Characterizing the genetic basis of copper toxicity in *Drosophila* reveals a complex pattern of allelic, regulatory, and behavioral variation

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Abstract

A range of heavy metals are required for normal cell function and homeostasis. Equally, the anthropogenic release of heavy metals into soil and water sources presents a pervasive health threat. Copper is one such metal; it functions as a critical enzymatic cofactor, but at high concentrations is toxic, and can lead to the production of reactive oxygen species. Using a combination of quantitative trait locus (QTL) mapping and RNA sequencing in the *Drosophila* Synthetic Population Resource (DSPR), we demonstrate that resistance to the toxic effects of ingested copper in *D. melanogaster* is genetically complex, and influenced by allelic and expression variation at multiple loci. Additionally, we find that copper resistance is impacted by variation in behavioral avoidance of copper and may be subject to life-stage specific regulation. Multiple genes with known copper-specific functions, as well as genes that are involved in the regulation of other heavy metals were identified as potential candidates to contribute to variation in adult copper resistance. We demonstrate that nine of 16 candidates tested by RNAi knockdown influence adult copper resistance, a number of which may have pleiotropic effects since they have previously been shown to impact the response to other metals. Our work provides new understanding of the genetic complexity of copper resistance, highlighting the diverse mechanisms through which copper pollution can negatively impact organisms. Additionally, we further support the similarities between copper metabolism and that of other essential and nonessential heavy metals.

Introduction

Anthropogenic release of heavy metals into soil and water sources presents a pervasive threat with long-lasting ecological, health, and economic impacts (Wu et al. 1975; Wuana and Okieimen 2011; Babin-Fenske and Anand 2011; Gall et al. 2015). Elevated heavy metals have been reported in dozens of organisms at all levels of the ecosystem (Neuberger et al. 1990; Georgieva et al. 2002; Sánchez-Chardi and Nadal 2007; Usmani 2011; Gall et al. 2015; Wright et al. 2015; Plessl et al. 2017; Ecke et al. 2017; Ilunga Kabeya et al. 2018), demonstrating that the toxic effects of heavy metal pollution are wide reaching, and can spread through food webs (Gall et al. 2015; Ilunga Kabeya et al. 2018). Although required for normal physiological function at low concentrations, copper is one of many common environmental heavy metal pollutants linked to mining (Ramirez et al. 2005; Wuana and Okieimen 2011; Wright et al. 2015), pipes used to provide drinking water (Harvey et al. 2016), and pesticide use (de Oliveira-Filho et al. 2004). In its essential role, copper helps bind oxygen, catalyzes enzymatic reactions, and promotes normal neurological development (Hart et al. 1928; Danks 1988; World Health Organization et al. 1996; Uriu-Adams and Keen 2005; Norgate et al. 2006; Navarro and Schneuwly 2017). However, excessive copper exposure ultimately leads to the accumulation of reactive oxygen species, which can cause cellular damage through oxidative stress (Uriu-Adams and Keen 2005; Tchounwou et al. 2008).

Evolutionarily conserved metal-responsive transcription factor 1 (MTF-1) and metallothionein (MT) proteins function as a first line of defense against toxic effects of excessive copper exposure in diverse organisms including humans, flies, fungi, and plants (Macnair 1993; Goldsbrough 2000; Bellion et al. 2007; Calap-Quintana et al. 2017). MTF-1 binds to metal responsive elements of MT genes, increasing MT abundance in copper accumulating cells and allowing excess heavy metal ions to be sequestered until they are removed from the system (Filshie et al. 1971; Stuart et al. 1985; Mokdad et al. 1987; Egli et al. 2003; Balamurugan et al. 2004; Southon et al. 2004). Metal chaperone and transporter proteins such as *Ccs* (Culotta et al. 1997), *Atox1* (Southon et al. 2004; Hatori and Lutsenko 2013), *ATP7* (Norgate et al. 2006), and *CTR1* family transporters (Petris et al. 2003; Guo et al. 2004; Balamurugan et al. 2007; Turski and Thiele 2007; Calap-Quintana et al. 2017; Navarro and Schneuwly 2017) play a crucial role in the

response to heavy metal toxicity as well (Egli et al. 2003; Petris et al. 2003; Yepiskoposyan et al. 2006; Janssens et al. 2009). For example, in *Drosophila melanogaster*, high copper exposure decreases translation of *Ctr1A* and *Ctr1B* via *MTF-1* to reduce influx of copper ions (Balamurugan et al. 2007; Turski and Thiele 2007; Calap-Quintana et al. 2017; Navarro and Schneuwly 2017), whereas high copper exposure in humans leads to degradation of the *hCTR1* protein (the human ortholog of *Ctr1A/B*) and a reduction in the intracellular concentration of copper (Petris et al. 2003; Guo et al. 2004).

Much of our understanding of the response to copper stress has come from studies that use genetic manipulation to define the roles of metal responsive genes (e.g. Egli et al. 2003, 2006a; Bellion et al. 2007; Kirby et al. 2008; Bahadorani et al. 2010). However, QTL (quantitative trait locus) and GWA (genome-wide association) studies have demonstrated the genetic complexity of the response to heavy metal stress (Willems et al. 2007; Courbot et al. 2007; Zhou et al. 2017). For example, QTL mapping using *Caenorhabditis elegans* recombinant inbred advanced intercross lines (RIAILs) showed that several regions of the genome are involved in the response to cadmium, copper, and silver exposure (Evans et al. 2018). GWA with the *Drosophila* Genetic Reference Panel (DGRP) revealed multiple candidate loci associated with the response to cadmium and lead stress (Zhou et al. 2017). QTL mapping of metal resistance in plants has further demonstrated the role that allelic variation plays in the response to heavy metal stress despite strong selection pressure against metal-sensitive alleles in natural populations (Willems et al. 2007; Courbot et al. 2007; Turner et al. 2010; Arnold et al. 2016). For instance, an interspecific QTL study of two closely related species of *Arabidopsis* (metal-tolerant *A. halleri* x metal-sensitive *A. lyrata petraea*) identified multiple regions of the genome that contributed to zinc and cadmium resistance, and demonstrated that metal resistant alleles had become fixed in the metal tolerant species in the populations sampled (Willems et al. 2007; Courbot et al. 2007). Similarly, sequencing of *A. arenosa* populations locally adapted to serpentine soils revealed strong selection for introgressed alleles from the more tolerant *A. lyrata* (Arnold et al. 2016). These and other examples from *Mimulus gutattus* growing in old copper mine tailings (Allen 1971; Macnair 1983; MacNair et al. 1993; Wright et al. 2015; Selby and Willis 2018) highlight the

utility of using a quantitative genomics approach with powerful mapping panels to examine the influence of allelic variation on metal tolerance.

D. melanogaster is an important model for understanding the mechanisms involved in the response to toxic heavy metal exposure due to the ease with which it can be genetically manipulated (e.g. Egli et al. 2006a; reviewed in Navarro and Schneuwly 2017) and to the extensive conservation of genes involved in the response to metal ions between flies and humans (Zhou et al. 2017). In addition, the existence of large *Drosophila* mapping panels makes this model system especially well-suited for examining the effect of naturally-occurring alleles on the response to heavy metal stress. In this study, we used the *Drosophila* Synthetic Population Resource (DSPR) (King et al. 2012a; b) to investigate the influence of allelic and expression variation on the response to toxic copper exposure through QTL mapping with more than 1500 strains, and RNA sequencing of copper-resistant and copper-sensitive strains. Because several genes, including *MTF-1* and MTs, respond to multiple heavy metals (e.g. copper, zinc, lead, and cadmium (Calap-Quintana et al. 2017)), and because other's work has suggested that pleiotropic QTL can underlie genetic variation for multiple metals (Evans et al. 2018), our focus on the genetic architecture of copper resistance has the potential to provide insight into a broader set of genes with allelic and expression variation that influence the response to heavy metal stress.

In this study, we mapped 12 QTL associated with variation in adult female copper resistance, and the implicated genomic regions include genes with known copper-specific functions. Of particular note are *Ccs*, which shuttles copper ions to superoxide dismutase 1 (SOD1) (Schmidt et al. 2000), *Sod3*, which binds extracellular copper ions and is involved in the response to oxidative stress (Blackney et al. 2014), and *CG11825*, a gene that has been previously linked to copper exposure in other studies (Norgate et al. 2007). Copper resistance QTLs include genes with functions linked to other metals, including *Catsup* (zinc, (Navarro and Schneuwly 2017)) and *whd* (lead and cadmium (Strub et al. 2008)), providing new evidence that these genes may also be involved in the response to copper stress. Gene expression differences between a subset of resistant and sensitive DSPR strains additionally showed that many other genes are involved in the response to copper stress. We showed that copper sensitive strains were characterized by increased expression of genes related to mitochondrial function and energy

synthesis relative to resistant strains. We tested 16 candidate genes that were implicated by QTL or differential expression analysis using RNAi knockdown in the whole animal and in the anterior region of the midgut. Knockdown of 9 of these genes, including both QTL and RNAseq candidates, influenced copper resistance. Overall, because several of our candidates have not been previously linked to copper stress, we were able to demonstrate that both genes with known copper specific function (such as *Ccs*) and genes that may influence regulation of other heavy metals (such as *trpl*) influence resistance to copper stress. Together, our study provides new insight into the diverse set of genes that impact copper resistance through allelic and expression variation.

Materials and Methods

Mapping panel

We reared and phenotyped the >1500 recombinant inbred lines (RILs) that comprise the DSPR (*Drosophila* Synthetic Population Resource) to measure variation in susceptibility to copper stress. The DSPR is a multiparental, advanced generation intercross mapping panel derived from 15 unique and fully-sequenced founder strains, which represent a global sampling of genetic diversity in D. melanogaster. The DSPR consists of two mapping panels (A and B), which are composed of two subpanels (A1 and A2, and B1 and B2). The subpanels were started from the same set of founders, but were maintained independently (see King et al. 2012b for additional details on the mapping panel).

Rearing and assay conditions

Strains from the DSPR were maintained, reared, and tested in the same incubator under a 12:12hr light:dark photoperiod at 25°C and 50% humidity. To obtain female flies for the adult copper response assay, RNA sequencing, and RNAi validation, adults were transferred to cornmeal-molasses-yeast food, allowed to oviposit for two days, then discarded. Experimental female, presumably mated, flies from the following generation were sorted over CO_2 and were placed into vials with new cornmeal-molasses-yeast media for 24 hours prior to the start of each assay before they were transferred to copper-supplemented food. All adult assays were performed on 3-5 day old individuals.

Adult female copper resistance

The adult female response to copper stress was measured as percent survival on media containing 50mM $CuSO_4$ following a 48-hour exposure period. As essentially no flies die on control food throughout the span of our assay (Highfill et al. 2016), we did not assess adult female survival on control food in this study. Experimental females were transferred without CO_2 anesthesia to vials containing 1.8g potato-based Instant *Drosophila* Medium (Carolina Biological Supply Company 173200) hydrated with 8mL 50mM $CuSO_4$ (Copper(II) sulfate, Sigma-Aldrich C1297). Instant *Drosophila* Medium is estimated to contain approximately 0.02mM Cu prior to hydration (Maroni and Watson 1985). Copper resistance was measured in a total of 11 batches across the A (N strains = 767) and B (N strains = 789) mapping panels of the DSPR. Each strain was measured in a single batch with 3 vial replicates each containing between 7 and 20 individuals (average number of flies per vial replicate = 19.4). The effect of copper on survival was reported as mean percent survival per strain across the three replicate vials. Retaining vials with fewer than 15 flies did not impact our QTL mapping results in a meaningful way (see below). Hereafter, the adult survival response to 48hr 50mM $CuSO_4$ is referred to as adult copper resistance.

Adult female feeding response to copper-supplemented media

A subset of strains evenly sampled throughout the distribution of B2 subpanel adult copper resistance (0% $\pm$ 0 S.E. – 98.4% $\pm$ 1.59 S.E.) were used to measure the effect of copper exposure on food intake. We measured food intake in 3 blocks with at least two vial replicates of 20 females per strain (N = 95) per treatment (control vs. copper), following a protocol modified from (Shell et al. 2018).

Briefly, we added 1% Erioglaucine Disodium Salt (Sigma-Aldrich 861146), a blue dye, to water and to 50mM $CuSO_4$ no more than 24 hours prior to the assay to avoid dye decomposition. We hydrated 0.9g Instant *Drosophila* Medium with 4mL liquid, and flies were allowed to consume dyed food for 24 hours before they were frozen for up to 5 hours. No flies died during the 24-hour period. Subsequently, flies were homogenized with 3-4 glass beads in 600µL distilled water for 45 seconds using a Mini-Beadbeater-96 (BioSpec Products). Homogenate was centrifuged for 5 minutes at 14,000rpm, and 200µL supernatant was transferred to a 96-well plate. Fly homogenate was frozen for up to 48 hours before absorbance at 630nm was measured with a

BioTek Multimode Microplate reader (Synergy 2 v.1.03). Two water blanks and 14 standards ranging from 6.25 x 10^{-5}% to 0.006% dye in water were prepared fresh for each block, and were included in each plate to determine the dye concentration of fly homogenate, and to assess consistency among blocks. Absorbance readings for standards were highly correlated across plates and blocks (Table S1). To calculate the estimated amount of dye consumed, we used a linear model to find the slope and intercept of the standard curve (Concentration of Standard ~ Absorbance * Block). Estimated percent dye in each fly homogenate sample was determined with the equation

$$\%\ Dye\ in\ Sample = (0.002443\ x\ absorbance) - 0.0001465$$

Variation in feeding behavior among DSPR strains on copper and control food was assessed with a two-way ANOVA with an interaction (% Dye Consumed ~ DSPR Strain * Treatment), and effect size was calculated using Cohen's F (R package sjstats) (Cohen 1988; Lüdecke 2018). The correlation between average percent dye consumed and average adult copper resistance was assessed with a linear model that included an interaction between percent dye consumed and treatment in addition to the additive effects of these factors.

Copper-specific developmental response

Developmental viability was estimated in the B panel from 100 strains that were evenly sampled from throughout the distribution of B1 and B2 subpanel adult copper resistance (0% ± 0 S.E. – 98.4% ± 1.59 S.E.). Approximately 100 females per strain were allowed to oviposit on cornmeal-molasses-yeast media for 17-20 hours in 6oz polypropylene *Drosophila* bottles (Genesee Scientific: 32-130) with yeast paste to encourage egg laying. Following oviposition, remaining yeast paste was removed and embryos were gently dislodged from the media surface by rinsing with 1X PBS and swirling with a small, bristled paintbrush. Subsequently, for each strain, we arrayed multiple aliquots of 10µL of embryos suspended in 1X PBS onto a petri dish containing 2% agar dyed blue with Erioglaucine Disodium Salt (Sigma-Aldrich 861146; 8mg/mL).

For each dish we aliquoted eggs into 14 cells (Figure S1), photographed the dish (Nikon D3200, 105mm 1:2.8 DG Sigma Macro lens), and the number of embryos within each cell was recorded with ImageJ (v. 1.51s). Embryos from each cell (30 – 306 embryos, average = 125 embryos) were then transferred with a rubber, bristleless paintbrush to vials containing control or 2mm $CuSO_4$ hydrated Instant *Drosophila* Medium (1.8g media plus 8ml liquid). The rubber paintbrush was examined after each egg transfer to ensure all eggs had been transferred to the vial. The developmental response to copper was assessed with 4-7 replicates per treatment for each strain (mean replicates per strain = 6.8). We used a lower copper concentration in this assay because previous reports have shown that the larval life stage is much more susceptible to copper toxicity compared to adults (Bahadorani and Hilliker 2009).

Copper stress has the potential to reduce the number of individuals that complete development from egg to adult as well as the time needed for individuals to complete development. To estimate the effect of copper exposure on development time, for each experimental vial we recorded the number of days between set up and the first emergence of adults. To assess the effect of copper on developmental viability, we calculated the proportion of embryos in each vial that eclosed as adults in the seven days following the day of first emergence. Developmental viability was square root transformed to improve deviation from normality within treatment (Shapiro Wilks Test on transformed data; W = 0.99, P = 0.04). From here forward, square root transformed developmental viability is simply referred to as developmental viability and all subsequent analyses were performed on square root transformed data. Vials were monitored daily for 30 days after set up. Of the 1,356 vials included in this assay, 100 copper treatment vials yielded zero flies. These vials were given a development time of 30 days.

