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Oxidative stress contributes to outcome severity in a *Drosophila melanogaster* model of classic galactosemia

Patricia P. Jumbo-Lucioni¹, Marquise L. Hopson¹, Darwin Hang², Yongliang Liang³, Dean P. Jones³ and Judith L. Fridovich-Keil^{1,*}

SUMMARY

Classic galactosemia is a genetic disorder that results from profound loss of galactose-1P-uridylyltransferase (GALT). Affected infants experience a rapid escalation of potentially lethal acute symptoms following exposure to milk. Dietary restriction of galactose prevents or resolves the acute sequelae; however, many patients experience profound long-term complications. Despite decades of research, the mechanisms that underlie pathophysiology in classic galactosemia remain unclear. Recently, we developed a *Drosophila melanogaster* model of classic galactosemia and demonstrated that, like patients, GALT-null *Drosophila* succumb in development if exposed to galactose but live if maintained on a galactose-restricted diet. Prior models of experimental galactosemia have implicated a possible association between galactose exposure and oxidative stress. Here we describe application of our fly genetic model of galactosemia to the question of whether oxidative stress contributes to the acute galactose sensitivity of GALT-null animals. Our first approach tested the impact of pro- and antioxidant food supplements on the survival of GALT-null and control larvae. We observed a clear pattern: the oxidants paraquat and DMSO each had a negative impact on the survival of mutant but not control animals exposed to galactose, and the antioxidants vitamin C and α -mangostin each had the opposite effect. Biochemical markers also confirmed that galactose and paraquat synergistically increased oxidative stress on all cohorts tested but, interestingly, the mutant animals showed a decreased response relative to controls. Finally, we tested the expression levels of two transcripts responsive to oxidative stress, *GSTD6* and *GSTE7*, in mutant and control larvae exposed to galactose and found that both genes were induced, one by more than 40-fold. Combined, these results implicate oxidative stress and response as contributing factors in the acute galactose sensitivity of GALT-null *Drosophila* and, by extension, suggest that reactive oxygen species might also contribute to the acute pathophysiology in classic galactosemia.

INTRODUCTION

Galactose is essential for life in metazoans. Derivatives of galactose in glycoconjugates are key elements of cell membrane structures, hormones, extracellular matrix, immunologic determinants and structural elements of the central nervous system, among other roles (Segal, 1995). For mammalian infants, galactose is also an important source of sugar calories as it represents half of the monosaccharide liberated from the digestion of lactose. For full catabolism, however, galactose must be converted into glucose-1-phosphate (glc-1P) via the Leloir pathway (Frey, 1996; Berg, 2002; Holden et al., 2003). In humans, a deficiency of the second enzyme of the Leloir pathway, galactose-1-phosphate uridylyltransferase (GALT, E.C. 2.7.7.12), results in the autosomal recessive, potentially lethal disorder classic galactosemia (OMIM 230400) (Fridovich-Keil and Walter, 2008; Bennett, 2010; Bosch, 2011).

Infants with classic galactosemia experience acute symptoms within days to weeks of beginning to nurse or drink a milk-based

formula. Symptoms can escalate rapidly from vomiting and failure to thrive to cataracts, hepatomegaly, *Escherichia coli* sepsis and neonatal death (reviewed in Fridovich-Keil and Walter, 2008). Dietary restriction of galactose, generally implemented by switching the infant from milk to a soy-based formula, prevents or resolves the acute symptoms. Unfortunately, despite early and rigorous dietary restriction of galactose, many patients grow to experience intellectual disability, speech difficulties, locomotor impairment and, for girls and women, primary or premature ovarian insufficiency, among other complications. We, and others, have reported that these long-term complications develop regardless of how early treatment is initiated, how rigorously galactose intake is restricted or how closely patients are followed clinically (Waggoner et al., 1990; Schweitzer-Krantz, 2003; Bosch, 2006; Fridovich-Keil, 2006; Hughes et al., 2009; Jumbo-Lucioni et al., 2012).

Despite decades of research, there is still no clear understanding of the pathophysiology that underlies either the acute or long-term complications of classic galactosemia (Tyfield and Walter, 2002; Leslie, 2003; Fridovich-Keil and Walter, 2008); however, a number of intriguing hypotheses have been put forward (reviewed in Tyfield and Walter, 2002; Leslie, 2003; Fridovich-Keil and Walter, 2008). These include ATP depletion via futile cycles of phosphorylation and dephosphorylation of galactose (Mayes and Miller, 1973), inhibition of key enzymes by galactose-1-phosphate (gal-1P) (Wells et al., 1969; Gitzelmann, 1995; Parthasarathy et al., 1997; Bhat, 2003) and depleted UDP-gal leading to impaired galactosylation of cerebrosides (Lebea and Pretorius, 2005).

Until recently, studies exploring factors contributing to pathophysiology in classic galactosemia have been limited by the lack of a genetic animal model that recapitulates the patient

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TRANSLATIONAL IMPACT

Clinical issue

Classic galactosemia results from profound loss of galactose-1P uridylyltransferase (GALT), the second enzyme in the Leloir pathway of galactose metabolism. More than 100 children are born in the USA each year with this condition. Affected infants appear normal at birth, but experience a rapid escalation of potentially lethal symptoms following exposure to milk, which contains high levels of galactose. Despite more than 50 years of research, the underlying basis of galactose sensitivity in GALT deficiency has remained unclear, hampering efforts at prognosis and improved intervention.

Results

Here, the authors apply a *Drosophila melanogaster* model of galactosemia to test whether oxidative stress contributes to the pathophysiology of GALT deficiency. They use nutritional, biochemical and genetic approaches, testing the impact of dietary oxidants and antioxidants on survival of developing animals, measuring the accumulation of biochemical markers of oxidative stress and response, and quantifying the expression levels of genes involved in the oxidative stress response. The results indicate that galactose exposure contributes to oxidative stress in *Drosophila* and demonstrate that GALT-null *Drosophila* are dramatically more vulnerable to this stress than GALT-expressing flies.

Implications and future directions

These findings indicate that oxidative stress and/or the physiological response to oxidative stress might play a role in classic galactosemia in humans, opening up new opportunities for diagnosis, prognosis and treatment.

outcome. Nonetheless, numerous studies have been reported using so-called experimental animal models (i.e. genetically normal animals exposed to high levels of dietary galactose) to explore the impact of galactose on animal physiology. These experimental mouse (Wei et al., 2005; Cui et al., 2006; Long et al., 2007), and *Drosophila melanogaster* (Jordens et al., 1999; Cui et al., 2004) models have provided compelling evidence that D-galactose exposure decreases lifespan and that this effect is galactose-specific (Jordens et al., 1999). High level galactose exposure of genetically normal mice and/or dogs has also been associated with negative long-term outcomes that include neurodegeneration, and cognitive disability (Shang et al., 2001; Shen et al., 2002), diminished immune response (Song et al., 1999; Shang et al., 2001) and retinal degeneration (Engerman and Kern, 1984). Reports on D-galactose-treated rodents (Yelinova et al., 1996; Kowluru et al., 1997) and flies (Cui et al., 2004) suggest that galactose metabolism in these systems leads to oxidative stress and that the resulting oxidative damage accounts for the life-shortening effect of the exposure. Galactose-dependent free-radical generation observed in rat brain homogenates was also reversible after antioxidant administration (Tsakiris et al., 2005). Paradoxically, despite heightened oxidative stress biomarkers, galactose-treated rodents, flies and tissue culture cells also demonstrated evidence of lower than expected antioxidant enzyme activities (Cui et al., 2004; Cui et al., 2006), suggesting that the normal defences might be compromised; no mechanism has yet been established. Of note, anecdotal studies have shown that galactosemic patients on poor dietary control also displayed lower total antioxidant status along with remarkably increased markers of oxidative stress (Schulpis et al., 2005; Schulpis et al., 2006).

