THE MECHANISMS OF α-AMANITIN RESISTANCE IN THE FRUIT FLY DROSOPHILA MELANOGASTER

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THE MECHANISMS OF $\alpha$-AMANITIN RESISTANCE IN THE FRUIT FLY DROSOPHILA MELANOGASTER

By
Chelsea L. Mitchell

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Table of Contents

List of Figures .................................................................................................................... vi
List of Tables .................................................................................................................... vii
Preface .............................................................................................................................. viii
Acknowledgements ............................................................................................................ ix
Abstract ............................................................................................................................... x
Introduction ......................................................................................................................... 1
Chapter 1 ............................................................................................................................. 6
  1.1 Abstract ......................................................................................................................... 6
  1.2 Introduction ................................................................................................................... 7
  1.3 Results ........................................................................................................................... 9
    1.3.1 After five decades without selective pressure, the three Asian fly stocks are still resistant to α-amanitin .......................................................................................... 9
    1.3.2 α-Amanitin delays larva-to-adult development in a concentration-dependent manner ....................................................................................................................... 14
    1.3.3 α-Amanitin reduces adult body size development in a concentration-dependent manner ....................................................................................................................... 16
    1.3.4 α-Amanitin in the larval food increases egg-lay performance in adult females....................................................................................................................... 18
    1.3.5 α-Amanitin in larval food exerts a negative long-term effect on adult lifespan....................................................................................................................... 21
  1.4 Discussion ................................................................................................................... 23
    1.4.1 α-Amanitin resistance has no apparent fitness cost ........................................... 23
    1.4.2 α-Amanitin causes developmental retardation phenotypes that resemble stress responses.................................................................................................................... 24
    1.4.3 α-Amanitin increases female fecundity ............................................................. 26
    1.4.4 Implications ...................................................................................................... 27
    1.4.5 Limitations ........................................................................................................ 28
    1.4.6 Future research ................................................................................................. 29
  1.5 Conclusions ................................................................................................................. 30
  1.6 Materials and Methods ............................................................................................ 31
1.6.1 Fly stocks .......................................................................................................... 31
1.6.2 Generation of the isochromosomal lines .......................................................... 32
1.6.3 Dose-response studies of the fly stocks to α-amanitin ..................................... 32
1.6.4 Thorax measurements, fecundity, and longevity measurements ...................... 34
1.6.5 Statistical analyses ............................................................................................ 35
1.7 Acknowledgements ..................................................................................................... 35
1.8 Author Contributions .................................................................................................. 35
Chapter 2 ........................................................................................................................... 36
2.1 Abstract ....................................................................................................................... 36
2.2 Introduction ................................................................................................................. 37
2.3 Results ......................................................................................................................... 40
  2.3.1 Experimental Design ......................................................................................... 40
  2.3.2 Genes Encoding Cytochrome P450s, GSTs, and UGTs are Differentially Expressed in Ama-KTT/M/2 ................................................................. 43
  2.3.3 Genes Encoding Cytochrome P450s and GSTs are Inducible by α-Amanitin ................................................................................................................. 46
  2.3.4 Mdr Genes are Neither Constitutively Up-Regulated nor Inducible in Ama-KTT/M/2 ........................................................................................................... 48
  2.3.5 Genome Enrichment Analysis Confirms 30-Year-Old QTL Mapping Data .... 50
  2.3.6 Gene Ontology Enrichment Analysis Suggests Additional α-Amanitin Resistance Mechanisms ............................................................................................. 52
  2.3.7 The Domain Enrichment Analysis Verifies the Gene Ontology Enrichment Analysis ............................................................................................................. 57
  2.3.8 The RT-qPCR Results Confirm the Microarray Data ...................................... 60
2.4 Discussion ................................................................................................................... 62
  2.4.1 Several Mechanisms Seem to Confer α-Amanitin Resistance ......................... 62
  2.4.2 Implications ...................................................................................................... 66
  2.4.3 Limitations ........................................................................................................ 67
  2.4.4 Future Studies ................................................................................................... 69
2.5 Conclusions ................................................................................................................. 71
2.6 Materials and Methods ................................................................................................ 71
  2.6.1 Fly Stocks ......................................................................................................... 71
  2.6.2 Generation of the isochromosome stock Ama-KTT/M/2 .................................. 72
2.6.3 Dose-Response Studies of the Fly Stocks to α-Amanitin ........................................ 72
2.6.4 Sample Preparation for the Microarray Analysis .................................................. 74
2.6.5 Affymetrix Array Target Preparation, Hybridization, and Scanning .................... 75
2.6.6 Microarray Data Normalization .............................................................................. 75
2.6.7 Genome Enrichment Analysis ................................................................................. 76
2.6.8 Identification of Differentially Expressed Genes (DEGs) ...................................... 77
2.6.9 Gene Ontology Enrichment Analysis ..................................................................... 78
2.6.10 Protein Domain Enrichment (PDE) Analysis ....................................................... 78
2.6.11 RT-qPCR Validation of the Microarray Results .................................................... 79
2.7 Acknowledgments .................................................................................................. 80
2.8 Author Contributions ............................................................................................. 80
Conclusions .................................................................................................................... 81
Future Research ............................................................................................................. 82
References ..................................................................................................................... 83
Appendix A .................................................................................................................. 92
Appendix B .................................................................................................................. 94
List of Figures