We used a 2-way ANOVA with an interaction to measure the effect of strain and treatment on each developmental response. The DSPR strains we used in this study varied in development time ($F_{99,579}$ = 11.8, P < 0.00001; Table S2A) and developmental viability on control food ($F_{(99,579)}$ = 31.6, P < 0.00001; Table S2B). Furthermore, regression analysis demonstrated that development time and developmental viability in control and copper conditions were correlated (development time: $F_{(1,98)}$ = 61.0, P < 0.0001, R^2 = 38%; Table S2C, Figure S2A; developmental

Viability: $F_{(1,98)} = 54.1$, $P < 0.0001$, $R^2 = 36\%$; Table S2D, Figure S2B). Therefore, we regressed out variation in control development time and control developmental viability with linear models to more directly assess the effect of copper stress on these metrics of development. Residual development time and residual developmental viability are referred to from hereafter as copper-specific development time and copper-specific developmental viability, respectively.

Heritability

We estimated the genetic and phenotypic variances of adult copper resistance, control and copper feeding responses, copper-specific development time, and copper-specific developmental viability using linear mixed models (lme and varcomp functions in R; (R package: APE Paradis et al. 2004; R package: nlme Pinheiro et al. 2017)). For all responses, panel-specific broad-sense heritabilities were calculated as the proportion of the total strain-specific variation in the response to copper explained by the estimated genetic variance component (Lynch and Walsh 1998).

QTL mapping of life stage-specific response to copper stress

We used the DSPRqtl package in R (github.com/egking/DSPRqtl; FlyRILs.org), to identify QTL associated with variation in adult copper resistance, adult feeding response on control and on copper-supplemented food, copper-specific development time, and copper-specific developmental viability. QTL mapping was performed for each mapping panel (A and B) and phenotype separately. At each position in the genome, for each strain, we can estimate the additive probability that the segment of the genome was inherited from each of the 8 DSPR founders. QTL were identified by regressing the strain mean phenotype on these probabilities, and significance thresholds were assigned following 1000 permutations of the data (King et al. 2012a; b). For adult copper resistance, peak positions for each QTL were estimated with 2-LOD support intervals (King et al. 2012a). Because fewer strains were used to measure the feeding and copper-specific development traits, we used a 3-LOD drop to determine peak support intervals for these traits (King et al. 2012a). Gene ontology (GO) analysis was performed without normalizing for gene length for genes included in peak intervals for each trait and mapping panel separately (FlyMine.org (Lyne et al. 2007)).

Adult copper resistance varied between the A1 and A2 subpanels but did not vary between the B subpanels (A panel: $F_{1,2289} = 12.64$; $p < 0.001$; B panel: $F_{1,2495} = 0.03$; $p = 0.86$; Figure S3; Table S2E,F). Therefore, subpanel was included as a model covariate only in the QTL analysis of panel A. Phenotyping batch also significantly influenced variation in adult copper resistance in both the A and B panels (Table S2E,F). However, including batch as a covariate in the QTL mapping model did not drastically alter the estimation of LOD scores for either panel (A panel correlation between LOD scores = 99%; B panel correlation = 98%; Figure S4A), so we only present data from the models lacking batch as a covariate. Because the development assay was conducted on 100 strains across 15 batches, DSPR strain was highly confounded with batch. Therefore, we did not include batch or subpopulation as a covariate in the QTL mapping models for copper-specific development time or copper-specific developmental viability. As each strain assessed in the feeding response assay was measured in each of three blocks, block was not included in the model for either average feeding response on control or copper food.

To determine whether including vials containing relatively few flies influenced QTL mapping results due to mis-estimated phenotype means, we additionally mapped variation in adult copper resistance using only data from vials containing at least 15 flies (removing 316 - 7% - of the vials). LOD scores for the full data set were highly correlated with those for the reduced dataset for each panel (A panel correlation = 99%; B panel correlation = 99%; Figure S4B), so we only present data from analyses using all vials.

Differential gene expression in high and low adult copper resistance strains

We examined gene expression variation in a subset of 10 strains (6 with high adult resistance: 76 – 98% survival, and 4 with low adult resistance: 0.0 – 18% survival) from the B panel to explore how adult copper resistance class and gene expression interact when individuals are exposed to 50mM $CuSO_4$. Twenty experimental females from each DSPR strain were transferred to Instant *Drosophila* Medium hydrated with either water as a control or 50mM $CuSO_4$ for 9 hours. No individuals died during the 9-hour exposure period. Following exposure, 10 females from each strain and treatment were flash frozen in liquid nitrogen, placed in TRIzol Reagent (Invitrogen, 15596018), and immediately stored at -80°C. RNA was extracted from each of the 20 samples with the Direct-zol RNA Miniprep kit (Zymo Research, R2050), eluted in 100μL

water and stored at -80°C. We prepared libraries with the TruSeq Stranded mRNA kit (Illumina, 20020595), and paired-end 37-bp mRNA libraries were each sequenced to ~20 million reads on an Illumina NextSeq 550 at the University of Kansas Genome Sequencing Core.

Sequence quality assessment and trimming were performed using fastp (Chen et al. 2018). We used kallisto to perform pseudoalignment-based mapping of reads (Ensembl transcriptome release 90) (Bray et al. 2016), and performed differential expression analysis with sleuth (v.0.30.0) using likelihood ratio tests (Pimentel et al. 2017). Gene expression is likely to vary between the different DSPR strains; however, we were primarily interested in understanding whether there are consistent differences in gene expression between high and low resistance classes of strains. Given this interest, we treated each strain as a biological replicate of the high and low resistance classes and did not include DSPR strain in differential expression models. After determining that the interaction between resistance class and treatment did not influence expression of any gene at a 5% FDR (False Discovery Rate), we tested the additive effects of resistance class and treatment on gene expression, referred to from here as the full model (full model: ~ TRT + RES vs reduced model: ~ 1). We also examined the influence of each term independently in two additional models. The effect of treatment alone was assessed by accounting for resistance class (treatment model: ~ TRT + RES vs. reduced model: ~ RES), and the effect of resistance class alone was assessed by accounting for treatment (resistance model: ~ TRT + RES vs. reduced model: ~ TRT). From here on, these term-specific models are referred to as the treatment model and the resistance model, respectively. Significantly differentially expressed (DE) genes for each model were identified with a 5% FDR threshold.

We generated three lists of significantly differentially expressed (DE) genes: full model DE genes, treatment model DE genes, and resistance model DE genes. Sleuth applies a filter against genes with low expression (Pimentel et al. 2017). We applied an additional filter following sleuth analysis to remove genes from DE gene lists with average expression of less than 1 TPM (Transcripts Per Million). Additionally, we eliminated genes with expression variance greater than or equal to 1 TPM in any of the following four categories: sensitive strains, control treatment; sensitive strains, copper treatment; resistant strains, control treatment; resistant strains, copper treatment (Figure S5). We used principal components analysis (PCA) to examine the effect of

treatment and resistance class using quantile normalized TPM data for DE genes. DE gene lists were examined for co-regulated clusters of genes using Clust (Abu-Jamous and Kelly 2018). Gene ontology (GO) analysis was performed for each cluster and for each of the DE gene lists in their entirety (FlyMine.org (Lyne et al. 2007)).

RNAi knockdown of candidate genes associated with adult copper resistance

QTL mapping and RNAseq of adult females provided several candidate genes that were implicated by one or both of these experiments. TRiP UAS-RNAi strains (Perkins et al. 2015) for candidate genes, as well as a control UAS-LUC.VALIUM10 strain, were obtained from the Bloomington Drosophila Stock Center (BDSC) (Table S3). Crosses involved 10 TRiP males and 10 virgin females from Gal4-expressing driver strains. Each TRiP strain was crossed to both a ubiquitous Gal4-expressing driver strain (BDSC 25374) and an anterior midgut specific Gal4-expressing RNAi driver strain (1099 from Nicholas Buchon, flygut.epfl.ch). Three candidate gene TRiP strains (*swm*, *Catsup*, and *CG11825*) produced too few flies to test when crossed to the ubiquitous Gal4-expressing driver, and were thus excluded from our analysis. We tested two TRiP UAS RNAi strains for the genes *CG5235*, *MtnC*, and *ZnT41F* to assess the consistency in the effect of gene knockdown on copper survival (Table S3). An average of 19.3 Gal4-UAS RNAi females (min 7) were transferred to Instant Drosophila Medium hydrated with 50mM $CuSO_4$ using an average of 16.8 (min 10) replicate vials per genotype (a total of 203-365 individuals per genotype) across four batches. We counted flies daily until all were dead, and the response to copper stress in these RNAi knockdown genotypes was quantified as average lifespan. We chose to measure lifespan on copper instead of percent survival at 48 hours (as in our DSPR screen) because we had no meaningful a priori expectation that survival would be variable at 48 hours among the RNAi genotypes, and knockdown in genes hypothesized to influence the response to copper toxicity could drastically reduce or extend survival. To establish that GAL4-UAS-RNAi genotypes were not inherently unhealthy, we additionally placed 20 such females from each cross on Instant *Drosophila* Medium hydrated with water to assess overall viability. No individuals died on copper-free media through the duration of the RNAi assay. We compared copper resistance for each RNAi knockdown to its respective control using per vial average lifespan controlling for batch

with a two-way ANOVA (Average Lifespan ~ Strain * Batch) with planned comparisons. These analyses were performed separately for each GAL4 driver.

Data availability

All raw data and images generated from this study including adult and developmental copper resistance traits, feeding data, raw QTL mapping data, normalized TPM expression data, and RNAi data are available at FigShare. File S1 contains descriptions for all accompanying data files. DSPR genotype information is publicly available at http://wfitch.bio.uci.edu/~dspr/. RNAseq reads will be submitted to the NCBI SRA prior to acceptance. Unless otherwise stated, all analyses were performed in R (v. 3.6.2) (R Core Team 2017).

Results

Abundant variation in adult female copper resistance

We measured adult copper resistance in females from over 1,500 DSPR RILs by exposing close to 60 flies (3 vials of 20 flies) from each strain to 50mM $CuSO_4$ for 48 hours. Phenotypic variation and heritability were high for female copper resistance in both the A and B panels of the DSPR (A panel: H^2 = 83.0%; B panel: H^2 = 78.8%; Figure 1).

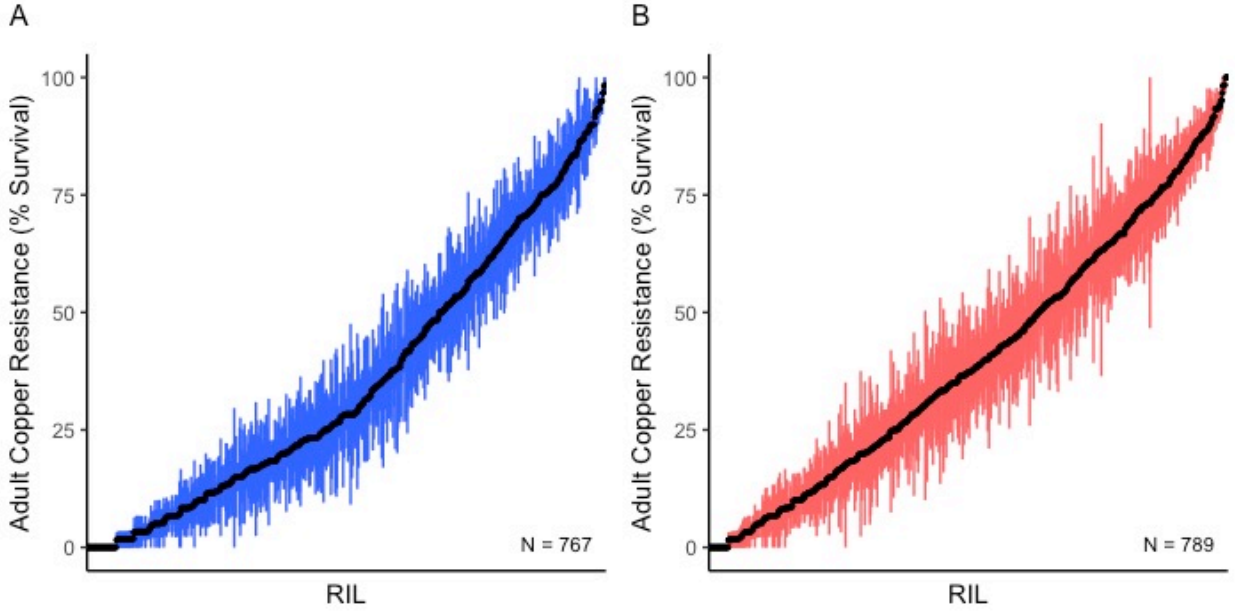

Figure 1. *Variation in mean female adult copper resistance (± SE) per DSPR strain in the A (A) and B (B) panels following 48-hour exposure to 50mM $CuSO_4$. Recombinant inbred line (RIL) is ordered by magnitude of response along the x axis.*

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358 **Adult female feeding response to copper-supplemented media**

359 Since the toxicity of copper in our assay likely stems from ingestion, we were interested
360 in flies' feeding response to copper-supplemented media. Using a sample of 95 strains from the
361 B2 subpanel that spanned the distribution of adult copper resistance (from 0% - 98.4% survival),
362 we tested the effect of 50mM $CuSO_4$ on feeding behavior. We estimated feeding by measuring
363 the amount of dye consumed by flies exposed to food hydrated with water or a copper solution
364 within a 24-hour period. Both DSPR strain and treatment significantly influenced feeding (DSPR
365 Strain: $F_{94,384} = 3.08$, $P < 0.00001$; Treatment: $F_{1,384} = 2306$, $P < 0.00001$, Table S2G; Figure 2A),
366 although treatment had a much larger effect on the feeding response than strain (Cohen's F:
367 Treatment = 2.57, DSPR strain = 0.91). We also observed an interaction between strain and
368 treatment (DSPR Strain x Treatment: $F_{94,384} = 2.77$, $P < 0.00001$, Cohen's F = 0.86, Table S2G),
369 indicating that the reduction in feeding due to copper is not uniform across strains (Figure 2).
370 Both feeding responses had high heritability (control feeding response: $H^2 = 87.6\%$; copper
371 feeding response: $H^2 = 87.6\%$).

372

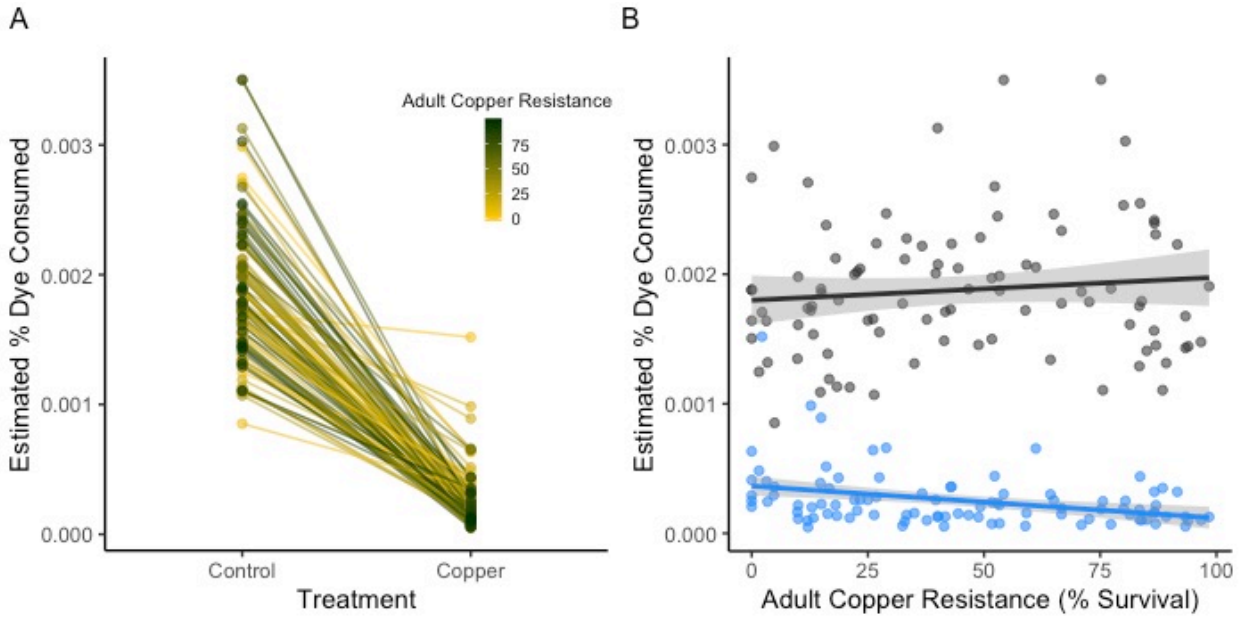


Figure 2. *Feeding behavior in 95 DSPR strains changed in response to 24-hour exposure to 50mM $CuSO_4$. A. Mean percent dye consumed varied among DSPR strains (P < 0.00001) and was much lower under copper conditions relative to control (water) conditions (Treatment: P < 0.00001). The interaction between strain and treatment (P < 0.00001) suggests that sensitivity to copper varies among the strains. B. Feeding behavior under control conditions was not correlated with adult copper resistance (P = 0.32); feeding behavior on copper was correlated with adult copper resistance (Adult Copper Resistance x Treatment: P = 0.03). Feeding response to copper is shown in blue; the control response is shown in black. Shading indicates the 95% CI of the regression.*

Overall, feeding behavior under copper conditions was weakly negatively correlated with adult copper resistance (R = -34.6%, $F_{3,186}$ = 263.7, P = 0.03; Table S2H, Figure 2B), while feeding behavior under control conditions was not correlated with adult copper resistance (R = 10.2%, $F_{1,93}$ = 0.98, P = 0.32, Figure 2B). This suggests that our adult copper resistance phenotype is partially influenced by a copper-specific behavior, where more sensitive strains tend to eat more copper food than more resistant strains in a 24-hour period. Equally, the limited strength of the relationship likely implies our resistance phenotype is primarily impacted by the physiological and metabolic response to copper and is not solely influenced by behavioral avoidance.