Recently, we established a *Drosophila melanogaster* genetic model of classic galactosemia that recapitulates significant aspects of the patient phenotype (Kushner et al., 2010). Like human

patients, GALT-null *Drosophila* succumb in development following galactose exposure but survive to adulthood under dietary galactose restriction or when rescued by expression of a wild-type human GALT transgene (Kushner et al., 2010). Also like patients, GALT-null flies, but not controls, accumulate significantly elevated levels of gal-1P following exposure to galactose (Kushner et al., 2010). Here we have used a threefold approach to test whether oxidative stress contributes to the acute galactose sensitivity of GALT-null *Drosophila*.

First, we tested the impact of dietary oxidants and antioxidants on the survival rates of GALT-null and control *Drosophila* exposed to galactose in development. Second, we monitored biochemical markers of oxidative stress response, including reduced and oxidized glutathione and cysteine intermediates, in representative samples. Finally, we tested the expression levels of two genes responsive to oxidative stress, *GSTD6* and *GSTE7*, in GALT-null and control larvae after acute exposure to galactose. Our results implicate oxidative stress in the mechanism of galactose toxicity in GALT-deficient *Drosophila*, and raise the intriguing possibility that oxidative stress might also play a role in the acute pathophysiology of classic galactosemia.

RESULTS

Oxidants paraquat and DMSO increase the acute galactose sensitivity of GALT-null *Drosophila*

We tested the impact of two oxidants, paraquat and dimethyl sulfoxide (DMSO), on the acute galactose sensitivity of GALT-null *Drosophila* by adding each compound at selected doses to vials of fly food either with or without galactose. Of note, all fly food also contained 555 mM glucose. To select an appropriate dose of galactose for these experiments, we first tested the relationship between galactose concentration in the food and survival of mutant and control *Drosophila* to adulthood under the conditions used here (see Methods). We saw a clear dose-dependent negative impact of galactose on survival of the mutant but not the control animals (supplementary material Fig. S1), and selected 200 mM galactose as the optimal dose for further experiments because the survival impact on GALT-null animals was robust but survival rates were not so low as to prevent us from seeing a potential further negative impact from other factors (e.g. dietary oxidants).

To test the impact of paraquat and DMSO, we monitored the survival rates of mutant and control *Drosophila* deposited in fixed numbers as first-instar larvae (L1) into replicate vials containing fly food that either did or did not include 200 mM galactose and that also either did or did not contain specified levels of paraquat or DMSO (see Methods). Of note, only oxidant levels that had no significant impact on survival rates of control animals were pursued.

For paraquat, these levels included 0, 50, 100 and 200 μ M. Although these levels had no significant impact on the survival rates of control larvae regardless of the presence or absence of galactose (Fig. 1A,B, open bars; supplementary material Fig. S2A,B), there was a marked impact on the survival rates of GALT-null larvae in the presence of galactose (Fig. 1A,B, shaded bars). Specifically, the three increasing levels of paraquat decreased the survival rates of GALT-null animals to pupation in galactose-supplemented food (Fig. 1A) by approximately 24, 37 and 58%, respectively, and to adulthood (Fig. 1B) by approximately 46, 55 and 73%, respectively. These differences were statistically significant, as indicated in Fig.

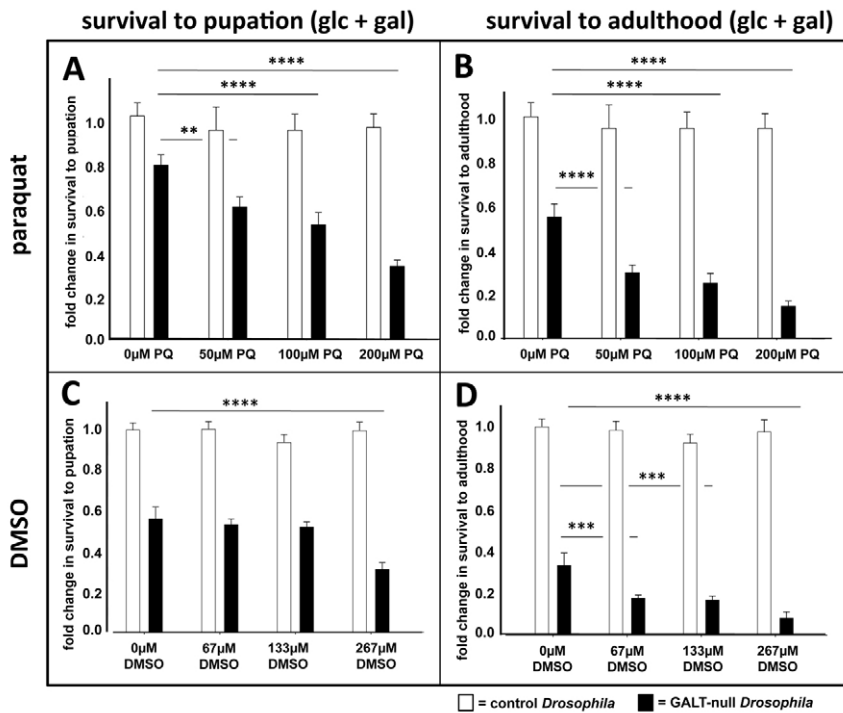


Fig. 1. Effects of oxidants on survival of control and GALT-null *Drosophila* exposed to galactose.

(A-D) Survival of control (open bars) and GALT-null (shaded bars) *Drosophila* larvae was monitored to pupation (A,C) and to adulthood (B,D) under the conditions indicated. PQ, paraquat; glc, glucose; gal, galactose. Values show mean + s.e.m. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. Values show mean + s.e.m. Corresponding data for animals raised in the absence of galactose are presented in supplementary material Fig. S2.

1. There was no apparent impact on the survival of animals maintained on food that did not contain galactose (supplementary material Fig. S2A,B).

For DMSO, the levels tested were 0, 67, 133 and 267 μM . As with paraquat, in the presence of galactose we saw a dose-dependent negative impact on survival of the mutant but not the control animals to adulthood (Fig. 1C,D). In the absence of galactose (supplementary material Fig. S2C,D), most vials showed unaffected survival rates although DMSO supplementation at 267 μM did decrease the survival of GALT-null *Drosophila* to pupation and eclosion by just over 10% compared with controls.