Figure 1.1 LC50 analyses for all fly stocks.........................................................11
Figure 1.2 Crossing scheme for the generation of the isochromosomal lines........12
Figure 1.3 Adult hatch time delay of the three original Asian stocks...............15
Figure 1.4 Adult thorax lengths of the three original Asian stocks..................18
Figure 1.5 Female fecundity of the three original Asian stocks.......................20
Figure 2.1 Ama-KTT/M/2 is not less resistant to α-amanitin than Ama-KTT........42
Figure 2.2 Graphical representation of the groups of larvae used for the microarray and qPCR analysis.................................................................42
Figure 2.3 Genome enrichment analysis for genomic correlates......................52
Figure 2.4 Gene ontology enrichment analysis for Ama-KTT/M/2 versus Canton-S on no toxin (group 2 versus 1).........................................................54
Figure 2.5 Gene ontology enrichment analysis for Ama-KTT/M/2 on α-amanitin versus Ama-KTT/M/2 on no toxin (group 3 versus 2).........................57
Figure 2.6 The qPCR results confirm the microarray data..............................61
Figure 2.7 A model of the four mechanisms that contribute to the resistance to α-amanitin in concert.................................................................63
List of Tables

Table 1.1 Comparison between current and historic resistance values.......................13

Table 1.2 Average total egg productivity of the three original Asian stocks in
response to α-amanitin in the larval food..................................................21

Table 1.3 Longevity of the three original Asian stocks........................................22

Table 2.1 Single gene analysis for Ama-KTT/M/2 versus Canton-S on no toxin
(group 2 versus 1)..................................................................................44

Table 2.2 Single gene analysis for Ama-KTT/M/2 on α-amanitin versus Ama-
KTT/M/2 on no toxin (group 3 versus 2)...................................................47

Table 2.3 Domain enrichment analysis for Ama-KTT/M/2 versus Canton-S on
no toxin (group 2 versus 1)........................................................................59

Table 2.4 Domain enrichment analysis for Ama-KTT/M/2 on α-amanitin versus
Ama-KTT/M/2 on no toxin (group 3 versus 2)...........................................59

Table A.1 Differentially expressed genes (DEGs) between Ama-KTT/M/2 on no toxin
versus Canton-S (group 2 versus 1), Ama-KTT/M/2 on toxin versus Canton-S
(group 3 versus 1), and Ama-KTT/M/2 on toxin versus Ama-KTT/M/2 on no
toxin (group 3 versus 2)..........................................................................92

Table A.2 Complete single gene analysis for Ama-KTT/M/2 versus Canton-S on no
toxin (group 2 versus 1)...........................................................................92

Table A.3 Complete single gene analysis for Ama-KTT/M/2 on toxin versus Ama-
KTT/M/2 on no toxin (group 3 versus 2)...................................................92

Table A.4 Genome enrichment analysis for group 2 versus 1 and group 3
versus 2........................................................................................................93

Table A.5 Comparison of qPCR and microarray fold-induction values...................93
Preface

The article presented in Chapter 1 of this thesis is used with permission from PLoS ONE as a collaborative work with co-authors Dr. Thomas Werner, Roger D. Yeager, Zachary J. Johnson, Stephanie E. D’Annunzio, and Kara R. Vogel. All data were collected at Michigan Technological University in the lab of Dr. Werner. I aided in designing the experiments, performed the majority of the experiments, analyzed most of the data, and assisted in writing the Materials and Methods and Results sections. I also contributed to the Introduction and Discussion sections by performing literature searches and analyses.