Copper-specific developmental response

In organisms with complex life cycles, the genetic control of physiological traits can be decoupled between life stages (Freda et al. 2017; Collet and Fellous 2019). To assess whether the strains with high resistance to copper as adults were also more resistant in other life stages, we sampled 100 strains from the B1 and B2 subpanels that span the range of adult copper resistance (from 0% – 98.4% survival). Embryos from these strains were placed on either control media, or media containing 2mM $CuSO_4$, and the day of first adult emergence (development time) and the proportion of embryos that emerged as adults (developmental viability) was recorded. Both development time and developmental viability were variable among strains on copper and control media (development time: $F_{99,1157} = 24.21$, $P < 0.00001$; developmental viability: $F_{99,1157} = 49.17.21$, $P < 0.00001$; Table S2I,J, Figure 3). Exposure to copper delayed emergence by nearly 4 days on average ($F_{1,1157} = 1293$, $P < 0.00001$; Table S2I, Figure 3A) and significantly reduced developmental viability ($F_{1,1157} = 3905$, $P < 0.00001$; Table S2J, Figure 3B). There was an interaction between strain and treatment for both measures of the developmental response to copper, indicating that although development time and developmental viability were negatively affected by copper exposure for the majority of strains, the magnitude of the effect of treatment varied among strains (development time: $F_{1,1157} = 11.75$, $P < 0.00001$; developmental viability: $F_{1,1157} = 13.13$, $P < 0.00001$; Table S2I,J, Figure 3A,B)

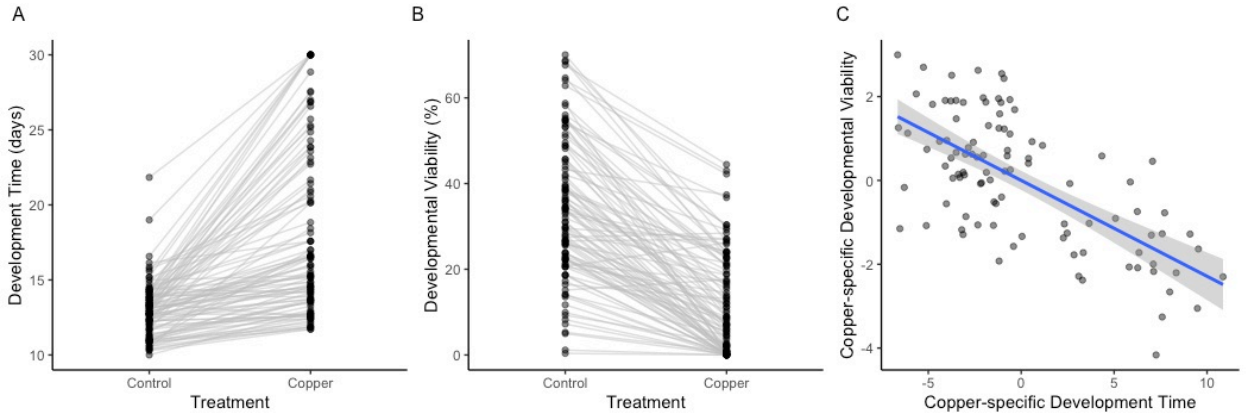


Figure 3. *Development time (A) and developmental viability (B) were reduced in most strains by exposure to 2mM $CuSO_4$. C. Copper-specific developmental viability and development time (corrected for strain-specific variation in these responses on control food) were correlated ($P < 0.00001$, $R^2 = 44\%$), indicating that strains with longer development time on copper-supplemented media also had lower viability. Points indicate the strain mean under each treatment condition. Grey shading indicates the 95% CI of the regression.*

Because we were primarily interested in the effects of copper on development time and developmental viability, we regressed out variation under control conditions from both developmental phenotypes (see Materials and Methods). Copper-specific development time and copper-specific developmental viability were correlated ($F_{1,98}$ = 76.4, P < 0.00001, R^2 = 44%; Table S2K, Figure 3C), demonstrating that strains with longer development time on copper also had lower viability. Heritability was similar between copper-specific development time (H^2 = 87.7%) and copper-specific developmental viability (H^2 = 87.7%).

Neither copper-specific development time nor copper-specific developmental viability were correlated with adult copper resistance at an alpha level of 0.05 (copper-specific development time: $F_{1,98}$ = 0.16, P = 0.69, R^2 = 0.02; Table S2L, Figure 4A; copper-specific developmental viability: $F_{1,98}$ = 2.71, P = 0.10, R^2 = 2.7%, Table S2M, Figure 4B). The lack of a significant correlation between both measures of the developmental copper response and adult copper resistance could imply that copper resistance is influenced by life stage-specific mechanisms. However, because several other aspects of our assays used to measure the adult and developmental response to copper differ (e.g. copper concentration and exposure time), the lack of a significant correlation between these life stage specific measures of the response to copper stress is likely also influenced by technical variation.

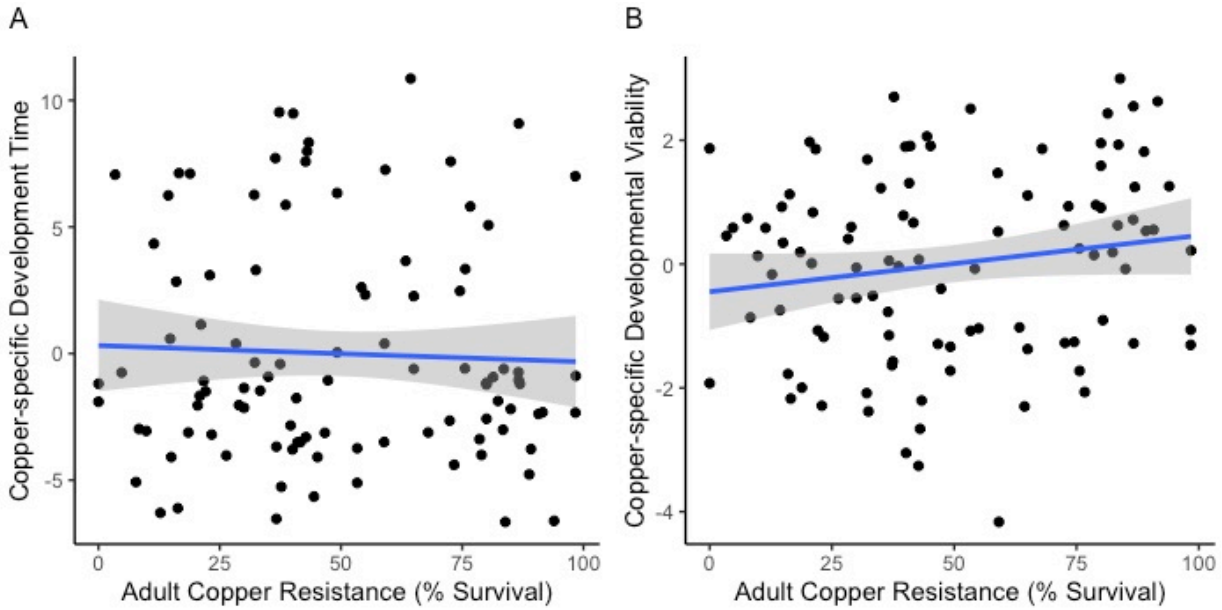
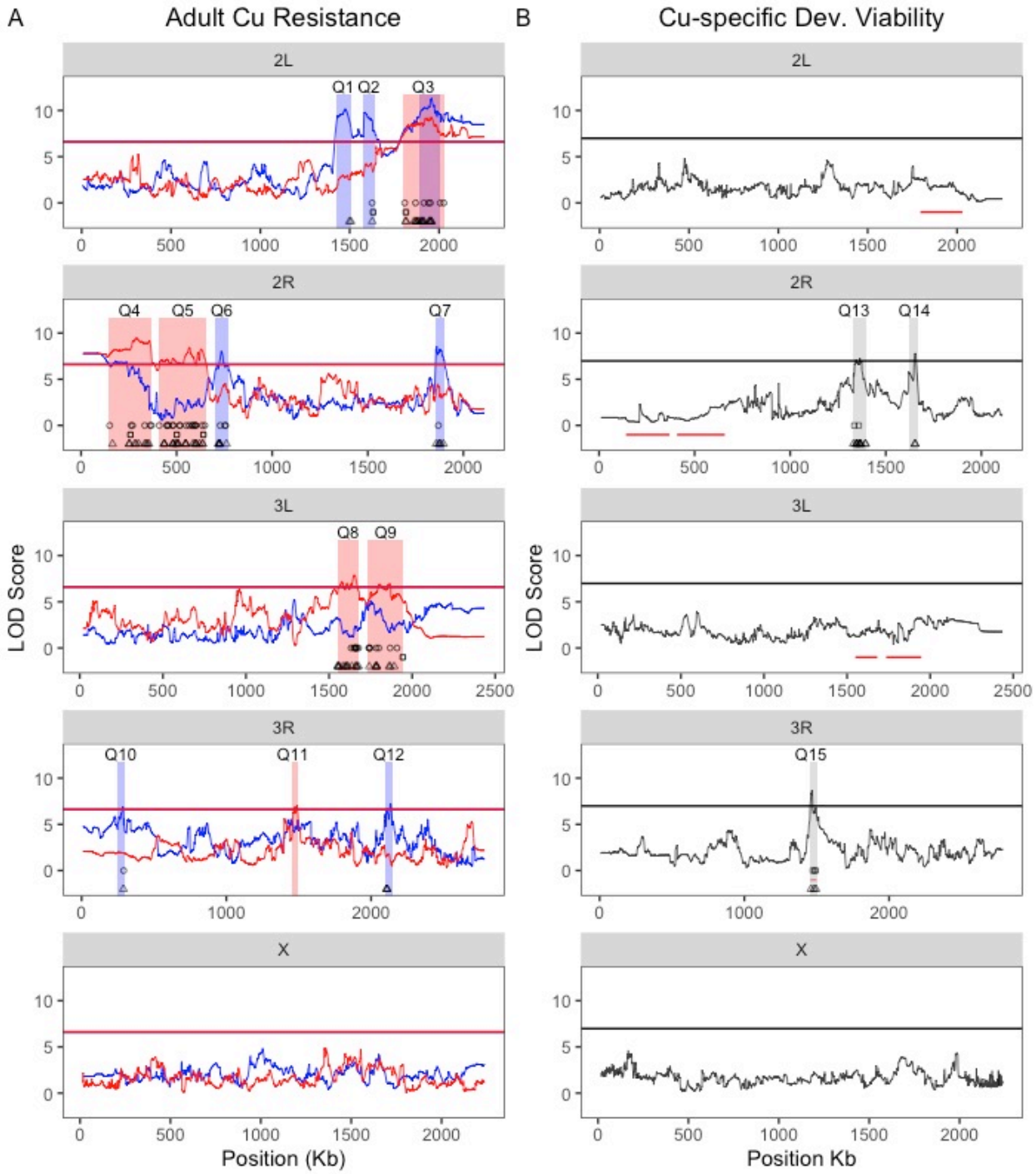


Figure 4. *Copper resistance is not correlated across life stages. A. Copper-specific development time was not correlated with adult copper resistance (P = 0.69). B. Copper-specific developmental viability and adult copper resistance were not correlated (P = 0.10). In both plots, points indicate strain means. Higher, positive values for copper-specific development time and developmental viability indicate longer or higher development time or viability on copper, respectively. Grey shading indicates the 95% CI of the regression between residual developmental response (corrected for variation in the response on control food) and adult female survival after 48hrs on 50mM* $CuSO_4$.

QTL mapping of life stage-specific response to copper stress

A principal goal of our study was to genetically dissect the response to copper stress, and using the DSPR we identified a total of 12 QTL between the A and B panels that were associated with variation in the adult copper resistance (Figure 5A, Table 1, Figure S6). Assuming that each QTL contributes to phenotypic variation in an additive manner, the QTL explained a substantial amount of variation in adult copper resistance (A panel: 36.19%; B panel: 27.93%; Table 1). The genetic architecture of adult copper resistance was largely panel-specific, with only one QTL (Q3) overlapping between the mapping panels. The 2-LOD drop interval of Q3 in the A panel fell entirely within the 2-LOD drop interval of Q3 in the B panel (Table 1, Figure 5A). Panel-specific genetic architecture of trait variation is consistent with several other studies that have mapped traits in both panels of the DSPR (Marriage et al. 2014; Najarro et al. 2017; Everman et al. 2019).

460 This lack of QTL peak overlap is likely the result of using a different set of founders to establish
461 each mapping panel (King and Long 2017) but may also reflect a lack of power (King et al. 2012a)
462 or epistatic effects that influence our ability to detect all QTL underlying adult copper resistance
463 in each panel.



464
465 ***Figure 5***. *QTL associated with variation in adult copper resistance (A) and copper-specific developmental*
466 *viability (B). A. We detected several QTL in the A (blue) and B (red) DSPR panels. Most QTL were panel*

specific with one QTL (Q3) overlapping between panels. Red and blue bars represent the 2-LOD drop intervals for each QTL. B. QTL mapped for copper-specific developmental viability. One QTL (Q15) for developmental viability overlapped with Q11 contributing to adult copper resistance. Red horizontal lines represent the 2-LOD drop intervals for the 6 QTL associated with the B panel adult survival response to copper, and grey bars represent the 3-LOD drop for the three QTL associated with copper-specific developmental viability (Table 1). The horizontal lines in each plot represent permutation-derived 5% critical thresholds (the thresholds for each panel in A. are nearly identical, leading to the lines overlapping.) Round points indicate DE genes influenced by resistance class, triangle points indicate DE genes influenced by treatment, and square points indicated DE genes that are shared between the treatment and resistance class models.

Table 1. Summary of QTL identified for response to copper stress by panel and life stage.

Adult Copper Resistance QTL: A panel						
QTL	**Peak LOD**	**Chr**	**Physical Interval (Mb)[a]**	**Genetic Interval (cM)**	**Variance Explained**	**No. Genes[b]**
Q1	10.13	2L	14.22-15.07	49.52-60.62	5.91	73
Q2	9.76	2L	15.77-16.38	51.35-51.88	5.70	68
Q3A	11.3	2L	18.87-20.09	53.39-53.85	6.56	154
Q6	8.01	2R	7.03-7.72	63.56-64.89	4.70	108
Q7	8.54	2R	18.54-19.03	100.24-102.17	5.01	92
Q10	6.88	3R	2.48-2.95	47.56-47.74	4.05	40
Q12	7.24	3R	21.01-21.46	86.55-88.14	4.26	78
Adult Copper Resistance QTL: B panel						
QTL	**Peak LOD**	**Chr**	**Physical Interval (Mb)[a]**	**Genetic Interval (cM)**	**Variance Explained**	**No. Genes[b]**
Q3B	9.21	2L	17.95-20.31	52.92-53.90	5.24	259
Q4	9.44	2R	1.41-3.69	54.99-57.07	5.36	247
Q5	8.46	2R	4.08-6.58	57.77-62.69	4.82	353
Q8	7.88	3L	15.50-16.82	42.90-44.06	4.49	240
Q9	6.97	3L	17.35-19.47	44.48-45.78	3.99	224
Q11	7.05	3R	14.57-14.98	63.92-65.00	4.03	41
Copper-specific Developmental Viability QTL: B panel						
QTL	**Peak LOD**	**Chr**	**Physical Interval (Mb)[a]**	**Genetic Interval (cM)**	**Variance Explained**	**No. Genes[b]**
Q13	7.11	2R	13.30-13.95	82.37-84.50	27.9	125
Q14	7.74	2R	16.19-16.66	90.86-92.45	30.0	60
Q15	8.65	3R	14.52-15.06	63.79-65.21	32.8	62

[a]Physical intervals are based on FlyBase release 5 of the D. melanogaster reference genome.

[b]Protein coding genes only. All genes including ncRNA and pseudogenes are included in Table S4.