Antioxidants vitamin C and α -mangostin are protective against the acute galactose sensitivity of GALT-null *Drosophila*

We also tested the impact of two antioxidants, vitamin C (ascorbate) (Rose and Bode, 1993; Duarte and Lunec, 2005) and α -mangostin (Bumrungpert et al., 2010), on the survival of GALT-null and control *Drosophila* larvae deposited on fly food containing either glucose or glucose plus galactose (see Methods). As with the oxidant exposures, these experiments were conducted using levels of antioxidant (20, 40 and 80 μM for vitamin C and 40, 120 and 360 μM for α -mangostin) that had no significant impact on the survival rates of control animals regardless of sugar exposure (Fig. 2; supplementary material Fig. S3). Unlike controls, which demonstrated no marked response (Fig. 2, open bars; supplementary material Fig. S3), we found a significant positive impact of antioxidant treatment on the survival rates of GALT-null larvae exposed to galactose (Fig. 2, shaded bars). In brief, the addition of vitamin C at 80 μM significantly ($P = 0.0022$) rescued the survival of GALT-deficient larvae to pupation (about 33% increase; Fig. 2A). vitamin C at both 40 and 80 μM produced a significant ($P < 0.0001$) and dose-dependent increase in the survival rates of mutant larvae to adulthood (approximately 77 and 127%

increases, respectively; Fig. 2B). Similarly, all doses of α -mangostin tested significantly ($P < 0.0001$) increased the survival rates of galactose-exposed mutant larvae to pupation (Fig. 2C), and the one dose (40 μM) that showed the strongest impact on survival to pupation also significantly ($P = 0.0026$) increased the survival of mutant animals to adulthood (about 87% increase; Fig. 2D). None of the vitamin C or α -mangostin doses tested significantly impacted the survival rates of either mutant or control larvae in the absence of galactose (supplementary material Fig. S3).

Gal-1P accumulation in GALT-null *Drosophila* is unaffected by oxidant or antioxidant exposures

Accumulation of gal-1P is a common marker of impaired Leloir function in patients and model systems, and all factors reported to date that relieve the lethal or growth-inhibitory effects of galactose exposure in the face of impaired GALT function have done so apparently by lowering the accumulation of gal-1P (Douglas and Hawthorne, 1964; Mehta et al., 1999; Kabir et al., 2000; Lai and Elsas, 2000; Ross et al., 2004). We therefore sought to test whether oxidants or antioxidants might also impact galactose sensitivity by modulating the accumulation of gal-1P in our GALT-null *Drosophila*. Toward this end, we extracted and quantified gal-1P from control and mutant third-instar (L3) larvae that had been exposed to food containing glucose or glucose plus galactose, with or without 100 μM paraquat or 80 μM vitamin C (see Methods).

As expected, when raised on food lacking galactose, control animals (Fig. 3A, open bars) accumulated only trace levels of gal-1P and GALT-null animals (Fig. 3A, solid bars) accumulated levels that were notably higher, probably reflecting the endogenous biosynthesis of galactose (Berry et al., 1995). In the presence of dietary galactose, GALT-null larvae accumulated levels of gal-1P that were more than 30-fold higher than those seen in their GALT-normal counterparts (Fig. 3B). What was most striking, however,

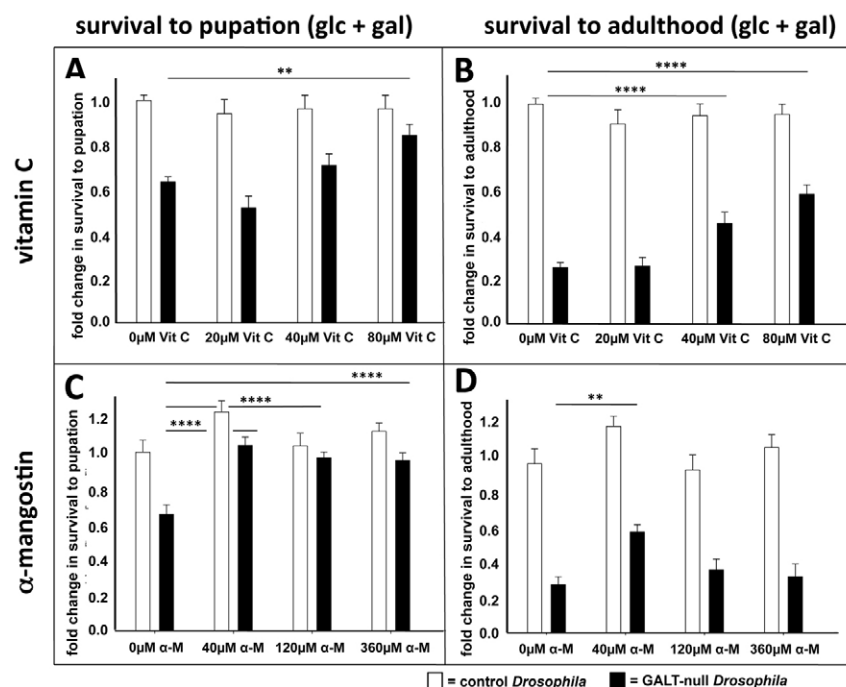


Fig. 2. Effects of antioxidants on survival of control and GALT-null *Drosophila* exposed to galactose.

(A-D) Survival of control (open bars) and GALT-null (shaded bars) *Drosophila* larvae was monitored to pupation (A,C) and to adulthood (B,D) under the conditions indicated. VitC, vitamin C; α-M, α-mangostin; glc, glucose; gal, galactose. Values show mean + s.e.m. ** $P < 0.01$; **** $P < 0.0001$. Corresponding data for animals raised in the absence of galactose are presented in supplementary material Fig. S3.

was that the mutant larvae demonstrated the same extremely high levels of gal-1P regardless of the presence or absence of either vitamin C or paraquat (Fig. 3B). In short, the marked impacts of paraquat and vitamin C on survival of galactose-exposed GALT-null *Drosophila* were not explained by changes in the levels of gal-1P that accumulated in those animals.

Impact of paraquat and vitamin C on oxidized and reduced glutathione and cysteine levels in GALT-null *Drosophila* exposed to galactose

As a biochemical approach to explore the impact of galactose exposure on oxidative stress in control and GALT-null *Drosophila*, we monitored the levels of reduced and oxidized glutathione (GSH and GSSG, respectively) and cysteine (Cys and CySS, respectively) in control and mutant larvae exposed to galactose. We also used the ratios of these reduced and oxidized moieties to estimate intracellular and extracellular redox potentials (E_h), respectively. As before, parallel cohorts of mutant and control larvae were exposed to food containing either glucose or glucose plus galactose

supplemented with no additive, with 100 µM paraquat or with 80 µM vitamin C, as described in Methods.