The article presented in Chapter 2 of this thesis is used with permission from PLoS ONE as a collaborative work with co-authors Dr. Thomas Werner, Michael C. Saul, Liang Lei, and Dr. Hairong Wei. All data were collected at Michigan Technological University in the lab of Dr. Werner. I aided in designing the experiments, performed the majority of the experiments, analyzed most of the data, and assisted in writing the Materials and Methods and Results sections. I also contributed to the Introduction and Discussion sections by performing literature searches and analyses.
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Abstract

Insect pesticide resistance has become a costly problem in the US. To make things more problematic, resistance to one pesticide is often associated with cross-resistance to other toxins, including pesticides that have not yet even been developed. In this study, we investigated a possible type of cross-resistance in the model fruit fly *Drosophila melanogaster*, some stocks of which are resistant to the very potent mushroom toxin α-amanitin. Because α-amanitin is solely produced by mushrooms, and because *D. melanogaster* does not feed on mushrooms in nature, the fruit flies should not be resistant to this toxin. In order to understand how this mushroom toxin resistance evolved, we first examined the physiological aspects of α-amanitin resistance in three *D. melanogaster* stocks, which were isolated in Asia half a century ago: Ama-KTT, Ama-MI, and Ama-KLM. We showed that all three fly stocks have not lost their α-amanitin resistance over time, even though they were maintained in the absence of selective pressure over the past ~1,200 generations. When we reared these flies on sub-lethal α-amanitin concentrations in our laboratory, the females unexpectedly doubled their fecundity. This effect could have dramatic ecological consequences by enabling *D. melanogaster* to invade the toxic mushroom niche. As a result of this potential niche switch, other mushroom-feeding flies could be driven to extinction. However, we also noted signs of developmental retardation and a shortened life span of the flies in response to increasing α-amanitin concentrations in the food, suggesting that the flies are not yet well adapted to the toxin. We next elucidated the molecular mechanisms causing α-amanitin resistance by performing a whole genome microarray study. Our data suggest that 1) cuticular proteins block α-amanitin from entering cells, 2) phase I and phase II detoxification enzymes modify α-
amanitin to prepare it for excretion, 3) intracellular lipid particles sequester $\alpha$-amanitin in the cytoplasm, and 4) peptidases cleave $\alpha$-amanitin. Because the most highly up-regulated genes in our microarray study were Cytochrome P450/phase I detoxification genes that are known to detoxify pesticides, we speculate that the use of pesticides was the primary cause for the observed cross-resistance to the mushroom toxin $\alpha$-amanitin.
Introduction

Dietary poison resistance is an interesting phenomenon found in animals. Different mechanisms are present in these organisms to help defend against various poisons. These mechanisms include avoidance of the poisonous parts of the food, excretion of toxins from the organism, sequestration of poisons inside cells, and enzymatic breakdown of poisons in the body \(^1\). Some examples of organisms that show remarkable toxin resistance phenotypes are plant alkaloid-sequestering caterpillars that can deter predators from consuming the caterpillars \(^2\), soft-shell clams that can store toxins from algae in their bodies to poison anyone who eats them \(^3\), and snakes that feed on highly poisonous amphibians that no other animal can use as a food source \(^4\).

One of the most notable examples of organisms that are resistant to dietary toxins are mushroom-feeding \(Drosophila\) species, which feed on mushrooms containing mixtures of various toxins, one of which is the notorious \(\alpha\)-amanitin \(^5\)\(^-\)\(^8\). It has been shown that the resistance to mushroom toxins protects these flies efficiently from parasitic nematodes that would otherwise render the flies sterile \(^9\)\(^,\)\(^10\).

\(\alpha\)-Amanitin is the primary toxin in Death Cap and Destroying Angel mushrooms \(^11\). These mushroom species are so deadly that they account for about 90% of the mushroom-related deaths in the United States \(^12\). \(\alpha\)-Amanitin inhibits RNA-polymerase II, which brings eukaryotic mRNA production to a halt \(^13\). In all tested wild-caught fruit fly species, including \(\alpha\)-amanitin resistant species, RNA-polymerase II has been found to be very susceptible to \(\alpha\)-amanitin \(^5\)\(^,\)\(^6\). RNA-polymerase II is active in the nucleus of cells and because of this, the resistant fruit fly species must employ mechanisms that protect
RNA-polymerase II from α-amanitin. During my work as a Master’s student, we discovered the first mechanisms that confer mushroom toxin resistance in fruit flies.