We did not find any QTL contributing to food consumption on either control or copper-supplemented food or copper-specific development time using both a strict (α = 0.05) and relaxed (α = 0.2) significance threshold, but we did find three QTL that contributed to variation

in copper-specific developmental viability (Figure 5B). Given we only phenotyped 100 DSPR strains, power deficits certainly contribute to the low numbers of QTL identified for these traits (King et al. 2012a). Additionally, due to Beavis effects (Beavis et al. 1991; King and Long 2017), estimates of QTL effects based on 100 DSPR strains are typically overestimated (King and Long 2017), so the relatively high estimates of the variance explained by the QTL mapped for developmental viability (Table 1) should be interpreted with care.

One copper-specific developmental viability QTL (Q15) overlapped with a QTL (Q11) associated with adult copper resistance in the B panel (Figure 5). The 2-LOD drop interval of Q11 fell entirely within the 3-LOD drop interval of Q15. Multiparental mapping panels allow estimation of the effects of the founder haplotypes at mapped QTL. To assess whether the founder haplotypes contributed to adult copper resistance and copper-specific developmental viability in similar ways, we tested the correlation between the estimated founder effects at the shared QTL. Given that the location of the copper-specific developmental viability Q15 peak may be poorly estimated due to the low sample size employed for mapping, we compared the haplotype effects from the adult and developmental datasets at the peak position of the Q11 adult copper resistance QTL. These estimated founder effects were significantly positively correlated ($F_{1,5}$ = 7.11, P = 0.04, R^2 = 59%; Table S2N, Figure 6), suggesting that the alleles at the estimated QTL peak position of Q15 and Q11 influence the response to copper stress in adults and developing individuals in a similar way. This result implies that the adult and developmental response to copper stress are not fully independent, as was suggested by the very weak phenotypic correlation between these traits (Figure 4).

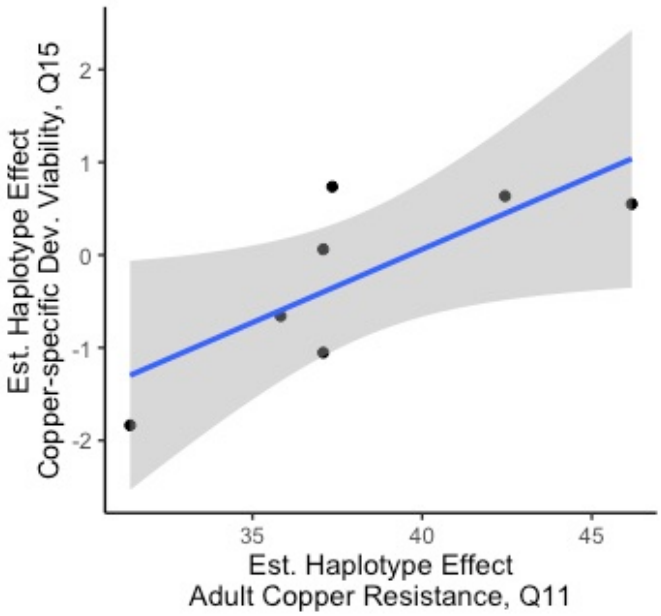


Figure 6. *Founder haplotype effects for copper-specific developmental viability and adult copper resistance estimated at a shared QTL position on chromosome 3R were significantly positively correlated (P = 0.04, R^2 = 59%). Grey shading indicates the 95% CI of the regression between estimated founder haplotype effects at Q11 for adult copper resistance and at the equivalent genomic position for copper-specific developmental viability, which resides within Q15.*

Genes implicated by mapped QTL

Combined across panels, the QTL regions associated with adult copper resistance include a total of 1823 unique protein coding genes. Of these, 10 genes have been previously associated with copper homeostasis, binding, chaperone activity, or copper cell development (Table S4). Promising copper-associated candidate genes include *Syx5*, *Grx1*, *CG11825*, *Ccs*, *Sod3*, and *CG5235*. *Syntaxin 5* (*Syx5*), associated with Q2, *Glutaredoxin 1* (*Grx1*), associated with Q9, and *CG11825*, associated with Q5, are all thought to be play a role in copper ion homeostasis (Norgate et al. 2007, 2010; Mercer and Burke 2016). *Syx5* is required for normal uptake of cellular copper, and it plays a critical role in copper ion homeostasis in *D. melanogaster* that is independent of other copper transporter proteins such as *Ctr1A/B* (Norgate et al. 2010). Similarly, *Grx1* knockdown results in copper deficiency, and this gene may function as a mediator of copper

528 transfer to chaperone proteins (Mercer and Burke 2016). *CG11825* has been identified as a
529 candidate for copper ion homeostasis in *D. melanogaster* by (Norgate et al. 2007), but functional
530 testing is lacking for this gene under copper stress conditions. The gene copper chaperone for
531 superoxide dismutase (*Ccs*), found under Q5, is an important chaperone protein that shuttles
532 copper ions to *Sod1* under normal conditions (Culotta et al. 1997; Schmidt et al. 2000). Genetic
533 ablation of *Ccs* in *D. melanogaster* resulted in increased sensitivity to oxidative stress following
534 paraquat exposure (Kirby et al. 2008); however, the effect of *Ccs* knockdown under copper stress
535 conditions has not been assessed. Genes previously associated with copper ion binding include
536 *Sod3* (Q6) and *CG5235* (Q8). While *Sod3* functions as an extracellular receptor for copper ions
537 and is protective against oxidative stress (Blackney et al. 2014), the link between *CG5235* and
538 copper is based only on prediction informed by gene ontology (Gaudet et al. 2011). In addition
539 to these copper-associated genes, we observed 64 genes with functions related to homeostasis
540 or detoxification of zinc, 2 genes involved with manganese regulation, and 19 genes involved in
541 binding unspecified metals. Of particular interest among these genes are *Catsup* (Q3), *ZnT41F*
542 (Q4), and *stl* (Q7), which are all associated with zinc transport or detoxification (Yepiskoposyan
543 et al. 2006; Ozdowski et al. 2009; Lye et al. 2013; Navarro and Schneuwly 2017), *trpl* (Q5) and
544 *DCP2* (Q8), which are hypothesized to be involved in manganese ion binding (Thurmond *et al.*
545 2019), and *swm* (Q3), *babo* (Q5), and *whd* (Q5), which are thought to be involved in binding of
546 unspecified metal ions based on gene ontology prediction (Gaudet *et al.* 2011; Thurmond *et al.*
547 2019).

548 The three QTL associated with copper-specific developmental viability spanned a total of
549 247 unique protein coding genes. Of these genes, none had functions previously linked to copper.
550 However, 8 genes were associated with zinc ion binding, and two were linked to metal ion binding
551 through gene ontology prediction (Table S4; (Gaudet *et al.* 2011; Thurmond *et al.* 2019)). Most
552 notable among the genes identified by copper-specific developmental viability QTL was *mekk1*
553 (Q15), which was demonstrated though gene knockdown to be the primary activator of JNK
554 signaling under cadmium stress in *Drosophila* S2 cells (Ryabinina et al. 2006). Although the Q15
555 developmental viability QTL overlaps with the adult copper resistance QTL Q11, *mekk1* is only

present within the interval implicated by Q15. Given that *mekk1* is within 11.2Kb of the Q11 2-LOD drop interval, this gene may still be a plausible candidate for adult copper resistance.

We performed GO enrichment analysis with genes implicated by adult copper resistance QTL in both the A and B panels as well as with genes implicated by QTL associated with copper-specific developmental viability with FlyMine (Lyne et al. 2007). No GO enrichment was observed for genes included in QTL intervals for either panel or life stage. This is unsurprising given that QTL intervals include many genes that are likely to be non-causative and potentially obscure any signal of enrichment.

Differential gene expression due to treatment and resistance class

Allelic effects on variation in complex traits are commonly mediated by regulatory variation (Roelofs et al. 2006; Ruden et al. 2009; Boyle et al. 2017; GTEx Consortium et al. 2018). We used an RNA sequencing approach to examine the effects of copper stress on gene regulation and to assess any differences in this response between genotypes with high or low adult copper resistance. We sequenced mRNA from whole females from 6 high (79 – 98%) and 4 low (0 – 18%) adult copper resistance strains from the B panel following a 9-hour exposure to control (water) and 50mM $CuSO_4$ conditions. A primary goal was to determine whether there are consistent differences in gene expression between high and low resistance classes of strains when exposed to copper stress, so we treated each strain as a replicate of the high and low resistance classes.

The interaction between treatment (control vs 50mM $CuSO_4$) and resistance class was not significant at a 5% FDR or at a relaxed cutoff of 20%, so this term was dropped from the model and the treatment (TRT) and resistance class (RES) terms were assessed additively. After additional filtering (see methods), we identified 1589 genes that were differentially expressed across treatment and adult copper resistance class with the full model (full model: ~ TRT + RES vs reduced model: ~ 1). We used PCA with quantile-normalized filtered TPM data from these 1589 genes to explore the patterns of gene expression among the sampled strains and treatments, finding a pronounced effect of gene expression on treatment, with a more subtle effect on resistance class (Figure 7, Table S5).

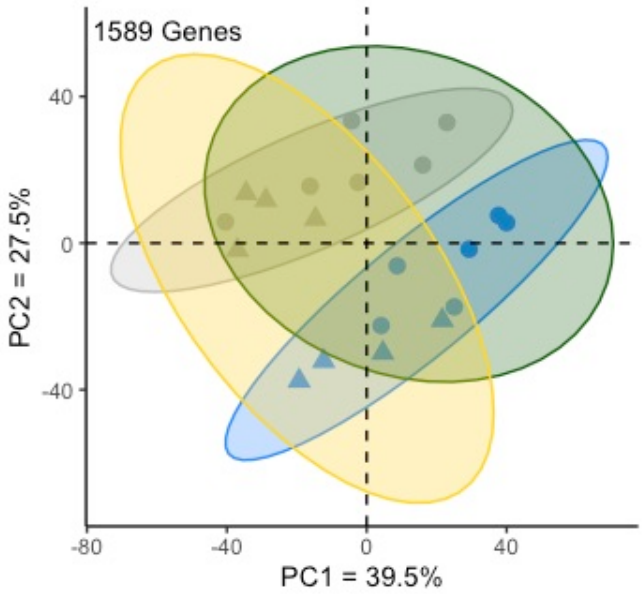


Figure 7. *Principal components analysis of significantly differentially expressed genes (quantile-normalized filtered TPM) identified by the full model (full model: ~ TRT + RES vs reduced model: ~ 1). The effect of treatment was pronounced among samples, while the effect of resistance level was more subtle. Ellipses indicate the equivalent of a 95% confidence interval. Blue indicates copper-exposed samples, grey indicates control-exposed samples, green indicates resistant strains, yellow indicates sensitive strains. Triangle points indicate sensitive strains; circles indicate resistant strains.*

To further explore how each of the main effects of the differential expression model influence gene expression, and to identify sets of genes that were influenced primarily by treatment or resistance class, we tested the effects of treatment and resistance class separately. Treatment alone (treatment model: ~ TRT + RES vs. reduced model: ~ RES) primarily contributed to differential expression in 848 genes, and adult copper resistance alone (resistance model: ~ TRT + RES vs. reduced model: ~ TRT) primarily contributed to differential expression in 466 genes. The vast majority of genes influenced by treatment and resistance class were included among the 1589 genes identified with the full model (92% of genes identified with the treatment model, 91% of genes identified with the resistance model). Of the 848 and 466 genes identified with the treatment and resistance class models, 58 genes were shared. The proportion of shared genes increased when a more relaxed significance threshold (20% FDR) was used in the treatment and

resistance class models, and the estimated effects of resistance class and treatment on gene expression in each DE gene list were weakly positively correlated (Treatment Model: R^2 = 8%; Resistance Class Model: R^2 = 2%). While the DE gene lists attributed to treatment and resistance class are not fully independent, they represent sets of genes for which the primary source of variation is either treatment or resistance class.

To broadly characterize DE genes identified in the treatment and resistance class models, we performed GO analysis with FlyMine (Lyne et al. 2007) for each gene list separately. GO analysis of each complete DE gene list is summarized in Table S6. Briefly, 111 GO terms were identified from the full model DE gene list and included terms related to cell organization, cell cycle, and metabolism among the top 10 (Table S6). Enrichment for 23 GO terms including those related to cytoplasmic translation, ribosome biogenesis, and RNA processing was observed for the 848 genes influenced by treatment (Table S6). Fifty-nine GO terms were identified from the 466 DE genes due to resistance class. Top among these GO terms were those related to ATP synthesis, cellular respiration, and mitochondrial function (Table S6). GO analysis of this set of 58 genes revealed enrichment for female gamete generation [*GO:0007292*] (P = 0.006), and no genes had any connection to copper or metal ion homeostasis to our knowledge. Many of the genes with DE due to treatment and/or resistance class fell within the QTL intervals for adult copper resistance or copper-specific developmental viability (Figure 5). Notably, no enrichment was observed for any GO term related to metal ion homeostasis or detoxification when the total DE gene lists were considered (but see below). Of the 848 genes with DE due to treatment, 87 (11%) overlapped with QTL intervals, and of the 466 genes with DE due to resistance class, 62 genes (13.3%) overlapped with QTL intervals. Of the 58 genes shared between the treatment and resistance class models, 12 genes (20.6%) overlapped with QTL intervals (Figure 5). DE genes from the treatment and resistance class models were not more likely than expected by chance to fall within QTL intervals (χ^2 = 14.5, df = 14, P = 0.41), although this does not preclude the possibility that those DE genes within mapped QTL are strong candidates to carry variation contributing to copper resistance.

To further explore the influence of resistance class on gene expression, we calculated the average change in gene expression following copper exposure for each of the 1589 DE genes

633 from the full model using the same filtered TPM data used for PCA for the high and low resistance
634 classes. The absolute values of these data were then log transformed to reduce spread, and the
635 sign of the change in gene expression was restored by multiplying the result by 1 or -1. Copper-
636 induced genes had higher expression under copper conditions, while copper-repressed genes
637 had lower expression under copper conditions.

638 Of the 848 DE genes identified in the treatment model, there was a roughly even split
639 between induced and repressed genes, with no difference between the resistance classes
640 (Kolmogorov-Smirnov (KS) test: D = 0.05, P = 0.33; Figure 8A). Among the top 20 most highly
641 induced genes under copper conditions in both resistant and sensitive classes were several MTs
642 (*MtnA*, *MtnC*, *MtnD*, *MtnE*) as well as two genes that comprise a major iron storage complex
643 (*Fer1HCH* and *Fer2LCH*). Because these genes and other genes with DE due to treatment were
644 induced in sensitive and resistant strains to similar degree, we suggest that sensitivity to copper
645 is not due to a failure to induce expression of genes with protective functions against copper ions.
646 Among the 466 DE genes identified in the resistance model, gene induction by copper was more
647 frequently observed in sensitive strains compared to resistant strains (KS test: D = 0.27, P <
648 0.00001; Figure8B). The top 20 most highly induced genes under copper conditions in sensitive
649 strains included several genes that are involved in mitochondrial structure, function, and energy
650 synthesis (e.g. *Ald1*, *levy*, *sesB*, *Mpcp1*, *COX5A*, *ATPsynb*), suggesting more sensitive strains may
651 be characterized by a greater susceptibility to oxidative stress.