Galactose supplementation alone produced a small but significant ($P < 0.0001$) increase in GSH levels in both mutant and control animals and this increase reverted to near normal levels in the presence of vitamin C (Fig. 4A). Interestingly, paraquat exposure of both genotypes in the presence of galactose dramatically decreased GSH levels (Fig. 4A). The converse was true for GSSG; paraquat exposure in the presence of galactose caused a marked increase ($P < 0.0001$) in GSSG levels in both mutant and control animals, but the magnitude of the increase for mutants was only about half that seen for controls (Fig. 4B). Multivariate analysis of variance (MANOVA) also revealed a significant ($P < 0.0001$) genotype by diet by treatment interaction for intracellular redox state (E_h). Specifically, paraquat exposure in the presence of galactose caused a significant increase in intracellular E_h for both mutant and control animals ($P < 0.0001$), but the magnitude of the change was diminished for mutants relative to controls (Fig. 4C). Notably, in the absence of galactose we saw no significant impact

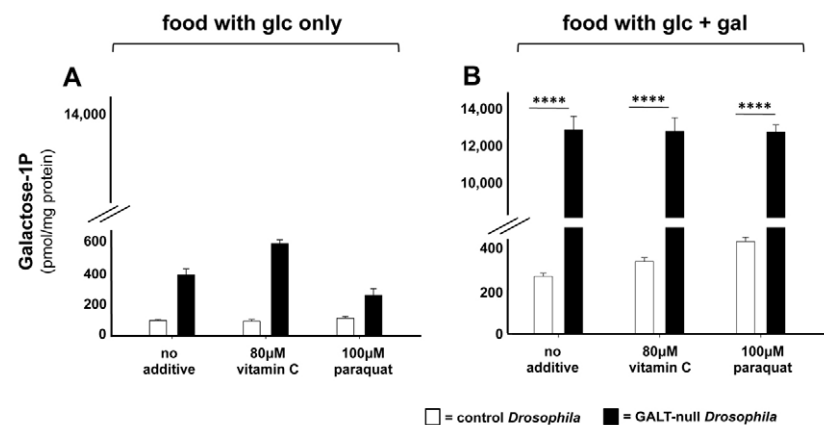


Fig. 3. Impact of vitamin C and paraquat on the accumulation of gal-1P in control and GALT-null *Drosophila* maintained in the absence and presence of galactose.

(A,B) Gal-1P was extracted from control (open bars) and GALT-null (shaded bars) larvae maintained under the conditions indicated. Gal-1P values in animals maintained on food (A) with glucose only, and (B) including both glucose and galactose. glc, glucose; gal, galactose. Values show mean + s.e.m. **** $P < 0.0001$.

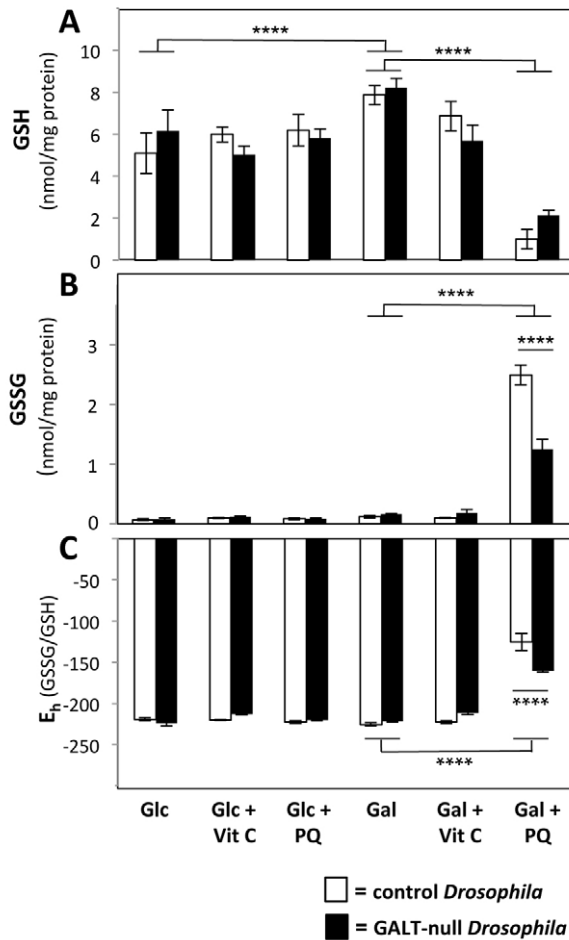


Fig. 4. Impact of vitamin C and paraquat on the levels of reduced and oxidized glutathione in control and GALT-null *Drosophila* maintained on glucose or glucose plus galactose food. (A-C) Samples tested were from control animals (open bars) or GALT-null animals (shaded bars). (A) Levels of reduced glutathione (GSH), (B) levels of oxidized glutathione (GSSG) and (C) intracellular E_h estimated from the GSH and GSSG levels. VitC, vitamin C; PQ, paraquat; Glc, glucose; Gal, galactose. Values show mean \pm s.e.m. **** P <0.0001.

of either vitamin C or paraquat on GSH, GSSG or intracellular E_h in mutants or controls (Fig. 4A-C, first three sets of bars in each panel).

Data analysis by MANOVA also revealed a significant (P <0.0001) interaction between GALT genotype, diet and exposure to vitamin C or paraquat for Cys (P <0.05), CySS (P <0.02) and Cys-GSH, a disulfide intermediate of glutathione metabolism (Fig. 5). Specifically, galactose exposure alone triggered a small but significant (P =0.0004) increase in Cys level in control but not mutant animals (Fig. 5A), and this increase was largely prevented by vitamin C. We saw no significant difference in Cys levels between mutant and control animals raised in the absence of galactose (Fig. 5A, first three sets of bars). In the presence of galactose; however, there was a greater than fivefold decrease in Cys levels observed in both mutant and control larvae exposed to paraquat as compared with all other conditions (P <0.0001; Fig. 5A). This change was accompanied by a commensurate rise in CySS in both mutant and

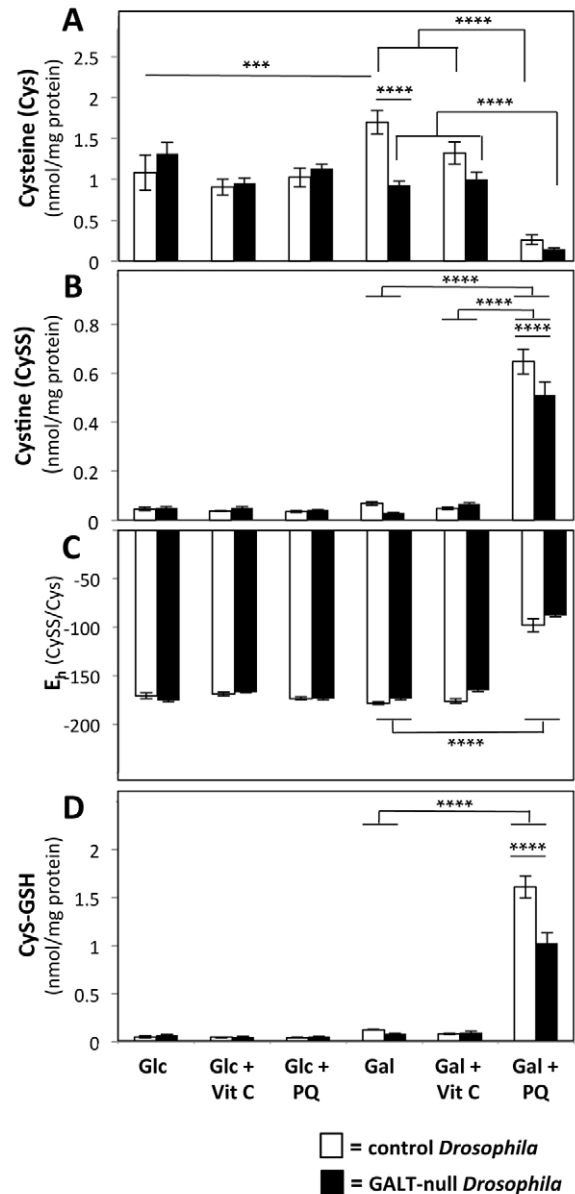


Fig. 5. Impact of vitamin C and paraquat on the levels of reduced and oxidized cysteine in control and GALT-null *Drosophila* maintained on glucose or glucose plus galactose food. (A-D) Samples tested were from control animals (open bars) or GALT-null animals (shaded bars). (A) Levels of reduced cysteine (Cys), (B) levels of oxidized cysteine (CySS), (C) extracellular E_h estimated from the Cys and CySS levels and (D) levels of Cys-GSH. VitC, vitamin C; PQ, paraquat; Glc, glucose; Gal, galactose. Values show mean \pm s.e.m. *** P <0.001; **** P <0.0001.