The model organism *D. melanogaster* is a non-mycophagous species, meaning that the flies do not consume mushrooms in nature. Therefore, there is no reason why this species should encounter mushroom toxins in nature. However, it has been shown on multiple occasions that some stocks of *D. melanogaster* display a moderate resistance to α-amanitin, though not enough to feed on very toxic mushrooms$^{14,15}$. The three most famous α-amanitin-resistant *D. melanogaster* stocks were isolated in the 1960s in Asia: Ama-KTT from Taiwan, Ama-MI from India, and Ama-KLM from Malaysia. In 1982, these stocks were found to be 29-fold, 25-fold, and 8.3-fold, respectively, more resistant to α-amanitin than the wild-type stock Oregon R, which has an extremely low tolerance towards α-amanitin$^{14}$. In the same study, the α-amanitin resistance phenotype was genetically mapped to two loci on the third chromosome. In another study performed two decades later, α-amanitin resistance of one North American *D. melanogaster* stock was mapped to the same two chromosomal loci$^{15}$. This second study also suggested that the Multidrug resistance pump gene *Mdr65*, which is on the left arm of chromosome 3, and *Protein kinase C98E (Pkc98E)*, which is on the right arm of chromosome 3, could be the genes responsible for α-amanitin resistance. This hypothesis is intriguing because PKC98E can phosphorylate MDR proteins$^{16}$, and MDR proteins may facilitate the excretion of α-amanitin from cells. However, no evidence has ever been shown that these two genes are the actual cause of α-amanitin resistance in any mycophagous or non-mycophagous species of *Drosophila*. Thus, the actual cause of α-amanitin resistance was still elusive at this point.
Insect pesticide resistance is a huge problem in the United States. It can cause damage to crops, causing farmers to lose billions of dollars to insect pests. Pesticide resistance can often be associated with a cross-resistance to other chemicals. Cross-resistance can occur when an organism is exposed to one chemical and then becomes resistant to other substances that may or may not be chemically related to each other, as it has been shown with pesticide resistance in cockroaches, whiteflies, diamondback moths, mosquitos, house flies, and fruit flies. Some members of the phase I detoxification gene family Cytochrome P450 (Cyp) have been shown to detoxify a broad range of pesticides, such as dicyclanil, imidacloprid, and DDT, and other chemicals including phenobarbital. The phase II detoxification gene families Glutathione-S-transferase (Gst) and UDP glucuronosyl transferase (Ugt) have also been linked to pesticide resistance. Because the same detoxification genes were most strongly up-regulated in our resistant D. melanogaster stock, we conclude that α-amanitin resistance may be a cross-resistance to pesticides.

In this study, we show that even after five decades of no selective pressure, the three Asian D. melanogaster stocks Ama-KTT, Ama-MI, and Ama-KLM have remained resistant to α-amanitin until today. We also show that an increase in α-amanitin concentration in the food increases female fecundity, but that it affects the larva-to-adult developmental time and longevity of the resistant fly stocks. Because the α-amanitin resistance phenotype has persisted over half a century, it seems like there is no major fitness cost associated with α-amanitin resistance. Normally, organisms lose toxin resistance after a few generations without selective pressure because there often is a fitness cost associated with resistance (“use it or lose it”). Mushroom toxin resistance,
however, is one of the first examples of toxin resistance that comes at no obvious fitness cost to an organism.

We conducted a microarray analysis to investigate the genes that confer $\alpha$-amanitin resistance across the genome of *D. melanogaster*. We used an isochromosome stock created to be homozygous for the second and third chromosomes for the Asian fly stock Ama-KTT. Based on the gene mapping data generated in 1982 $^{14}$ and 2000 $^{15}$, we had expected to see *Mdr* genes and the *Pkc98E* gene to be up-regulated in our $\alpha$-amanitin-resistant flies. However, this was not the case. Instead, we saw genes from the *Cyp* (phase I detoxification), *Gst* (phase II detoxification), and *Ugt* (phase II detoxification) families up-regulated in the resistant flies. In particular, a several hundred fold up-regulation of the *Cyp* genes *Cyp6a2*, *Cyp12d1-d*, and *Cyp12d1-p* was associated with the $\alpha$-amanitin resistance phenotype in our study, which are known players in pesticide detoxification. We also showed evidence that perhaps peptidases, lipid particles, cuticular proteins, and salivary gland secretion proteins could have some involvement in $\alpha$-amanitin resistance, perhaps by altering $\alpha$-amanitin or by blocking $\alpha$-amanitin so that it cannot enter the cells. Because of the known functions of our *Cyp* and *Gst* candidate genes in insecticide resistance, we believe that a cross-resistance may have evolved in response to pesticide exposure in Asia five decades ago.