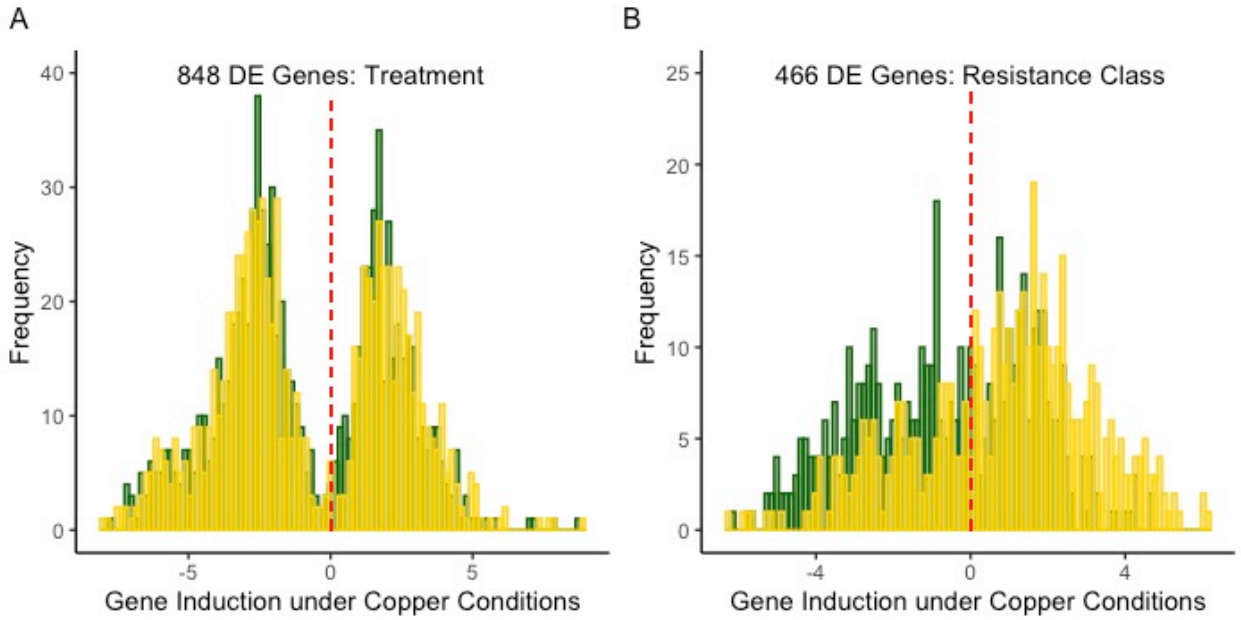


Figure 8. *Induction of genes under copper conditions with DE identified from the treatment model (A) andf the resistance model (B). A. The effect of treatment on DE highlights that roughly equal numbers of genes were induced or repressed under copper conditions (KS-test: D = 0.05, P = 0.33). B. Among DE genes identified by the resistance model, genes were more likely to be induced by copper exposure in sensitive strains compared to resistant strains (KS-test: D = 0.27, P << 0.001). In each plot, yellow bars indicate gene expression in sensitive strains and green bars indicate gene expression in resistant strains.*

Cluster analysis of differentially expressed genes

The patterns of copper-induced gene expression across treatment and resistance class observed in Figure 8A and B raise the question of whether there are co-regulated sets of genes that distinguish resistant and sensitive strains under copper and control conditions. For example, the presence of metal-associated genes among the those induced by treatment may signal a larger network of genes that are co-regulated in response to heavy metal stress. To identify any such co-regulated groups, we used Clust (Abu-Jamous and Kelly 2018) to identify non-overlapping clusters of genes from the 848 genes influenced by treatment and the 466 genes influenced by resistance class using quantile-normalized, filtered TPM data. We then performed GO analysis on any resulting clusters formed from the two sets of genes with FlyMine (Lyne et al. 2007).

672 Clust identified 3 clusters of co-regulated genes with DE due to treatment (Figure S7A).
673 Treatment clusters 1 (101 genes) and 2 (17 genes) consisted primarily of genes that were induced
674 by copper exposure, while treatment cluster 3 (17 genes) consisted solely of genes that were
675 copper-repressed (Figure S7A). While top GO terms for treatment clusters 1 and 3 revealed
676 enrichment for genes involved in processes unrelated to metal ion homeostasis or response (e.g.
677 cell cycle, RNA processing; Table S6), treatment cluster 2 was enriched for genes involved in iron
678 import, transport, and detoxification of iron and inorganic compounds (Table S6). Gene
679 enrichment in treatment cluster 2 is consistent with our expectation that copper-induced genes
680 will include those that are involved in the response to toxic metal ion exposure. Interestingly,
681 only one of the genes (Gclc) in treatment cluster 2 has been previously directly associated with
682 copper through its interactions with the copper transport proteins *Ctr1A* and *ATP7*. Also included
683 in treatment cluster 2 are *Fer1HCH* and *Fer2LCH*, which are primarily involved with iron storage
684 but may interact with copper ions during protein assembly (Huard et al. 2013). Treatment clusters
685 1 and 3 included 17 and four genes, respectively, that were implicated by adult copper resistance-
686 associated QTL (Figure S7A). The gene *dnk* in treatment cluster 1 was also implicated by the
687 copper-specific developmental viability QTL Q15 (Figure S7A). Two of the genes (*CG11878* and
688 *CG5506*) identified in treatment cluster 2 were implicated by adult copper resistance-associated
689 QTL intervals (Figure S7). One gene, *CG5506*, was empirically demonstrated to interact with
690 *Fer2HCH* (Guruharsha et al. 2011); however, neither gene has been previously associated with
691 copper exposure.
692 Clust identified 2 clusters of co-regulated genes that were differentially expressed due to
693 resistance class (Figure S7B). Resistance cluster 1 (75 genes) was primarily enriched for genes
694 involved in cell cycle processes (Table S6), and 11 genes were also implicated by adult copper
695 resistance QTL. Resistance cluster 2 (56 genes) included genes that were more often copper-
696 induced in sensitive strains and copper-repressed in resistant strains (Figure S7B). Resistance
697 cluster 2 was enriched for two broader categories of GO terms including several related to muscle
698 structure (e.g. myofibril assembly [*GO:0030239*], $P < 0.00001$) and mitochondrial function and
699 energy synthesis (e.g. ATP metabolic process [*GO:0046034*], $P < 0.00001$). Resistance cluster 2
700 also included genes involved in inorganic ion homeostasis [*GO:0098771*], although enrichment

for this GO term was weak (P = 0.05). Of the genes involved in inorganic ion homeostasis, three (*CG14757*, *trpl*, and *sesB*) are particularly noteworthy given that all three genes are copper-induced in sensitive strains and copper-repressed in resistant strains, and two have been previously linked to metal ion homeostasis (Figure 9). Exposure of the Drosophila S2 cell line to 2mM $CuSO_4$ resulted in increased expression of *CG14757*, indicating that this gene is responsive to copper stress (Norgate et al. 2007); however, its exact function relative to the toxic effects of copper has not been elucidated. The gene *trpl* is predicted to be involved in manganese ion binding (Thurmond *et al.* 2019) and was included in the adult copper resistance-associated QTL Q5 in this study. *sesB* is a mitochondrial transporter gene that was demonstrated to be important for protection against oxidative stress through gene knockdown in D. melanogaster (Terhzaz et al. 2010). Other genes included in this group (*up*, *SERCA*, and *nrv3*; Figure 9) are involved in transport of calcium, sodium, and potassium (Domingo et al. 1998; Gaudet et al. 2011) or are thought to be involved in ATP metabolism (*Vha68-1*) (Thurmond *et al.* 2019). In addition to *trpl*, 2 other genes from resistance cluster 2 were implicated by adult copper resistance QTL.

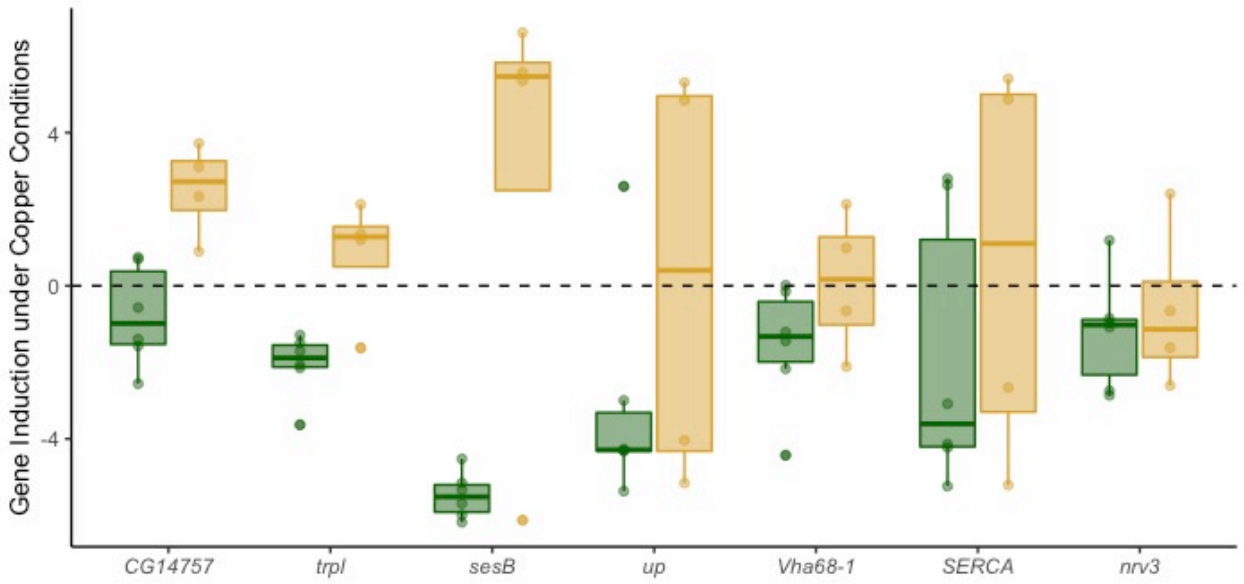


Figure 9. *Copper-induced expression of genes involved in inorganic ion homeostasis that were included in resistance cluster 2. Resistant strains are shown in green, sensitive strains are shown in yellow.*

RNAi knockdown of candidate genes associated with adult copper survival

Several genes with links to copper or metal ion homeostasis were implicated by QTL or were differentially expressed due to treatment or resistance class (see above) (Table S3). We picked 16 genes to functionally test using RNAi knockdown. QTL-implicated genes included *Catsup* and *swm* (Q3), *ZnT41F* (Q4), *CG11825*, *whd*, *babo*, and *Ccs* (Q5), *stl* (Q7), *DCP2* and *CG5235* (Q8). We also tested *trpl* (Q5), which, along with *Mvl*, was among the DE genes influenced by resistance class. Because *Ccs* and both *Sod1* and *Sod2* closely interact, we tested *Sod1/2* even though these genes were not implicated by either QTL or RNAseq. From genes with DE due to treatment, we tested *MtnC* and *CG10505*. Of these candidate genes, only *Sod1*, *MtnC*, and *Mvl* have been previously specifically linked to copper stress (Calap-Quintana et al. 2017). *Ccs*, *CG5235*, *Sod1*, *CG11825*, and *CG10505* are all associated with copper transport or binding. The remaining candidate genes (*trpl*, *DCP2*, *whd*, *stl*, *swm*, *babo*, *Catsup*, and *ZnT41F*) have not been experimentally linked to copper stress, but are associated with metal ion binding or homeostasis.

Genes were tested using TRiP UAS RNAi strains (Perkins et al. 2015) that were crossed to a background with a ubiquitously expressed Gal4-expressing driver resulting in knockdown in the whole animal and to a background with an anterior midgut-specific Gal4-expressing driver resulting in knockdown in this specific region of the midgut. We measured average lifespan of all knockdown genotypes on 50mM $CuSO_4$ with at least 10 replicates.

In general, more genes influenced copper resistance when they were knocked down in the whole animal compared to when candidates were knocked down in the anterior midgut (Figure 10). Of the candidate genes with known associations with copper, *Ccs*, *CG5235 (b)*, *MtnC (b)*, and *Sod1* reduced copper resistance relative to the control when knocked down in the whole animal using the ubiquitous driver (Figure 10A). Inconsistent effects of ubiquitous *CG5235* knockdown may be influenced by vector efficiency; the knockdown vector for *CG5235 (a)* is a long dsRNA vector (VALIUM10), while the knockdown vector for *CG5235 (b)* is a shRNA vector (VALIUM20). Both TRiP strains for *MtnC* used the same vector (VALIUM20) (Perkins et al. 2015); however, these two strains target *MtnC* at different locations within the gene, and knockdown efficiency may differ between the two sites. Knockdown of copper-associated genes in the anterior midgut did not influence copper resistance relative to the control, suggesting that

reduced expression of *Ccs*, *CG5235*, *Sod1*, *CG11825*, and *CG10505* in this limited region of the midgut does not hinder the fly's ability to cope with copper stress (Figure 10B).

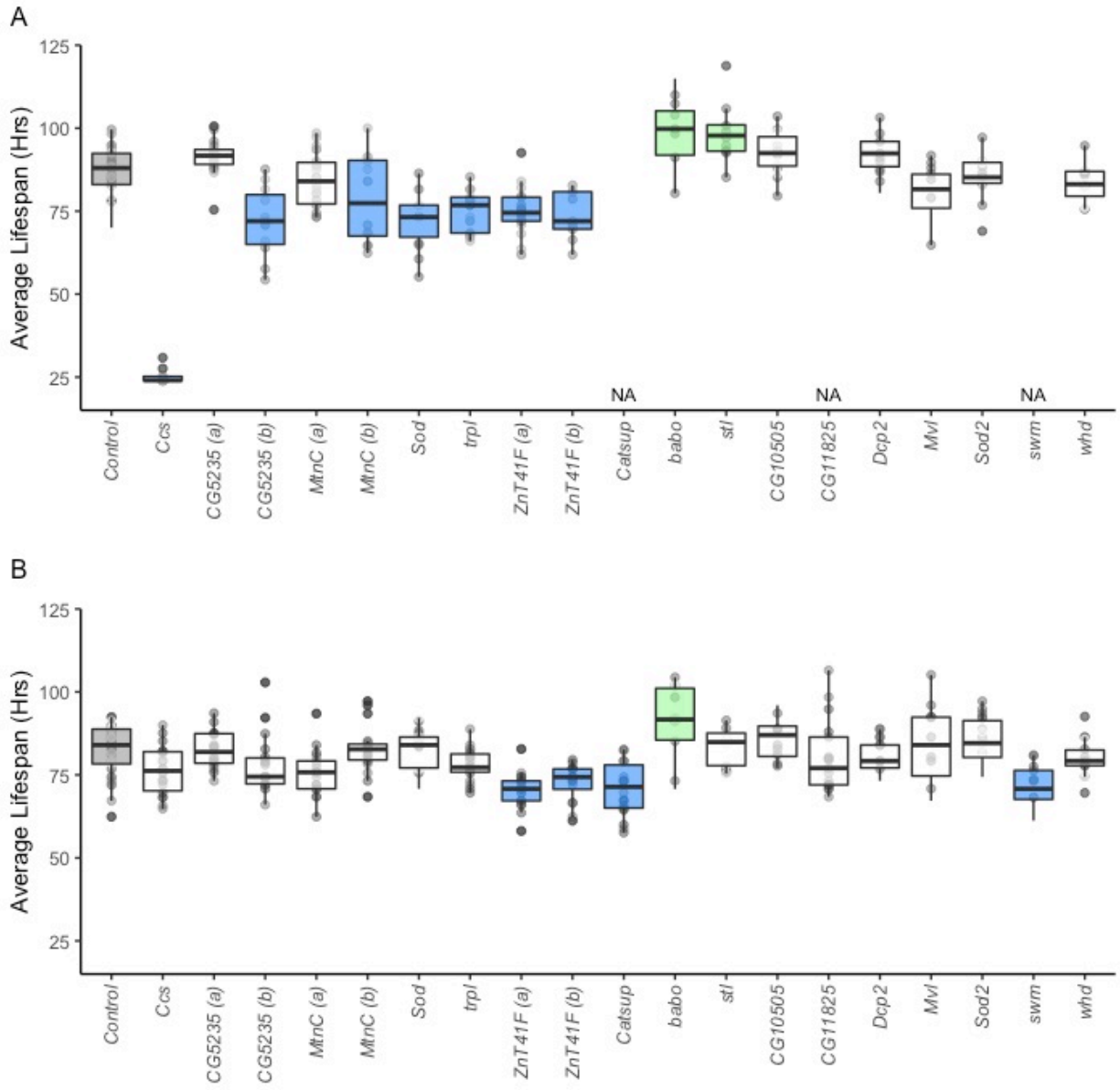


Figure 10. *Average lifespan of TRiP UAS RNAi knockdown strains crossed to a ubiquitous Gal4-expressing driver (A) and to an anterior-specific Gal4-expressing driver (B). A. Increased susceptibility was observed with knockdown of* Ccs*,* CG5235 (b)*,* MtnC (b)*,* Sod*,* trpl*, and* ZnT41F *with the ubiquitous Gal4 driver. Knockdown of* babo *resulted in increased resistance to copper toxicity relative to the control. B. Knockdown in the anterior midgut of* Catsup*,* swm*, and* ZnT41F *resulted in increased susceptibility to copper toxicity, while knock down of* babo *increased resistance relative to the control. In each plot, grey shading indicates the control, green shading indicates increased resistance to copper, blue shading indicates decreased resistance, and no shading indicates lack of a significant difference based on an experiment-wide a = 0.05. Three candidate gene TRiP strains (*swm*,* Catsup*, and* CG11825*) produced too few flies to test when crossed to the ubiquitous Gal4-expressing driver, and were thus excluded from our analysis. We tested multiple*

TRiP UAS RNAi strains for genes CG5235 *(*CG5235 (a)*,* CG5235 (b)*),* MtnC *(*MtnC (a)*,* MtnC (b)*), and* ZnT41F *(*ZnT41F (a)*,* ZnT41F (b)*) to assess the consistency in the effect of gene knockdown on copper survival (Table S3).*

The candidate gene *ZnT41F* consistently reduced copper resistance relative to control when knocked down in the whole animal and in the anterior midgut. While *ZnT41F* was previously shown to indirectly affect zinc homeostasis (Yin et al. 2017), the role it plays in copper ion homeostasis has not been described. Similarly, *Catsup* and *swm*, which have not been previously linked to copper, reduced copper resistance when knocked down in the anterior midgut. That knockdown of these genes in the whole animal did not influence copper resistance suggests these genes interact with copper soon after ingestion, although this would require additional follow-up to confirm. Interestingly, knockdown of *babo* in both the whole animal and the anterior midgut increased copper resistance relative to the controls (Figure 10). Knockdown of *stl* in the whole animal had a similar effect. Both genes are predicted to be involved in metal ion binding (Gaudet *et al.* 2011; Thurmond *et al.* 2019), but any additional evidence linking them to the detoxification of heavy metal ions under stressful conditions is lacking.