control animals (P <0.0001; Fig. 5B). However, as with GSSG, the magnitude of the increase for mutants was less than that seen for controls (P <0.0001; Fig. 5B). Extracellular E_h , calculated from the levels of Cys and CySS, was also affected by diet and treatment (P <0.0001), with both mutant and control larvae showing a significant twofold increase when maintained on food supplemented with both galactose and paraquat (Fig. 5C). Strikingly, the disulfide Cys-GSH also increased more than tenfold in both mutant and control animals exposed to the combination

of paraquat and galactose ($P<0.0001$), and again the levels in mutants were significantly lower than those seen in controls ($P<0.0001$; Fig. 5D).

Finally, we compared the levels of total glutathione (GSH + GSSG) and total cysteine (Cys + CySS + CyS-GSH) in lysates prepared from mutant and control larvae exposed to food containing either glucose or glucose plus galactose with no additive, with 80 μ M vitamin C or with 100 μ M paraquat. Galactose exposure alone slightly increased the level of total glutathione in both mutants and controls ($P=0.0003$); this increase was prevented by vitamin C (Fig. 6A). Total glutathione was decreased by about a factor of two in both mutants and controls exposed to galactose plus paraquat as compared with galactose alone; this difference was highly significant ($P<0.0001$; Fig. 6A). By contrast, total cysteine revealed a differential impact of galactose plus paraquat on mutants and controls. Specifically, galactose exposure alone resulted in a 60% increase in the total cysteine level in controls ($P=0.0005$) but not mutants. Total cysteine tended to decrease in these animals in the presence of vitamin C (Fig. 6B), but the effect was not statistically significant. Similarly, galactose plus paraquat exposure resulted in a 33% increase in total cysteine relative to galactose alone for controls and a 61% increase relative to galactose alone for mutants (Fig. 6B). Despite this difference, control animals exhibited significantly higher total cysteine levels compared with mutants when exposed to galactose plus paraquat ($P=0.0003$; Fig.

6B). There were no significant changes in total glutathione or cysteine for either mutant or control animals in the absence of galactose (Fig. 6, first three sets of bars in both panels).

***Drosophila* larvae exposed to galactose show a striking induction of genes responsive to oxidative stress**

As a final test of whether galactose exposure causes oxidative stress in *Drosophila*, we used quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to monitor the expression levels of two genes, *GSTD6* and *GSTE7*, both involved in glutathione metabolism and known to function in the response to oxidative stress (Alias and Clark, 2007; Li et al., 2008), and also a housekeeping gene (*ACT5C*) that encodes actin. We quantified the levels of all three transcripts in cohorts of mutant and control larvae maintained under glucose or glucose plus galactose conditions (see Methods). Of note, all of the larvae were between 48 and 52 hours old at the time of harvest, and the period of galactose exposure was limited to the final 12 hours of life so that even the GALT-null larvae were still very much alive at the time of harvest. Both mutant and control larvae exposed to galactose demonstrated dramatic increases ($P<0.0001$) in the expression levels of both *GSTD6* and *GSTE7* relative to *ACT5C* (Table 1). Specifically, *GSTE7* was induced by galactose exposure about fivefold in controls and about eightfold in GALT-null larvae; *GSTD6* was induced by galactose exposure >40-fold in controls and >80-fold in GALT-null larvae (Table 1).

DISCUSSION

The underlying basis of pathophysiology in classic galactosemia has remained a mystery for more than 50 years; the work described here brings us one important step closer to unraveling that mystery. Using a *Drosophila melanogaster* genetic model of classic galactosemia, we looked at whether oxidative stress and response contribute to the mechanism of acute galactose toxicity in GALT deficiency; our results provide compelling evidence that the answer is yes.

The hypothesis tested here is based on a preponderance of evidence amassed over decades from studies of genetically wild-type animals exposed to high levels of galactose in what have been called 'experimental' models of galactosemia (Yelinova et al., 1996; Jordens et al., 1999; Ho et al., 2003; Wei et al., 2005; Cui et al., 2006;

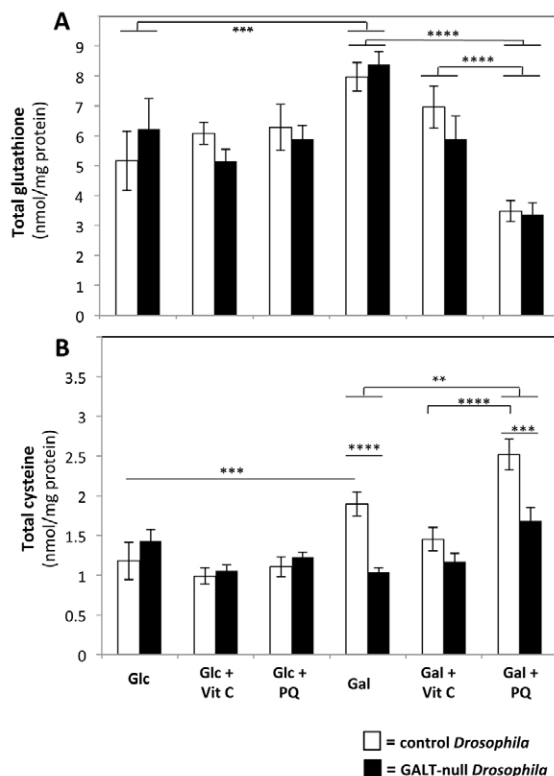


Fig. 6. Impact of vitamin C and paraquat on the levels of total glutathione and total cysteine in control and GALT-null *Drosophila* maintained on glucose or glucose plus galactose food. (A,B) Samples tested were from control animals (open bars) or GALT-null animals (shaded bars). (A) Total glutathione (GSH + GSSG) and (B) total cysteine (Cys + CySS + CyS-GSH). VitC, vitamin C; PQ, paraquat; Glc, glucose; Gal, galactose. Values show mean \pm s.e.m. ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$.

Table 1. Impact of 12 hours of galactose exposure on the expression levels of genes in GALT-null and control *Drosophila* larvae

Larvae tested	Galactose exposure time (hours)	Expression level relative to <i>ACT5C</i>	
		<i>GSTD6</i>	<i>GSTE7</i>
Control	0	0.344 \pm 0.052	0.591 \pm 0.114
Control	12	14.794 \pm 0.456 (>40-fold)	3.122 \pm 0.195 (>5-fold)
GALT-null	0	0.354 \pm 0.058	0.456 \pm 0.131
GALT-null	12	29.467 \pm 1.189 (>80-fold)	3.885 \pm 1.004 (>8-fold)

GSTD6 and *GSTE7* each encode proteins (glutathione *S*-transferase) responsive to oxidative stress; *ACT5C* encodes a housekeeping protein (actin). Values presented are mean \pm s.e.m. ($n=3$). The fold change in expression level for each gene attributable to the galactose exposure is indicated in parenthesis; each of these changes was statistically significant at $P<0.0001$.