This research has far-reaching implications on the ecology and physiology of animals, but also on agriculture. Cross-resistance associated with pesticide resistance currently is and will present a major biological problem. For example, a change in the egg-lay preference of *D. melanogaster* females from rotten fruit towards mushrooms
could lead to dramatic changes in the occupation of the toxic mushroom niche, which may drive mushroom-feeding flies entirely out of their niche, especially considering the extremely high female fecundity (egg-lay production rate) that further increases in response to α-amanitin in *D. melanogaster*. In terms of agricultural problems, it is alarming that pest species can and do develop cross-resistance to pesticides that aren’t even on the market yet. Our research is important because we elucidated the molecular mechanisms of cross-resistance to poisons, which is broadly applicable to insect pesticide resistance.
Chapter 1

Long-Term Resistance of *Drosophila melanogaster* to the Mushroom Toxin Alpha-Amanitin

The material contained in this chapter was previously published in *PLoS ONE*


1.1 Abstract

Insect resistance to toxins exerts not only a great impact on our economy, but also on the ecology of many species. Resistance to one toxin is often associated with cross-resistance to other, sometimes unrelated, chemicals. In this study, we investigated mushroom toxin resistance in the fruit fly *Drosophila melanogaster* (Meigen). This fruit fly species does not feed on mushrooms in nature and may thus have evolved cross-resistance to α-amanitin, the principal toxin of deadly poisonous mushrooms, due to previous pesticide exposure. The three Asian *D. melanogaster* stocks used in this study, Ama-KTT, Ama-MI, and Ama-KLM, acquired α-amanitin resistance at least five decades ago in their natural habitats in Taiwan, India, and Malaysia, respectively. Here we show that all three stocks have not lost the resistance phenotype despite the absence of selective pressure over the past half century. In response to α-amanitin in the larval food, several signs of developmental retardation become apparent in a concentration-dependent manner: higher pre-adult mortality, prolonged larva-to-adult developmental time, decreased adult body size, and reduced adult longevity. In contrast, female fecundity

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1 The material contained in this chapter was previously published in *PLoS ONE.*
nearly doubles in response to higher α-amanitin concentrations. Our results suggest that
α-amanitin resistance has no fitness cost, which could explain why the resistance has
persisted in all three stocks over the past five decades. If pesticides caused α-amanitin
resistance in *D. melanogaster*, their use may go far beyond their intended effects and
have long-lasting effects on ecosystems.

### 1.2 Introduction

Insect pesticide resistance costs the United States billions of dollars in crop losses
and pesticide design every year \(^1\). Oftentimes, pesticide resistance is associated with
cross-resistance to several other chemicals, such as in mosquitoes \(^{20,21}\), potato beetles \(^{48}\),
whiteflies \(^{18}\), diamondback moths \(^{19}\), cockroaches \(^{17}\), house flies \(^{22}\), and fruit flies \(^{23-25}\). In
this study, we describe a curious mushroom toxin resistance phenotype in the fruit fly *D.
melanogaster*, which may have evolved from pesticide exposure in their natural habitats.

α-Amanitin is the principal toxin of several deadly poisonous mushrooms, such as
the Death Cap and Destroying Angel \(^{11}\). These two mushroom species account for about
90% of the mushroom-related deaths in the United States \(^{12}\). α-Amanitin exerts its toxic
function by inhibiting RNA-polymerase II, thereby interfering with messenger RNA
production in eukaryotic organisms \(^{13}\). Because RNA-polymerase II in all tested wild-
captured fruit fly species is very susceptible to this toxin \(^{5,6}\), the flies must employ unique
mechanisms that prevent the toxin from entering the nucleus of the cells, where the RNA-
polymerase II is active.

Mushroom-feeding (mycophagous) *Drosophila* species are super-resistant to all
mushroom toxins, allowing them to breed in virtually all toxic mushrooms \(^{5-8}\). This
unusual ability provides these flies with access to a unique food source and protection
from parasitic nematodes, which would render the flies sterile.\textsuperscript{9,10}

Paradoxically, mushroom toxin resistance is even found in some mushroom-
avoiding fruit flies, such as certain stocks of the genetic model organism \textit{D. melanogaster}.
Because \textit{α}-amanitin is solely produced by mushrooms,\textsuperscript{49-51} these flies should never
encounter this mushroom toxin in nature. In the 1960s, the first three \textit{α}-amanitin-resistant
\textit{D. melanogaster} stocks were isolated in Asia: Ama-KTT from Taiwan, Ama-MI from
India, and Ama-KLM from Malaysia. In 1982, they were shown to be 29-fold, 25-fold,
and 8.3-fold, respectively, more resistant to \textit{α}-amanitin than the susceptible wild type
stock Oregon-R.\textsuperscript{14} These three resistant stocks are, however, not resistant enough to
survive a poisonous mushroom diet.\textsuperscript{14,52}