Discussion

Variation in heavy metal stress is influenced by a complex genetic architecture

D. melanogaster has been a particularly important model for elucidating the roles of genes involved in the response to copper and other heavy metals (e.g. Egli et al. 2003, 2006a; b; Balamurugan et al. 2004, 2007; Yepiskoposyan et al. 2006; Calap-Quintana et al. 2017). In our study, we used this model to investigate the role of genetic diversity in resistance to the heavy metal copper. We used a combination of QTL mapping and RNA sequencing to characterize allelic and gene expression variation that influences resistance to copper stress in strains from the multiparental DSPR mapping panel. In comparison with previous reports investigating the genetic architecture of copper and resistance to other heavy metals in plants (Macnair 1993; Selby and Willis 2018), the genetic architecture of copper resistance in *D. melanogaster* appears to be more

792 complex. Where one to three QTL were identified for heavy metal resistance in several plant
793 species including *Mimulus guttatus*, wheat, and corn (Allen 1971; Macnair 1983, 1993; MacNair
794 et al. 1993; Bálint et al. 2007; Selby and Willis 2018), we identified 12 QTL that underlie variation
795 in adult copper resistance (Figure 5A), and found that the diverse DSPR strains varied widely in
796 survival following exposure to copper stress (Figure 1).

797 Part of the difference in apparent complexity underlying the response to copper is likely
798 due to the higher power of our mapping panel, which employs a much larger number of
799 genetically diverse strains coupled with higher genetic marker density compared to the mapping
800 populations used in plant studies (e.g. Willems et al. 2007; and Courbot et al. 2007; King et al.
801 2012a). Secondly, the structure of the DSPR may be particularly conducive for detecting allelic
802 variation in genes that influence the response to copper stress given the global sampling of
803 founder strains used to generate the DSPR (King et al. 2012a), which may capture more of the
804 natural variation for copper resistance than that present in any one natural population. Third, in
805 contrast to natural populations which are often of interest because of their proximity to heavy
806 metal pollution (e.g. Allen 1971; Macnair 1983; Ramirez et al. 2005; Turner et al. 2010; Wuana
807 and Okieimen 2011; Wright et al. 2015; Arnold et al. 2016), the DSPR is naïve to any form of heavy
808 metal selection or stress. Strong selection for heavy metal resistance could reduce variation at
809 causative genes and lead to an apparent reduction in the complexity of resistance (Arnold et al.
810 2016).

811 The level of genetic complexity for copper resistance described in our study is consistent
812 with reports of metal resistance in flies, yeast, and worms where measures of resistance were
813 conducted in other heavy metal-naïve mapping populations. The DGRP (*Drosophila* Genetic
814 Reference Panel (Mackay et al. 2012)), another large D. melanogaster mapping panel, was used
815 to demonstrate a complex genetic architecture for heavy metal exposure through GWA (genome
816 wide association) and extreme QTL mapping (i.e. sequencing and comparing pools of individuals
817 with divergent phenotypes) in adult and developing life stages (Montgomery et al. 2014; Zhou et
818 al. 2016, 2017). In these studies, tens to hundreds of genes have been implicated in natural
819 genetic screens for lead (Zhou et al. 2016, 2017), cadmium (Zhou et al. 2017), and methylmercury
820 (Montgomery et al. 2014). In *Saccharomyces cerevisiae* using several extreme QTL mapping

pools, Ehrenreich et al. (2012) demonstrated that more than 20 distinct loci were associated with resistance to cadmium and nearly 40 loci were associated with copper resistance. In *Caenorhabditis elegans*, Evans et al. (2018) found 4, 6, and 6 QTL associated with the response to cadmium, copper, and silver, respectively.

Interestingly, each of the studies mentioned above generated largely distinct lists of genes associated with the responses to the heavy metals tested, and overlap between candidate genes from Zhou et al. (2016) and (2017), Montgomery et al. (2014), Ehrenreich et al. (2012), and Evans et al. (2018) and the current study is minimal as well (Table S4, Table S5). It is worth noting that none of these shared genes (Table S4, Table S5) have been previously associated with heavy metal toxicity or homeostasis and would require additional validation to determine whether they are causative for resistance to any heavy metals. However, overlap in candidate genes identified for the response to different heavy metals raises the possibility that a single gene or gene network may influence the response to multiple heavy metals. Consistency in genetic architectures underlying the response to chemical stressors has been examined using large *S. cerevisiae* and *C. elegans* mapping panels (Ehrenreich et al. 2010; Evans et al. 2018), and while there is some evidence for shared genetic architectures between traits, it is clear that each toxin response is influenced by a largely trait-specific genetic architecture. For example, out of 82 QTL that were identified for 16 different toxins ranging from heavy metals to cancer therapy drugs, Evans et al. (2018) found three QTL hotspots that were implicated for multiple types of toxins. Similarly, Ehrenreich et al. (2010) showed that ~20% of QTL detected in their study were shared between 2 – 5 of 17 different chemical stressors in yeast. Only one QTL was shared between more than five different chemical stressors (Ehrenreich et al. 2010). In a later study Ehrenreich et al. (2012) demonstrated similarly low levels of consistency in genetic architecture through extreme QTL mapping of 13 chemicals. Although we did not measure resistance to multiple heavy metal stressors in our study, we functionally validated several candidate genes implicated by QTL and DE gene lists which had functions linked to other metals including zinc, lead, manganese, and cadmium, or that were not linked to a specific metal (e.g *swm* (Q3), *babo* (Q5), and *stl* (Q5); Figure 10). Pleiotropic gene effects inferred from our RNAi analyses may be the result of metal-sensitive genes responding to a generalized set of cytotoxic effects stemming from production of reactive

oxygen species caused by heavy metal toxicity (Uriu-Adams and Keen 2005). This hypothesis is further supported by evidence of copper-induced expression of genes involved in oxidative stress response in sensitive strains that are repressed in resistant strains (e.g. *sesB*, Figure S7B, Figure 9). However, additional tests of the response of DSPR strains to a diverse set of heavy metals is needed to fully understand whether the non-copper candidate genes we identified have correlated effects on resistance to other heavy metals.

Consistency in the genetic architecture of Copper resistance across life stages

Genes that are involved in copper homeostasis in *D. melanogaster* adults have in some cases also been shown to regulate copper in larvae. For example, exposure of larvae to $CuSO_4$ induces expression of MTs (Egli et al. 2003, 2006b), and we demonstrated a copper-induced increase in MT expression in adults (Table S5). Knockdown of copper transporter genes in the CTR family alters copper homeostasis in both larvae and adult flies as well (Zhou et al. 2003; Turski and Thiele 2007). Given the similar genetic responses to copper in adults and larvae, we expected that the physiological response to copper stress would be correlated between life stages. It was previously established that developmental life stages are more susceptible to copper stress compared to adults; whereas adults were shown to survive for 80 days on 1mM $CuSO_4$, developmental viability from egg to adult on 1mM $CuSO_4$ was less than 10% in the same strain (Bahadorani and Hilliker 2009). Our goal was to understand the relationship between adult copper resistance and the effect of copper stress on development in a set of genetically diverse *D. melanogaster* strains.

Similar to previous reports, we found that copper stress delayed development and reduced viability (Zhou et al. 2003; Bahadorani and Hilliker 2009; Pölkki 2016) although to differing degrees among the DSPR strains (Figure 3), suggesting that as with adult copper resistance, copper-specific development time and developmental viability are genetically variable. Despite the lack of a statistically significant correlation between the developmental responses to copper stress and adult copper resistance (Figure 4), we did observe evidence of partially shared genetic architectures between copper-specific developmental viability and adult copper resistance (Figure 5B). Additional testing would be needed to determine whether the same genes implicated by developmental viability QTL Q15 and adult copper resistance QTL Q11

879 influence copper resistance at each life stage. Our detection of fewer QTL associated with copper-
880 specific developmental viability may be directly related to the reduced number of genotypes
881 sampled in our assay. Power to detect a 10% QTL with 100 DSPR strains is less than 20% (King et
882 al. 2012a), so the actual level of overlap in the genetic architecture between the adult and
883 developmental viability responses may be higher than we observe. Notably, founder haplotype
884 frequencies in the full set of lines and the subset of 100 are very similar across the genome (Figure
885 S8), so it is unlikely the case that the subset fails to capture the same allelic diversity present in
886 the full set.
887 Because the ecology of the developing and adult stages of *D. melanogaster* are quite
888 distinct, that copper resistance might be influenced by largely life stage-specific mechanisms is
889 not unexpected. For example, *D. melanogaster* adults and larvae avoid copper-supplemented
890 food when given the opportunity (Balamurugan et al. 2007; Bahadorani and Hilliker 2009);
891 however, in natural populations, higher mobility of adults would allow the adult life stage to
892 avoid heavy metal contaminated food more effectively. Life-stage specific genetic architectures
893 were observed in *D. melanogaster* for cold tolerance (Freda et al. 2019), and the decoupling of
894 the genetic mechanisms that influence survival and fitness have been reported in diverse
895 organisms with complex life cycles (Moran 1994; Ragland and Kingsolver 2008). However, a
896 number of other factors including the large difference in copper dose and the nature of the
897 response tested at each life stage may obscure or complicate the relationship between the
898 developmental and adult responses we observed. We used a much lower dose in our assessment
899 of the effect of copper on development time and developmental viability compared to the adult
900 copper resistance phenotype, and differences in dose can alter the genetic architecture for a
901 trait. For example, in the yeast *S. cerevisiae*, Wang and Kruglyak (2014) demonstrated that the
902 overall genetic architecture of haloperidol resistance was dose-dependent. While one QTL was
903 consistently detected for each of the 5 doses tested, several QTL were only detected at a single
904 dose (Wang and Kruglyak 2014). With the added complexity of assessing the effects of copper in
905 different life stages, it is difficult to fully determine whether the effects of copper in the adult
906 and developmental assays are analogous. Further confounding this comparison, our adult copper
907 assay was implemented over a 48-hour time period in contrast to exposing developing flies from

egg to adult to copper over a period of 30 days at most. The harmful effects of copper on development may be constant or variable across different stages (egg, larvae, pupation), and this represents an area of ongoing research.

Copper sensitivity is influenced by gene expression variation and behavior

Differences in expression levels of genes that have protective functions against toxins, or that are co-opted by toxins can lead to variation in resistance levels. For instance in humans, natural variation in expression levels of the gene *CMG2* is associated with variation in resistance to anthrax (Martchenko et al. 2012), and in the fungus *Suillus luteus*, selection pressure from heavy metal pollution quickly led to copy number variation in transport genes with protective functions against heavy metal toxicity (Bazzicalupo et al. 2019). Sensitivity to copper in adult *D. melanogaster* DSPR strains does not appear to be due to insufficient expression of genes involved with copper or metal detoxification such as MTs or CTR family transporters (Figure S7, Table S5). Instead, we found that genes associated with metabolism and mitochondrial function were copper-induced in sensitive strains and copper-repressed in resistant strains (Figure S7B, Figure 9). Given that we also observed that sensitive strains are more slightly likely to consume copper in larger amounts in a 24-hour period compared to resistant strains (Figure 2), sensitive strains may be under greater metabolic stress as they cope with exposure to behaviorally-mediated higher levels of ingested copper. Copper resistance in *D. melanogaster* may not be simply a function of how well the organism is able to detoxify food; more likely, copper resistance is a combination of behavioral aversion to copper and the metabolic stress induced by the amount of metal consumed in addition to detoxification ability.

In general, food consumption rate has a complex genetic basis in *D. melanogaster* (Garlapow et al. 2015), and when given a choice, both *D. melanogaster* adults and larvae tend to avoid copper-supplemented food at much lower concentrations relative to those tested in this study (Balamurugan et al. 2007; Bahadorani and Hilliker 2009). Bahadorani and Hilliker (2009) showed that adult copper avoidance was observed at 1mM $CuSO_4$, and avoidance in third-instar larvae was observed at 0.25mM $CuSO_4$ (Balamurugan et al. 2007). Similarly, adult *D. melanogaster* avoid pupation and oviposition on copper-supplemented food (Bahadorani and Hilliker 2009). While this behavioral component likely plays an important role in mediating

copper stress in natural populations, these studies focused on only one or few genetic strains, making it difficult to extrapolate how a genetically variable population would behave in response to copper. The correlation between adult copper resistance and copper food consumption in the 100 DSPR strains tested in our study suggests that variation in copper avoidance may play an important role in overall adult copper resistance. At this point, the specific relationship between copper consumption rates, metabolic stress, and genetic resistance to copper has not been characterized, but doing so in future studies has to potential to more clearly define resistance to ingested toxins compared to an assessment based solely on survival. Important remaining questions include whether behavioral avoidance and sensitivity to heavy metals are influenced by variation in chemosensory detection ability (e.g. Arya et al. 2015; He et al. 2016) or variation in preference for metal-supplemented food (e.g. Highfill et al. 2019). Addressing these questions with a large panel such as the DSPR will help support our efforts to characterize the relationship and potential interaction between behavior and genetic capacity for copper resistance.

Conclusions

Copper resistance in *D. melanogaster* is genetically complex, is influenced by allelic and expression variation as well as by variation in behavioral avoidance of copper, and may be controlled by distinct sets of loci in different life stages. Several genes that have known copper-specific functions as well as genes that are involved in the regulation of other heavy metals were identified as potential candidates for variation in adult copper resistance and copper-specific developmental viability. We demonstrated that nine of these candidates influenced adult copper resistance, providing evidence of pleiotropic effects of genes previously thought to be associated with other heavy metals. Copper is just one of many heavy metals that pollute the environment with negative impacts on humans, fungi, plants, and insects at a global scale. Understanding the complexity of the genetic basis of copper resistance and the potential sources of variation that interact with resistance is important for understanding the diverse mechanisms through which copper pollution can negatively impact organisms.

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Supplemental Figures

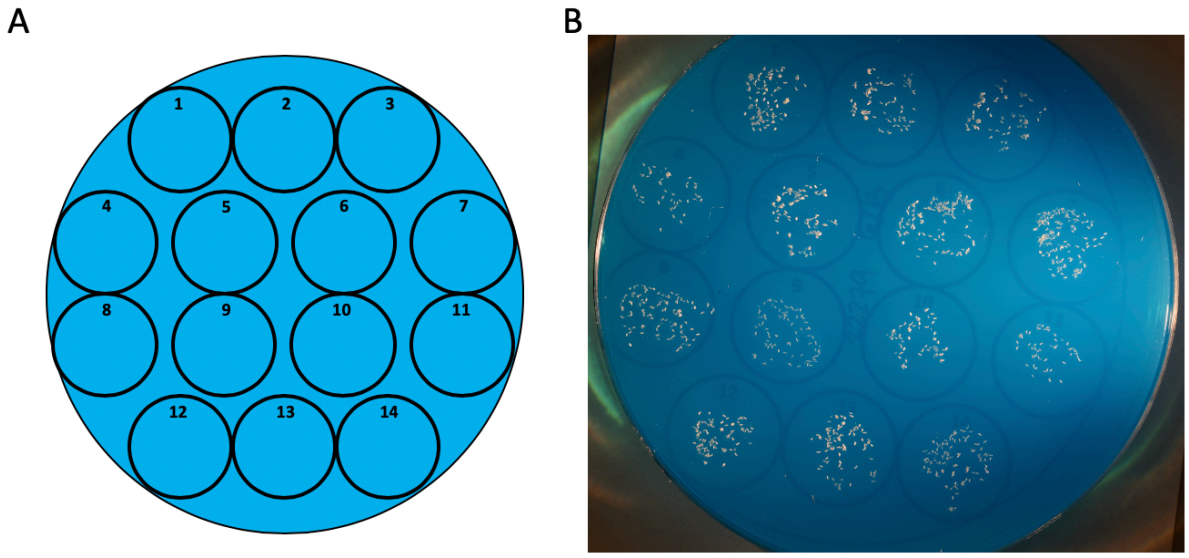

Figure S1. *Embryos from each strain were arrayed on a 100mm petri dish containing 2% agar with dye for contrast. A. Template used for arraying embryos. B. An example of embryos arranged in each cell of the template. After taking a picture of each dish, embryos were transferred to vials containing water or 2mM* $CuSO_4$*. All embryos from each cell on the template were transferred to one vial, resulting in up to 7 control vials and 7 copper vials per strain.*