Long et al., 2007). Collectively, these studies demonstrated that exposure to high levels of galactose result in accelerated aging and decreased lifespan as a result of oxidative stress and damage. This result was specific to galactose and, paradoxically, although galactose exposure caused oxidative stress, it also compromised antioxidant defenses, leaving the organism especially vulnerable to damage (Cui et al., 2004). Here we used genetically modified GALT-null animals exposed to biologically relevant levels of dietary galactose (e.g. 25% of the monosaccharide present) to test the potential role(s) of the oxidative stress response in galactose sensitivity. Our approach was threefold.

First, we quantified the impact of two oxidants and two antioxidants on survival of control and GALT-null (mutant) *Drosophila* raised from an early larval stage on food containing either glucose as the only sugar or glucose plus galactose. The oxidants we used were DMSO and paraquat; the antioxidants were vitamin C and α -mangostin. DMSO, commonly used as a solvent and in cryogenic preservation of mammalian cells, is also a potent oxidant that leads to the formation of superoxide anion when exposed to air (oxygen) and hydroxide (OH⁻) (Hyland and Auclair, 1981). Paraquat (1,1'-dimethyl-4-4'-bipyridinium dichloride) has long been used as a pesticide; its toxicity derives from the generation of superoxide anions and oxidation of the NADPH pool with the subsequent disruption of biochemical processes requiring NADPH (Bonilla et al., 2006). Vitamin C (ascorbate) is regarded as a potent antioxidant and is capable of scavenging a wide array of reactive oxygen and nitrogen radicals; it is particularly protective of DNA and low-density lipoproteins (Rose and Bode, 1993; Duarte and Lunec, 2005). Vitamin C also has the ability to recycle other cellular antioxidant defenses, such as glutathione, from their respective free radical forms (Duarte and Lunec, 2005). Finally, α -mangostin is one of the major xanthenes found in the tropical fruit mangosteen (Bumrungpert et al., 2010; Larson et al., 2010). Potent antioxidant (Williams et al., 1995; Jung et al., 2006; Martinez et al., 2011) and anti-inflammatory potentials (Udani et al., 2009; Bumrungpert et al., 2010) have been ascribed to this compound. Interestingly, α -mangostin is able to scavenge singlet oxygen, superoxide and peroxynitrite anions, but not hydroxyl radicals or hydrogen peroxide under in vitro conditions (Pedraza-Chaverri et al., 2009). Other studies (Williams et al., 1995) suggest that α -mangostin enhances the initial free-radical scavenging potential and prolongs the early resistance to oxidative stress until all antioxidants are exhausted.

From our oxidant and antioxidant experiments we observed a clear pattern: both oxidants exacerbated the galactose sensitivity of GALT-null but not the control *Drosophila* and, conversely, both antioxidants had a protective effect. Of note, the ability of α -mangostin to enable galactose-challenged GALT-null larvae to survive to pupation (Fig. 2C) but not always to adulthood (Fig. 2D) might reflect the reported strong initial, but not sustained, antioxidant impact of this compound (Williams et al., 1995).

Next, we quantified biochemical markers of the oxidative stress response in mutant and control larvae maintained on glucose-only or glucose plus galactose food and exposed to no additive, vitamin C or paraquat. The markers tested included oxidized and reduced glutathione and cysteine, and again a pattern was clear: galactose and paraquat synergized to create heightened markers of oxidative stress in both mutant and control animals. However, there were

also notable quantitative differences evident in the responses of mutants and controls to oxidative stress; namely, in many instances mutants showed a significantly diminished response relative to controls. The implications for mechanism are discussed below.

Finally, we used qRT-PCR to monitor the expression levels of two genes known to function in response to oxidative stress, *GSTD6* and *GSTE7* (Alias and Clark, 2007; Li et al., 2008). We tested RNA levels in both mutant and control larvae maintained in either the presence or absence of galactose for a short window of time (12 hours). The levels of both genes were induced dramatically and differentially by galactose exposure in control and mutant animals; for *GSTE7* the induction was >fivefold for controls and >eightfold for mutants, and for *GSTD6* the induction was >40-fold for controls and >80-fold for mutants. Combined, these data provide compelling evidence that galactose exposure leads to oxidative stress in both GALT-null and control *Drosophila*, but that GALT-null larvae respond differently in a way that leaves them unusually vulnerable to the stress.

Implications for mechanism

The data presented here raise two important and distinct points with regard to mechanism. First, galactose exposure both causes oxidative stress and sensitizes to other sources of oxidative stress. Second, although GALT-null and control *Drosophila* clearly both experience oxidative stress as a result of galactose exposure, the impacts of that stress might differ qualitatively and quantitatively. For example, the survival rates of galactose-exposed mutant animals are dramatically affected by the presence of other oxidants and antioxidants whereas the survival rates of control animals are not. In isolation, these data could suggest that galactose exposure causes higher oxidative stress in mutants than in controls, but combined with the biochemical data presented here, the results suggest that there are differences in how the animals respond to oxidative stress rather than in the level of stress itself.

The observation that both oxidants and antioxidants impact the survival of GALT-null *Drosophila* exposed to galactose without substantially impacting the levels of gal-1P is also important because it implies either that the oxidant and antioxidant modifiers each act downstream of gal-1P in the ostensible pathway of galactose toxicity or, alternatively, that each acts independently of it. Either way, these data run counter to the common assumption that gal-1P accumulation is central to the negative outcomes associated with classic galactosemia (Pesce and Bodourian, 1982; Gitzelmann and Steinmann, 1984; Gitzelmann, 1995). Of note, the oxidant and antioxidant modifiers of acute outcome described here represent the first modifiers of outcome in GALT deficiency that do not appear to work by either preventing the synthesis of gal-1P or promoting its catabolism (Wierenga et al., 2008; Boxer et al., 2010; Tang et al., 2011). It is important to note that the data presented here do not address the question of whether oxidative stress might also contribute to long-term, apparently galactose-independent, outcomes in GALT-deficient flies; that question will be a focus of future attention.

Why might galactose exposure promote oxidative stress in GALT-deficient *Drosophila*?