In two studies, \textit{α}-amanitin resistance of four resistant Asian and North American
\textit{D. melanogaster} stocks was mapped to virtually the same two dominantly acting loci on
the third chromosome,\textsuperscript{14,15} suggesting that the resistance phenotype may have spread
globally. Begun and Whitley\textsuperscript{15} suggested that the Multidrug resistance pump gene
\textit{Mdr65} (on the left arm of chromosome 3) and the \textit{Protein kinase C98E (Pkc98E)} gene
(on the right arm of chromosome 3) confer \textit{α}-amanitin resistance, thus, protecting the
susceptible RNA-polymerase II in the nucleus. In our previously published work, we re-
investigated this case by performing a microarray analysis, using the Ama-KTT-derived
isochromosomal line Ama-KTT/M/2. We found that four molecular mechanisms, but
probably not a multidrug resistance pump, may contribute to \textit{α}-amanitin resistance in this
\textit{D. melanogaster} stock: cuticular proteins block the entry of \textit{α}-amanitin into cells,
Cytochrome P450 and Glutathione-S-transferase enzymes detoxify \textit{α}-amanitin,
peptidases cleave α-amanitin, and lipid particles sequester α-amanitin in the cytoplasm. Remarkably, three Cytochrome P450 genes were at least 200-fold constitutively up-regulated in the resistant larvae: Cyp6a2, Cyp12d1-d, and Cyp12d1-p. These genes have been shown to respond to, or detoxify, various chemically unrelated substances, including the pesticides DDT, imidacloprid, dicyclanil, atrazine, and the drug phenobarbital. Thus, α-amanitin resistance in D. melanogaster may have evolved as cross-resistance to pesticides applied to the habitats of these flies, such as gardens, vineyards, and other fruit plantations.

In the present study, we show that the three Asian D. melanogaster stocks Ama-KTT, Ama-MI, and Ama-KLM are still resistant to α-amanitin, even after five decades of being maintained in a stock center without any selective pressure (~1,200 generations). Furthermore, the addition of α-amanitin to the larval food increases female fecundity, but also affects larva-to-adult development and longevity of the resistant fly stocks. We conclude that α-amanitin resistance has no obvious fitness costs in the three Asian D. melanogaster stocks, explaining why the resistance phenotype has persisted in these populations for such a long time.

1.3 Results

1.3.1 After five decades without selective pressure, the three Asian fly stocks are still resistant to α-amanitin

The three Asian D. melanogaster stocks Ama-KTT from Taiwan, Ama-MI from India, and Ama-KLM from Malaysia were collected from their natural habitats in the
1960s. In 1982, i.e. two decades after their isolation, these stocks were shown to be 29-fold (Ama-KTT), 25-fold (Ama-MI), and 8.3-fold (Ama-KLM) more resistant to the mushroom toxin $\alpha$-amanitin than the susceptible wild type stock Oregon-R $^{14}$. In this study, we tested if these three Asian fly stocks have retained their resistance after five decades of being reared in the stock center without selective pressure. We first calculated the current lethal concentration 50 (LC$_{50}$) values of Ama-KTT, Ama-MI, Ama-KLM, and Oregon-R, which are the $\alpha$-amanitin concentrations in the larval food in [µg/g] that cause 50% of the individuals to die before the adults emerge. Additionally, we included the wild type stock Canton-S in our comparison because it has recently become a more widely used control in many studies. For each dose-response curve, we placed 100 freshly hatched first-instar larvae per concentration on $\alpha$-amanitin-containing food. Eleven toxin concentrations (including the 0-toxin control) were used, and three replicates were performed for each dose-response experiment. We counted hatching flies as survivors, followed by ANOVA analysis. From this experiment, we established that all three Asian fly stocks are still more resistant than Oregon-R: Ama-KTT is currently 22-fold, Ama-MI 10-fold, and Ama-KLM 11-fold more resistant than the Oregon-R control flies (Figure 1.1, Table 1.1). We note that the resistance differences observed between 1982 and today may be due to the slightly different methodologies used in both studies: for higher accuracy, we manually placed healthy, counted first-instar larvae on toxic food, while in the 1982 study, females laid uncontrolled numbers of eggs on non-toxic food that was later supplemented with $\alpha$-amanitin.
**Figure 1.1. LC50 analyses for all fly stocks.** A) Oregon-R, Canton-S, and multi-balancer stock; B) Ama-KTT, Ama-KTT/M/2, and Ama-KTT/M/5; C) Ama-MI, Ama-MI/M/2, and Ama-MI/T/6; D) Ama-KLM, Ama-KLM/M/5, and Ama-KLM/M/7 LC50 analyses are shown. All analyses contain three experimental replicates (100 larvae in each experiment for each concentration) and were normalized, using 0-toxin concentration as a control. The error bars represent the standard error of the mean (s.e.m.).