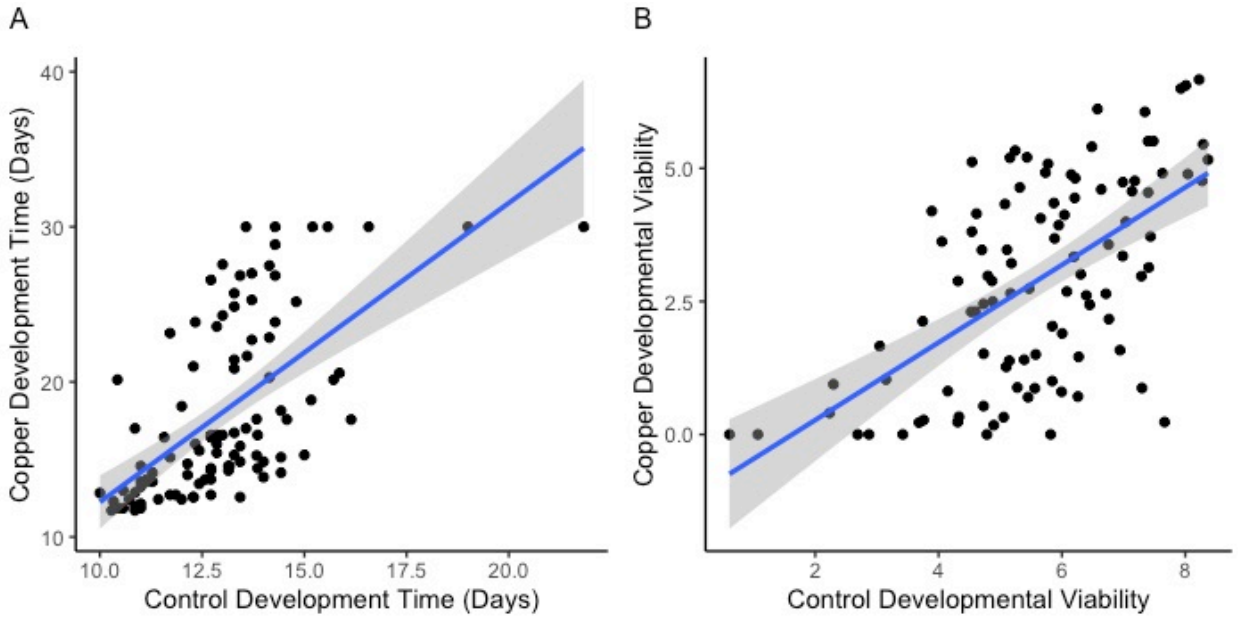

Figure S2. *Correlation between developmental responses on control and copper media. A. Development time on control food was correlated development time on copper food ($F_{(1,98)} = 61.0$, $P < 0.0001$, $R^2 = 38\%$). Vials in which no individuals emerged were given a value of 30 days. B Square root transformed developmental viability on control and 2mM $CuSO_4$ was also correlated ($F_{(1,98)} = 57.1$, $P < 0.0001$, $R^2 = 36\%$). Each point represents the strain mean response. Grey shading indicates the 95% CI of the regression.*

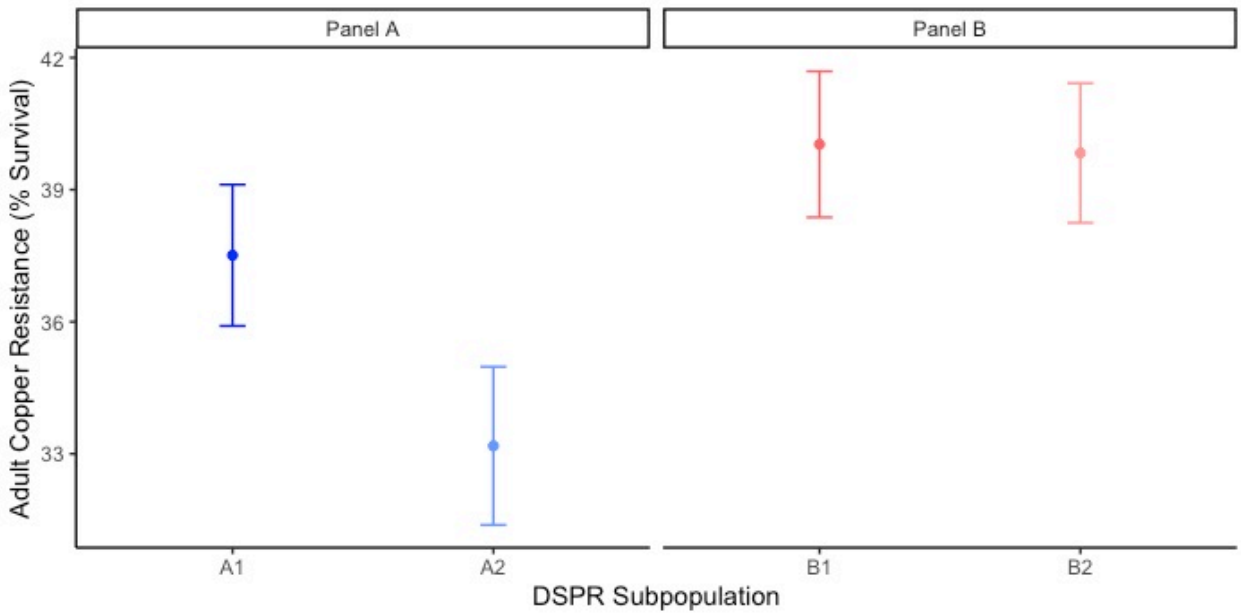

Figure S3. *Mean (± 95% CI) female copper resistance per subpanel. Subpanel influenced percent survival in the pA ($F_{1,2289}$ = 12.64; p < 0.001) but not pB panel ($F_{1,2495}$ = 0.03; p = 0.86).*

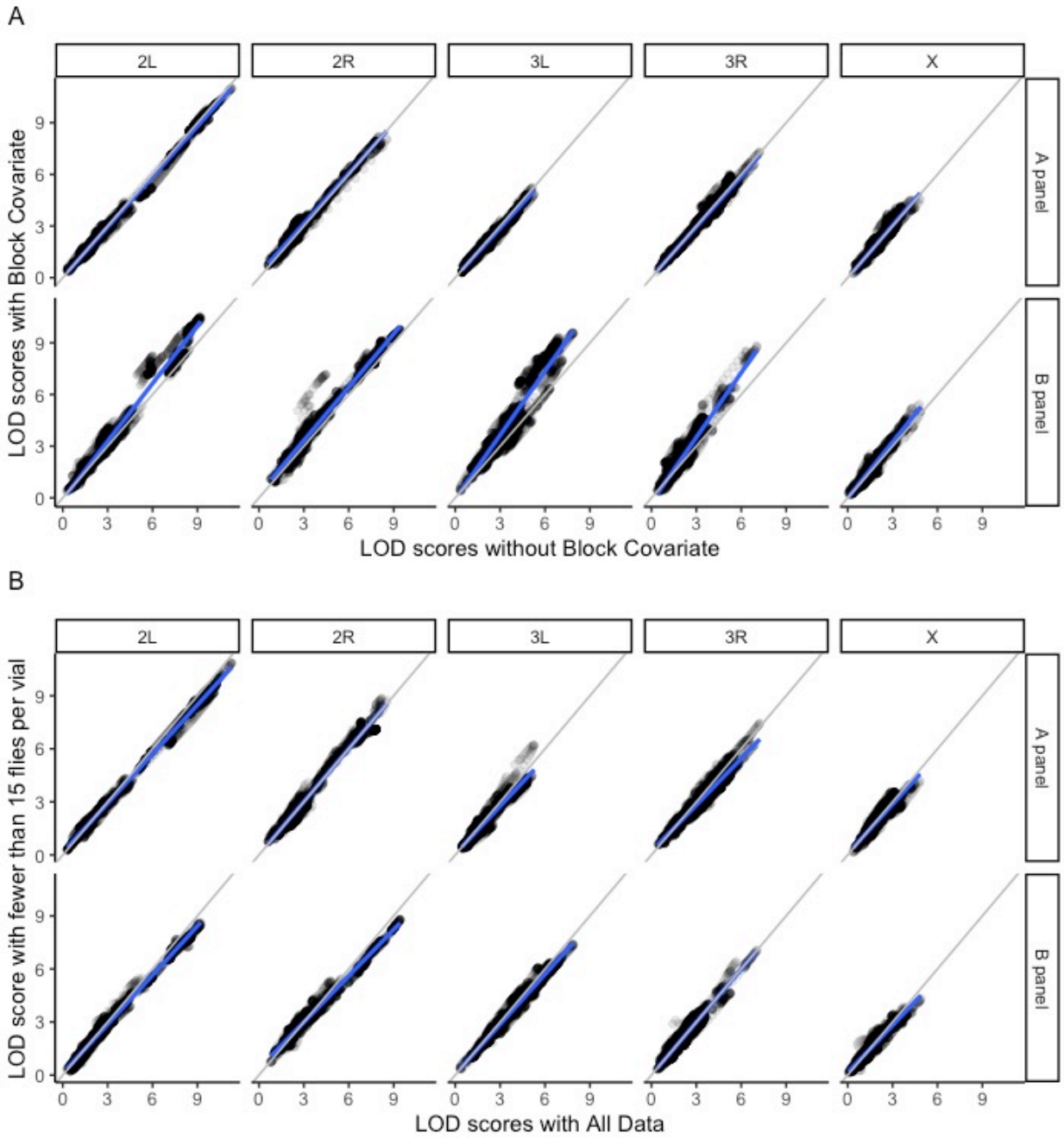

Figure S4. *Comparison of LOD scores between a model with batch included as a covariate (A) and when vials with fewer than 15 flies were removed from the calculation of mean survival prior to mapping (B). Best fit lines are shown in blue in each plot; grey lines indicate the 1:1 line for comparison.*

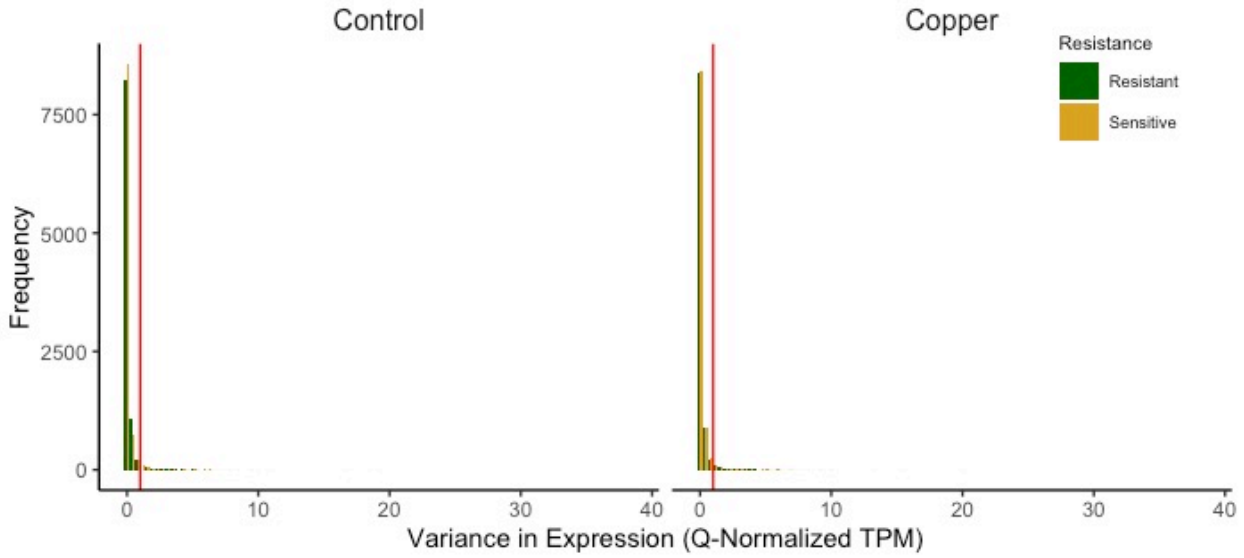

Figure S5. *Variance in quantile-normalized TPM by within each sample group (resistant vs. sensitive) and treatment group (copper vs. control). The red vertical line indicates the cutoff of 1. All genes with a variance equal to or greater than 1 were excluded from all downstream analyses of DE gene from the three DE models.*

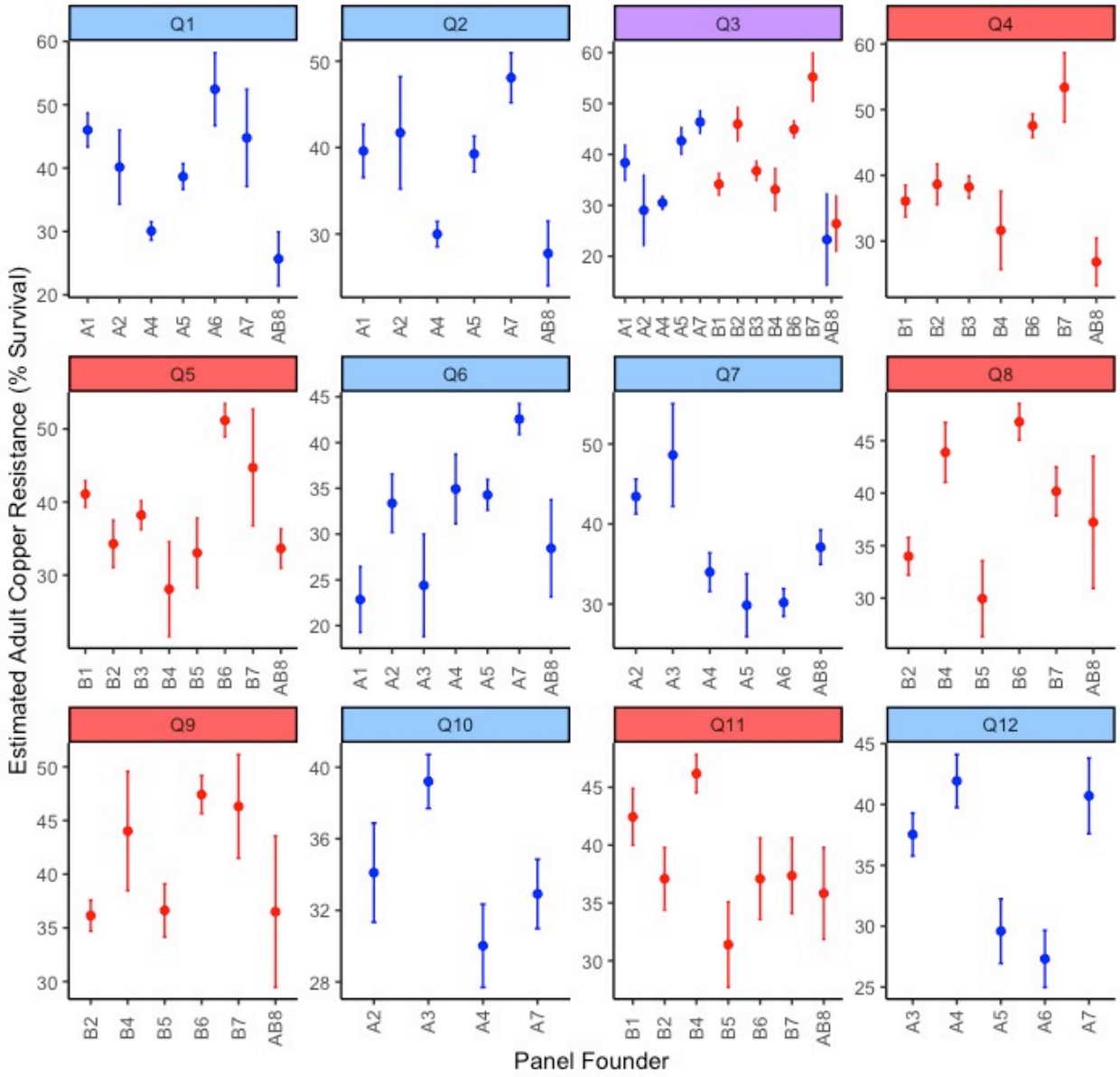

Figure S6. *Estimated founder haplotype effects at each QTL associated with adult copper resistance. Data are presented as estimated founder means (±SE) when the founder haplotype was present in more than 7 DSPR strains. Plots are colored by panel; panel A is plotted in blue, panel B is plotted in blue. Purple indicates the shared QTL (Q3) between the A and B panels.*