The results presented here confirm that galactose exposure leads to oxidative stress and demonstrate that GALT-null animals show

heightened sensitivity to that stress. But why? A number of possibilities exist. For example, the production of ATP via metabolism of galactose is by definition a more indirect process than is the production of ATP via glycolysis of glucose, because to be fully metabolized galactose must first be 'converted' into glc-1P by the Leloir pathway. This reality might lead cells to rely more heavily on mitochondrial oxidative phosphorylation to produce energy (Aguer et al., 2011). Indeed, studies from yeast demonstrate that 'Leloir competent' yeast consume substantially more oxygen when cultured in medium containing galactose as the carbon source than when cultured in medium containing glucose as the carbon source (De Deken, 1966). Furthermore, as has been suggested (Obrosova et al., 1997), accumulated gal-1P might inhibit key glycolytic enzymes such as phosphoglucosmutase (Gitzelmann, 1995), and futile cycles of phosphorylation and dephosphorylation of galactose (Mayes and Miller, 1973) might further deplete ATP stocks, putting increased energy strain on cells. Again, this could stress mitochondrial function, which could potentially both lead to and sensitize to oxidative stress. That paraquat and vitamin C both alter survival rates of galactose-exposed GALT-null *Drosophila* without altering their gal-1P levels suggests that the gal-1P level might not be what is important here. This favors the hypothesis of dynamic phosphorylation and dephosphorylation of galactose, although it does not rule out the possibility that gal-1P might also inhibit key enzymes and thus exacerbate the problem. Future studies with *Drosophila* and other model systems will be required to distinguish between the possible explanations for the mechanism(s) behind the observations reported here.

Implications for patients

The studies presented here were performed using control and GALT-null *Drosophila* and therefore the results might or might not translate to the human condition. That said, anecdotal studies have shown that galactosemic patients on poor dietary control display increased markers of oxidative stress yet lower total antioxidant status (Schulpis et al., 2005; Schulpis et al., 2006). Considering that antioxidant supplements (e.g. vitamin C) and supplements designed to improve mitochondrial function (e.g. creatine) are apparently well tolerated, at least in healthy people, it is tempting to speculate whether such supplements might prove beneficial for patients with classic galactosemia.

METHODS

Drosophila stocks and maintenance

We used two excision alleles of *Drosophila melanogaster* GALT, *dGALT^{ΔAP2}* and *dGALT^{C2}*, generated by mobilizing an existing SUPor-P insertion in the 5'-UTR of the CG9232 locus (KG00049) as previously detailed (Kushner et al., 2010). The *dGALT^{ΔAP2}* allele carries a 1647 base pair deletion that removes almost the entire *dGALT* gene. Flies homozygous for this allele demonstrate no detectable GALT activity. By contrast, the *dGALT^{C2}* allele carries a precise excision of the P element and flies homozygous for this allele demonstrate wild-type GALT activity. Both stocks have been characterized and we have reported previously that flies homozygous for *dGALT^{ΔAP2}* mimic aspects of classic galactosemia including a significant galactose-dependent decrease in survival and considerable accumulation of a metabolic intermediate, gal-1P (Kushner et al., 2010).

For this study, fly stocks were maintained at 25°C on molasses-based food that contained 43.5 g/l cornmeal, 17.5 g/l yeast extract, 8.75 g/l agar, 54.7 ml/l molasses, 10 ml/l propionic acid and 14.4 ml/l Tegosept mold inhibitor (10% w/v in ethanol). For experiments that measured galactose sensitivity, animals were reared under non-overcrowding conditions on a diet that consisted of 5.5 g/l agar, 40 g/l yeast, 90 g/l cornmeal, 555 mM glucose (Fisher Scientific, Pittsburgh, PA), 10 ml/l propionic acid, 14 ml/l Tegosept mold inhibitor (10% w/v in ethanol) and the indicated amount of D(+)-galactose (Sigma-Aldrich, St Louis, MO) measured from a 20% w/v galactose stock solution.

Survival assays

To test the impact of varying dietary exposures on survival of developing *Drosophila*, we established the following protocol. First, both *dGALT^{ΔAP2}* and *dGALT^{C2}* were raised under non-overcrowding conditions in parallel on foods containing either glucose only (555 mM) or glucose (555 mM) plus galactose. To control for larval density, parents of the desired genotypes were allowed to mate and deposit embryos for 24 hours on grape juice agar medium to generate embryo collections. After 24 hours, cohorts of 20 first-instar larvae were collected under the microscope and transferred to replicate 12×55 mm polystyrene vials each containing 2 ml of the appropriate fly food. Each vial was plugged with cotton and maintained under conditions of controlled temperature (25°C) and humidity (60%), and monitored for about 19 days. Over the course of this time, the numbers of pupa and adults were recorded. Replicate vials (10-20 replicates) were monitored for each genotype and condition. Initial studies testing the impact of galactose at 0 mM, 150 mM, 175 mM, 200 mM and 225 mM revealed that 200 mM galactose was the preferred amount (supplementary material Fig. S1).

This same protocol was applied to test the impacts of oxidants and antioxidants on GALT-null and control animals. The additives tested included dimethyl sulfoxide (DMSO; Sigma-Aldrich), paraquat (methyl viologen dichloride hydrate; Sigma-Aldrich), vitamin C (Fisher Scientific) and α -mangostin (Gaia Chemical, Gaylordsville, CT). These supplements were selected on the basis of their established or predicted roles as oxidants (paraquat and DMSO) or antioxidants (α -mangostin and vitamin C). Different stock solutions of each additive were prepared by dissolving each compound into the appropriate solvent (water for vitamin C and paraquat, DMSO for α -mangostin) so the same volumes of solvent and additive were added to each batch of food. We were careful to avoid exposing supplements to high temperatures or excessive light, as recommended by the manufacturers (Naidu, 2003). For food containing α -mangostin, a comparable amount of DMSO was added to the control food to counter the impact of DMSO alone. Doses for vitamin C and paraquat were selected on the basis of earlier reports on *Drosophila* (Bahadorani et al., 2008; Rzezniczak et al., 2011). α -mangostin had not been previously studied in flies so we tested a broader range: 40, 120 and 360 μ M. Doses for DMSO were selected on the basis of the observed survival rates of animals exposed to DMSO as compared with animals raised in food containing no DMSO.

Of note, survival rates for specific genotypes and food exposures were highly reproducible within experiments, and relative survival rates were also reproducible between experiments. However,

absolute survival rates sometimes differed between experiments, presumably reflecting the impact of varying cryptic environmental factors such as moisture content of the food. All comparisons described here involved mutant and control cohorts tested side by side in the same experiment, and with experiments replicated by each of two different experimenters.

Metabolite extraction and measurement

Newly eclosed adult flies were allowed to lay eggs for 5-7 days in vials containing 10 ml of glucose-only (555 mM) or glucose (555 mM) plus galactose (200 mM) food with and without vitamin C (80 μ M) or paraquat (100 μ M). Doses of vitamin C and paraquat were selected on the basis of their impact on survival of mutant animals. Cohorts of 20 third-instar wandering larvae were collected from appropriate vials. Each cohort was placed into 125 μ l of ice-cold high performance liquid chromatography (HPLC)-grade water and ground for 15 seconds using a Teflon micropestle and handheld motor (Kimble Chase Life Science and Research Products LLC, Vineland, NJ). A sample was taken from each lysate for protein quantification (using the BioRad DC Assay with BSA as a standard). Metabolites were extracted from the remaining lysate as previously described (Ross et al., 2004; Openo et al., 2006). The aqueous phase was dried under vacuum with no heat (Eppendorf Vacufuge). All samples were diluted with HPLC-grade water to normalize for protein concentration and then centrifuged through 0.22- μ m Costar Spin-X centrifuge tube filters (Corning, Lowell, MA) at 4000 g for 4 minutes to remove any particulates. The soluble phase from each sample was then transferred to a glass HPLC vial. Metabolites were separated and quantified using a Dionex ICS-2500 Ion Chromatograph fitted with a CarboPac PA10 4 \times 250 mm analytical column as previously described (Ross et al., 2004). At least five replicates were tested for each genotype-diet combination.