We further investigated the α-amanitin resistance level of the commonly used control stock Canton-S. Our data show that Canton-S is three times more susceptible to the toxin than Oregon-R (Figure 1.1A, Table 1.1). Comparing the various resistance levels of all five stocks that we tested, it seems that α-amanitin resistance is a more variable genetic trait among *D. melanogaster* stocks than it was previously anticipated.
Figure 1.2. Crossing scheme for the generation of the isochromosomal lines. One highly resistant virgin female of each original Asian fly stock was mated with two males of the multi-balancer stock. F1 generation males that carried an Ama chromosome 2 balanced over CyO and an Ama chromosome 3 balanced over TM6B, Tb or MKRS were crossed back to one multi-balancer virgin female. F2 generation males carrying an Ama chromosome 2 balanced over CyO and an Ama chromosome 3 balanced over TM6B, Tb were back-crossed to one multi-balancer virgin female. Virgin siblings of the F3 generation were then crossed to produce the isochromosomal lines.

Over the past ~50 years, allelic drift and/or reverse mutations of resistance-conferring alleles could have occurred in the stock center. Therefore, we wanted to make sure that the three Asian stocks are still largely homozygous for the resistance-conferring alleles/loci. We thus created isochromosomal lines by using one toxin-selected, highly
resistant virgin female of Ama-KTT, Ama-MI, and Ama-KLM, following the crossing scheme outlined in Figure 1.2. Although Phillips et al. \textsuperscript{14} suggested that only two dominantly acting third chromosome loci underlie \(\alpha\)-amanitin resistance in all three Asian fly stocks, we did not exclude the possibility that genes located on other chromosomes contribute to the resistance. Thus, we created isochromosomal lines that are isogenic for both major autosomes: chromosomes 2 and 3. We preliminarily tested all resulting isochromosomal lines for \(\alpha\)-amanitin resistance, with the result that all of them were approximately as resistant to the toxin as the original stocks (Figure 1.1, Table 1.1). We then focused on two randomly chosen isochromosomal lines that descended from each original Asian stock (Ama-KTT/M/2, Ama-KTT/M/5, Ama-MI/M/2, Ama-MI/T/6, Ama-KLM/M/5, and Ama-KLM/M/7) and calculated their exact LC\(_{50}\) values. As a result, the isochromosomal lines showed similar resistance levels to their parental stocks, suggesting that no major genetic changes have reversed the resistance phenotype over time. We note that the small differences that we detected in our assay may be due to experimental noise.

Table 1.1. \textbf{Comparison between current and historic resistance values.} Our calculated LC\(_{50}\) values and how they compare to the values calculated in 1982 \textsuperscript{14} are shown. Oregon-R served as the normalization control for the relative resistance values between today and 1982. LC\(_{50}\) values are given in [\(\mu\)g of \(\alpha\)-amanitin per g of larval food]. All values are averages of three experimental replicates.
<table>
<thead>
<tr>
<th>Stock</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (± s.e.m)</th>
<th>Current comparison to Oregon-R</th>
<th>1982 Comparison to Oregon-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canton-S</td>
<td>0.028 (± 0.001)</td>
<td>0.34-fold</td>
<td>-</td>
</tr>
<tr>
<td>Oregon-R</td>
<td>0.082 (± 0.005)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Multi-balancer</td>
<td>0.042 (± 0.001)</td>
<td>0.51-fold</td>
<td>-</td>
</tr>
<tr>
<td>Ama-KTT</td>
<td>1.843 (± 0.054)</td>
<td>22-fold</td>
<td>29-fold</td>
</tr>
<tr>
<td>Ama-KTT/M/2</td>
<td>2.167 (± 0.074)</td>
<td>26-fold</td>
<td>-</td>
</tr>
<tr>
<td>Ama-KTT/M/5</td>
<td>3.522 (± 0.120)</td>
<td>43-fold</td>
<td>-</td>
</tr>
<tr>
<td>Ama-MI</td>
<td>0.797 (± 0.094)</td>
<td>10-fold</td>
<td>25-fold</td>
</tr>
<tr>
<td>Ama-MI/M/2</td>
<td>1.600 (± 0.038)</td>
<td>20-fold</td>
<td>-</td>
</tr>
<tr>
<td>Ama-MI/T/6</td>
<td>1.518 (± 0.035)</td>
<td>19-fold</td>
<td>-</td>
</tr>
<tr>
<td>Ama-KLM</td>
<td>0.924 (± 0.052)</td>
<td>11-fold</td>
<td>8.3-fold</td>
</tr>
<tr>
<td>Ama-KLM/M/5</td>
<td>0.855 (± 0.052)</td>
<td>10-fold</td>
<td>-</td>
</tr>
<tr>
<td>Ama-KLM/M/7</td>
<td>0.912 (± 0.057)</td>
<td>11-fold</td>
<td>-</td>
</tr>
</tbody>
</table>