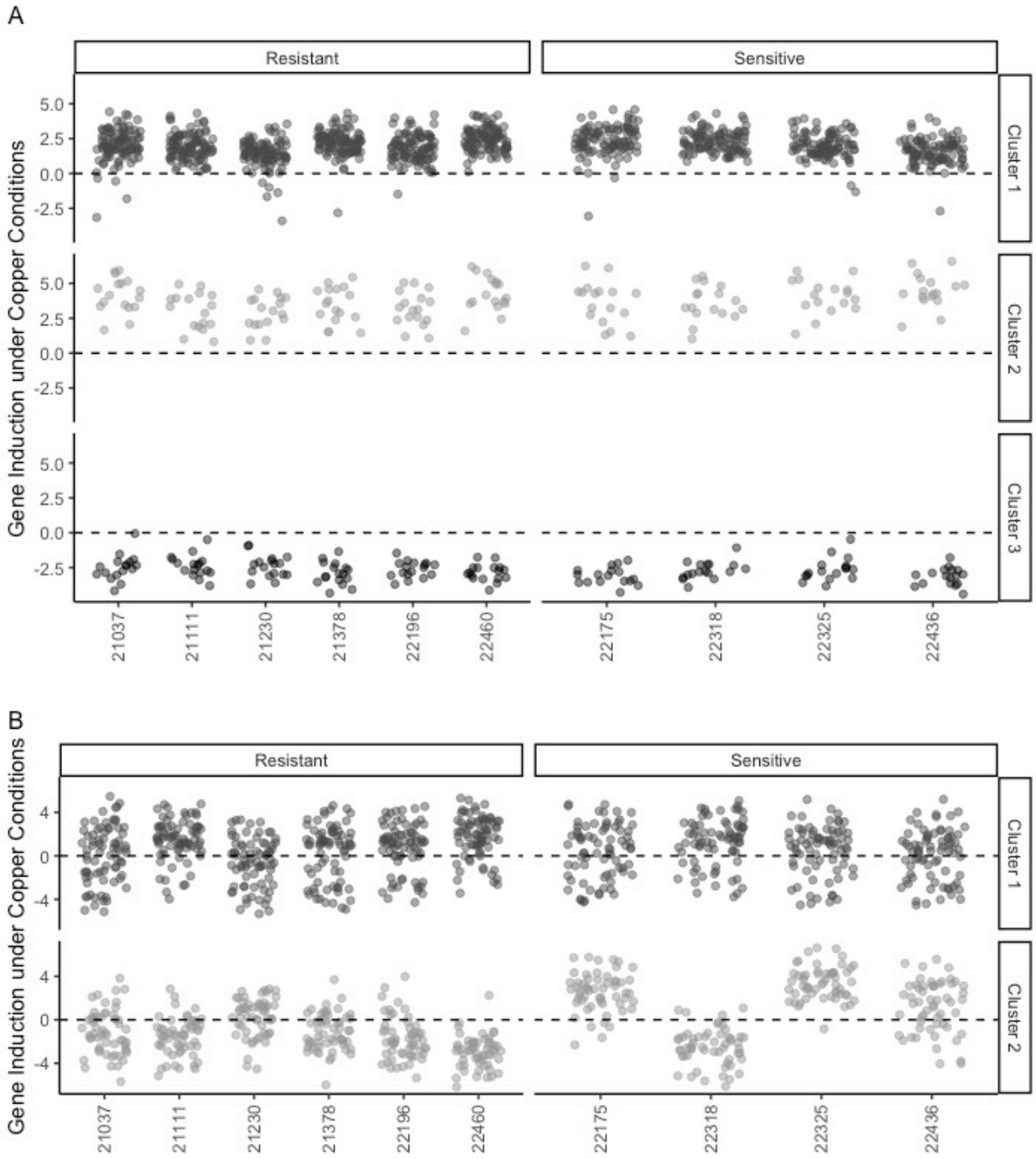

Figure S7. *Clust analysis of genes influenced by treatment (A) or resistance class (B). A. Clust identified 3 clusters from genes with DE due to treatment. 17 genes from cluster 1 were also implicated by adult copper resistance QTL; one gene was also implicated by copper-specific developmental viability QTL Q15. From treatment clusters 2 and 3, 2 and 4 genes, respectively, were also implicated by adult copper resistance QTL. B. Clust identified 2 clusters from genes with DE due to resistance class. In cluster1, 11 genes were also implicated by adult copper resistance QTL; 4 genes from cluster 2 were also implicated by adult copper resistance QTL. One gene from cluster 2 was implicated by copper-specific developmental viability QTL Q15 as well. Points are shaded to help distinguish clusters.*

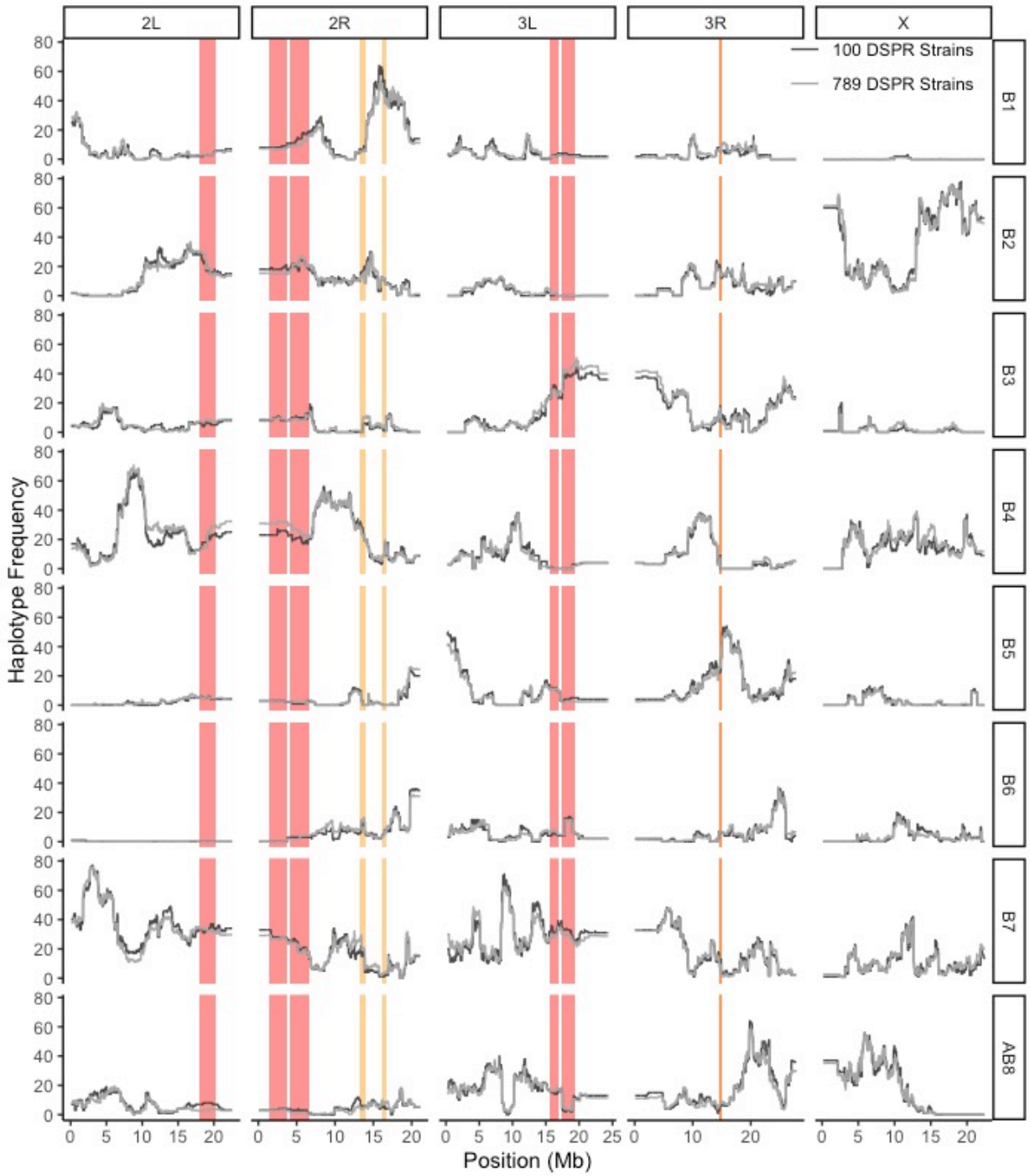

Figure S8. *Founder haplotype frequencies are shown at each marker position (every 10,000 bp) through the genome for the 789 DSPR strains sampled for the adult copper resistance phenotype (light grey) and the 100 DSPR strains sampled for the copper-specific developmental viability phenotype (dark grey). Representation of founder haplotypes in the DSPR strains sampled for the developmental phenotype is similar to founder haplotype representation in the 789 strains sampled for the adult phenotype. In each panel, QTL intervals for adult copper resistance and copper-specific developmental viability are shown as red and yellow bars, respectively.*

Supplemental Tables

Table S1. *Correlations between standards across each of plate and block were high and consistent.*

	block1_P1	block1_P2	block1_P3	block2_P1	block2_P2	block2_P3	block3_P1	block3_P2	block3_P3
block1_P1	--	99.99	99.99	99.97	99.97	99.91	99.96	99.94	99.94
block1_P2		--	99.99	99.98	99.98	99.90	99.97	99.96	99.96
block1_P3			--	99.98	99.98	99.92	99.97	99.95	99.96
block2_P1				--	99.99	99.86	99.99	99.98	99.97
block2_P2					--	99.87	99.98	99.97	99.97
block2_P3						--	99.84	99.86	99.88
block3_P1							--	99.98	99.97
block3_P2								--	99.99
block3_P3									--

Table S2. *Summary of analysis of variance and regressions.*

A. Effect of DSPR strain on development time

Source	df	SS	MS	F value	P value
DSPR Strain	99	1573	15.89	31.61	< 0.00001
Residuals	579	291.1	0.50		

B. Effect of DSPR strain on developmental viability

Source	df	SS	MS	F value	P value
DSPR Strain	99	2046	20.66	11.77	< 0.00001
Residuals	579	1016	1.75		

C. Correlation between copper and control development time

Source	df	SS	MS	F value	P value
Treatment	1	1214	1214	61.0	< 0.00001
Residuals	98	1950	19.9		

D. Correlation between copper and control developmental viability

Source	df	SS	MS	F value	P value
Treatment	1	128.4	128.4	54.1	< 0.00001
Residuals	98	232.8	2.38		

E. Variation in adult copper resistance among DSPR Mapping Panel A

Source	df	SS	MS	F value	P value
Subpanel	1	10621	10620.7	12.64	< 0.001
Batch	5	38679	7735.8	9.20	< 0.001
Subpanel x Batch	2	919	459.5	0.55	0.58
Residuals	2289	1923750	840.4		

F. Variation in adult copper resistance among DSPR Mapping Panel B

Source	df	SS	MS	F value	P value
Subpanel	1	25	24.5	0.03	0.86
Batch	5	30334	6066.7	7.18	< 0.001
Subpanel x Batch	3	2774	924.6	1.09	0.35
Residuals	2495	2107619	844.7		

G. Effect of dye consumption in 95 DSPR strain

Source	df	SS	MS	F value	P value
DSPR Strain	94	4.46e-5	4.70e-7	3.08	< 0.00001
Treatment	1	3.55e-4	3.55e-4	2306	< 0.00001
DSPR Strain x Treatment	94	4.01e-5	4.30e-7	2.77	< 0.00001
Residuals	348	5.36e-5	1.5e-7		

H. effect of adult copper resistance on dye consumption

Source	Estimate	SE	t value	P value
Intercept	1.80e-03	7.26e-05	24.8	< 0.00001

Adult Copper Resistance	1.80e-06	1.35e-06		1.31	0.19
Treatment	-1.40e-03	1.03e-04		-14.0	< 0.00001
Adult Copper Resistance x Treatment	-4.24e-06	1.91e-06		-2.22	0.027

I. Effect of copper on development time

Source	**df**	**SS**	**MS**	**F value**	**P value**
DSPR Strain	99	15505	156.6	24.21	< 0.00001
Treatment	1	8366	8366	1293	< 0.00001
DSPR Strain x Treatment	99	7525	76.0	11.75	< 0.00001
Residuals	1157	7485	6.5		

J. effect of copper on developmental viability

Source	**df**	**SS**	**MS**	**F value**	**P value**
DSPR Strain	99	3169	32.01	49.17	< 0.00001
Treatment	1	2543	2543	3905	< 0.00001
DSPR Strain x Treatment	99	846.0	8.55	13.13	< 0.00001
Residuals	1157	753.3	0.65		

K. correlation between copper-specific development time and copper-specific developmental viability

Source	**df**	**SS**	**MS**	**F value**	**P value**
Copper-specific Development Time	1	102.0	102.0	76.4	< 0.00001
Residuals	98	130.8	1.34		

L. Correlation between adult copper resistance and copper-specific development time

Source	**Estimate**	**SE**	**t value**	**P value**
Intercept	0.32	0.91	0.35	0.72
Adult Copper Resistance	-0.0006	0.02	-0.40	0.69

M. Correlation between adult copper resistance and copper-specific developmental viability

Source	**Estimate**	**SE**	**t value**	**P value**
Intercept	-0.45	0.31	-1.44	0.15
Adult Copper Resistance	0.009	0.006	1.65	0.10

N. Correlation between estimated haplotype effects

Source	**df**	**SS**	**MS**	**F value**	**P value**
Estimated Haplotype Effect	1	3.41	3.41	7.11	0.04
Residuals	5	2.40	0.48		

Table S3. *RNAi stocks for candidate genes.*

Gene	Stock ID	Association in This Study	Proposed Metal Association	Driver	N
trpl	RRID:BDSC_26722	Q5B, RNAseq	Mn (Gaudet et al. 2011)	Ubiquitous	17
				Midgut	18
CG5235	RRID:BDSC_27694	Q8B, RNAseq	Cu (Gaudet et al. 2011)	Ubiquitous	18
				Midgut	18
	RRID:BDSC_66964			Ubiquitous	11
				Midgut	18
ZnT41F	RRID:BDSC_65382	Q4B	Zn (Lye et al. 2013)	Ubiquitous	11
				Midgut	16
	RRID:BDSC_28638			Ubiquitous	17
				Midgut	18
MtnC	RRID:BDSC_53292	RNAseq	Cu, Zn, Cd (Egli et al. 2006a; Calap-Quintana et al. 2017)	Ubiquitous	NA
				Midgut	17
	RRID:BDSC_63008			Ubiquitous	12
				Midgut	18
Catsup	RRID:BDSC_55396	Q3A	Zn (Lye et al. 2013)	Ubiquitous	4
				Midgut	18
CG11825	RRID:BDSC_58199	Q5B	Cu (Norgate et al. 2007)	Ubiquitous	8
				Midgut	17
Ccs	RRID:BDSC_62919	Q5B	Cu, Zn (Kirby et al. 2008; Gaudet et al. 2011)	Ubiquitous	12
				Midgut	18
Sod1	RRID:BDSC_34616	NA	Cu, Zn (Gaudet et al. 2011)	Ubiquitous	17
				Midgut	18
Sod2	RRID:BDSC_32496			Ubiquitous	17
				Midgut	18
whd	RRID:BDSC_33635	Q5B, RNAseq	Fe, Cd (Strub et al. 2008)	Ubiquitous	18
				Midgut	18
DCP2	RRID:BDSC_34806	Q8B	Mn (Thurmond *et al.* 2019)	Ubiquitous	17
				Midgut	18
CG10505	RRID:BDSC_38317	RNAseq	Cu, Zn, Cd (Yepiskoposyan *et al.* 2006; Thurmond *et al.* 2019)	Ubiquitous	16
				Midgut	18
babo	RRID:BDSC_40866	Q5B	(Thurmond *et al.* 2019)	Ubiquitous	15
				Midgut	18
swm	RRID:BDSC_52935	Q3B	(Thurmond *et al.* 2019)	Ubiquitous	NA
				Midgut	13

Mvl	RRID:BDSC_55316	RNAseq	Fe, Cu, Mn, Cd (Southon et al. 2008; Bettedi et al. 2011)	Ubiquitous	14
				Midgut	18
stl	RRID:BDSC_57811	Q7A, RNAseq	Zn (Ozdowski et al. 2009)	Ubiquitous	18
				Midgut	18

Table S4. *Genes mapped to regions associated with each QTL. Data from FlyBase release FB2020_01. Grey text indicates non-protein coding genes. Red text indicates genes that overlap between QTL intervals.*

See additional file Supplemental Table 4

Table S5. *DE genes identified with the resistance class model (~ TRT + RES vs. reduced model: ~ TRT), treatment model (~ TRT + RES vs. reduced model: ~ RES), and the full model (~ TRT + RES vs reduced model: ~ 1). Gene position data is from FlyBase release FB2020_01.*

See additional file Supplemental Table 5

Table S6. *GO terms and associated gene IDs identified for the DE genes from the full model (~ TRT + RES vs reduced model: ~ 1), treatment model (~ TRT + RES vs. reduced model: ~ RES), resistance model (~ TRT + RES vs. reduced model: ~ TRT), and the clusters formed for the treatment and resistance sets of DE genes. GO analysis was performed using FlyMine.*

See additional file Supplemental Table 6

Supplemental Files

File S1. README file for datafiles accompanying this study.