E.K.D., H.-F.F., B.J.V., K.L.S., and M.G. analyzed the data. M.G. and R.E.S. wrote the manuscript, and R.E.S., M.R.M., B.C.S., E.K.D., B. J.V., K.L.S., S.D.P., and M.G. edited the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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