Measuring oxidized and reduced glutathione and cysteine

Newly eclosed *dGALT^{ΔAP2}* and *dGALT^{C2}* flies were allowed to lay eggs for 5-7 days in vials containing 10 ml of glucose-only (555 mM) or glucose (555 mM) plus galactose (200 mM) fly food with and without vitamin C (80 μ M) or paraquat (100 μ M). Doses of vitamin C and paraquat were selected on the basis of their impact on survival of mutant animals. Cohorts of 30 third-instar wandering larvae (approximately 50 mg of fresh tissue) were collected in Eppendorf tubes containing 500 μ l ice-cold 50 g/l perchloric acid solution containing 0.2 M boric acid and 10 μ M γ -Glu-Glu and placed on ice. Larvae were homogenized for 15 seconds using a Teflon micropestle and handheld motor (Kimble Chase Life Science and Research Products) and the homogenate centrifuged at 14,000 g for 2 minutes. Aliquots of 300 μ l of the supernatant were transferred to fresh tubes for further analysis. The remaining supernatant fluid was discarded and the protein pellet resuspended in 200 μ l of 1 N NaOH. Aliquots of 10 μ l of this suspension were taken to measure the amount of acid-insoluble protein using the BioRad DC Assay with BSA as a standard. Samples were stored at -80°C until they were derivatized with 60 μ l of 7.4 mg/ml sodium iodoacetic acid. The pH was adjusted to 8.8-9.2 with 1 M KOH-saturated K₃B₄O₇ and 300 μ l of 20 mg/ml dansyl chloride, followed by incubation in the dark at room temperature for 16-24 hours. Analysis by HPLC with fluorescence detection was performed as previously described (Jones et al., 1998; Miller et al., 2002).

Concentrations of thiols and disulfides were determined by integration relative to an internal standard (Jones et al., 2000). Redox potential (E_h) was calculated from the cellular GSH and GSSG concentrations using the Nernst equation as described (Kirlin et al., 1999). Whole-body total cysteine and glutathione levels were calculated by adding the levels of all cysteine and glutathione intermediates, respectively.

Quantitative RT-PCR

GALT-null and control larvae were prepared for harvest as follows. First, stocks of control and GALT-null flies were allowed to deposit embryos on grape juice agar plates at 25°C for 4 hours, after which the adults were removed and the plates were maintained at 25°C for another 24 hours. Early stage larvae (L1s) from each plate were then picked and transferred to fresh plates containing fly food with 555 mM glucose. After 12 hours, the larvae were collected by floatation in a 20% glucose solution, rinsed with phosphate buffered saline (PBS), and transferred again, either to a fresh plate with glucose fly food or to a fresh plate with fly food containing 555 mM glucose plus 225 mM galactose. After 12 hours on the new food, the larvae were again collected by floatation and stored at -20°C until the RNA was extracted using the RNeasy Mini Kit (Qiagen) as recommended by the manufacturer, with DNase digestion performed on the column. The resulting RNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit with random hexamers as primers (Applied Biosystems), followed by RNase digestion. The cDNA was then purified using a Qiaquick PCR Purification Kit (Qiagen) and quantified using the Nanodrop. The targets for real time PCR amplification were *Actin5C*, *GSTD6* and *GSTE7*. The primers for *Actin5C* amplification were actin 5C F, 5'-GCCCATCTACGAGGGTTATGC-3', and actin 5C R, 5'-CAAATCGCGACCAGCCAG-3', which defined an amplicon of 66 base pairs (Guenin et al., 2010). The primers for *GSTD6* were *dGSTD6* F1, 5'-TCCCCAGAAGCAAGCGCTGA-3', and *dGSTD6* R1, 5'-GGGTTTGCCCGTCCGAAGCA-3', which defined an amplicon of 106 base pairs. Finally, the primers for *GSTE7* were *dGSTE7* F1, 5'-ACCTTGGCTGCCCTGGAGGT-3', and *dGSTE7* R1, 5'-CGTCTCCAACGTGGGCACC-3', which defined an amplicon of 121 base pairs.

Prior to use, each of the primers was verified for specificity using BLAST (NCBI) to look for unintended matches in the *Drosophila melanogaster* genome sequence. Primer sets were also confirmed by the appearance of a single band of the anticipated size following traditional PCR amplification off a cDNA template followed by gel electrophoresis and staining.

Real-time PCR was performed using the Lightcycler 480 SYBR Green I Master Kit (Roche) in 20 μ l reactions. The reactions were set up in 96-well plates covered with optical tape (Genesee Scientific). The amplification was performed on a CFX96 Real Time System (Bio-Rad). The cycling conditions for the real-time PCR were: initial denaturation at 95°C for 5 minutes followed by 35 cycles of 92°C for 10 seconds, 55°C for 20 seconds, and 68°C for 10 seconds. This was followed by a melting curve analysis from 65°C to 95°C at 0.5°C increments to confirm the amplification of single products. Statistical significance was determined by two-way ANOVA with genotype and diet as independent variables.

Statistical analyses

Experiments to determine the relationship between galactose exposure and survival of GALT-null *Drosophila* were carried out in at least ten replicate vials; two-way ANOVA with genotype and diet as independent variables was used to determine significant differences in survival to adulthood for genotypes *dGALT^{ΔAP2}* and *dGALT^{C2}* raised on food containing 555 mM glucose plus 0, 150, 175, 200 or 225 mM galactose. Survival rate was calculated as the proportion of animals that survived to adulthood. For each additive tested, we analyzed survival for each diet (i.e. glucose-only and glucose plus galactose) separately. For this purpose, we used two-way ANCOVA to compare significant differences in survival to pupation and adulthood for both *dGALT^{ΔAP2}* and *dGALT^{C2}* animals with genotype and treatment as independent variables and with experimenter as covariate. Experiments were performed by two experimenters each loading comparable numbers of replicate vials per treatment group. Survival rate for each replicate was calculated as the fold-change relative to the average survival of control animals raised under control conditions (i.e. no additive). Two-way ANOVA with genotype and treatment group as independent variables was used to compare differences in metabolite accumulation for each diet separately. The interaction of genotype and treatment was tested for each dependent variable. We used MANOVA to determine the significance of differences in the levels of oxidative stress biomarkers for the different 'genotype by diet by treatment' groups. In all cases, post-hoc tests were performed on the least-square means to determine differences between groups. The criterion for statistical significance was $P < 0.05$ but P -values were adjusted for multiple comparisons as applicable. All statistical analyses were performed using SAS (Version 9.2; SAS Institute Cary, NC).

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

P.P.J.-L. and M.L.H. performed or contributed to all experiments except those involving qRT-PCR. Y.L. and D.P.J. quantified the levels of oxidized and reduced glutathione and cysteine in samples provided by P.P.J.-L. and M.H. and interpreted those data. D.H. performed and interpreted the experiments involving qRT-PCR. J.L.F.-K. and P.P.J.-L. conceived of and directed the project, and all authors contributed to writing and editing of the manuscript.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at <http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.010207/-/DC1>

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