1.3.2 α-Amanitin delays larva-to-adult development in a concentration-dependent manner

Mycophagous *Drosophila* species are usually super-resistant to mushroom toxins and show no deleterious developmental effects when breeding in most toxic mushrooms. Only at extremely high α-amanitin concentrations (250 - 1000 µg α-amanitin per g of mushroom), some mycophagous *Drosophila* species can show signs of developmental retardation, i.e., the larvae develop more slowly and the adults are smaller and have sometimes reduced or missing eyes. We were curious to see if the three resistant Asian *D. melanogaster* stocks Ama-KTT, Ama-MI, and Ama-KLM show similar developmental retardation symptoms in response to increasing α-amanitin concentrations and at what toxin concentrations these symptoms become apparent. First, we investigated the effect of α-amanitin on the larva-to-adult developmental time of Ama-KTT, Ama-MI, and Ama-KLM. For these experiments, we used the same animals that gave rise to the LC<sub>50</sub> data, followed by ANOVA analysis. Once every day, we recorded the numbers of hatched flies from each toxin concentration. We then compared the days on which the
hatching activity peaked. Our results (Figure 1.3) show that all three Asian stocks behaved similarly: increased α-amanitin concentrations caused concentration-dependent hatch time delays. For all three fly stocks, the lowest toxin concentrations delayed the peak of fly hatching by one day, while the highest tolerable concentrations caused up to three days of hatch delay, as compared to the 0-toxin concentration. Thus, unlike mycophagous *Drosophila* species, the three resistant Asian *D. melanogaster* stocks showed a developmental retardation phenotype that became apparent even at low toxin concentrations and became more severe as the toxin concentrations increased.
Figure 1.3. Adult hatch time delay of the three original Asian stocks. A) Ama-KTT, B) Ama-MI, and C) Ama-KLM. The first-instar larvae were laid on day 0. The data resulting from three experimental replicates were pooled. The error bars represent the s.e.m.

1.3.3 α-Amanitin reduces adult body size development in a concentration-dependent manner

We then tested how α-amanitin affects the adult body size of the three Asian stocks Ama-KTT, Ama-MI, and Ama-KLM (Figure 1.4), using again the same flies that were used in the previous LC50 and hatching time analyses. Because thorax lengths of flies are fixed throughout life and directly correlate with overall body size53-57, we measured the thorax lengths of all of the flies as a proxy for overall body size. Each experiment was performed in three replicates, and the data underwent ANOVA analysis. Our results show that all three Asian D. melanogaster stocks responded in similar ways to increasing α-amanitin concentrations, but differently from how mycophagous species respond to the same toxin. We observed seven trends that the three resistant D. melanogaster stocks shared (Figure 1.4): 1) on toxin-free control food, the emerging flies were somewhat smaller than flies that hatched from the lowest α-amanitin concentrations. 2) With increasing toxin concentrations, the thorax lengths first increased until a "sweet spot" was reached, which was always slightly above the LC50 of the respective stock (Figure 1.4, Table 1.1). This paradoxical thorax length increase may be an indirect effect due to reduced larval crowding, so that the surviving larvae had more food and could grow larger. 3) Above the "sweet spot" concentration, the thorax lengths then started to gradually decline in a toxin concentration-dependent manner. 4) In all three Asian D.
melanogaster stocks, the female's onset of thorax length decline started exactly at one concentration increment lower than in males, indicating that males may be slightly more resistant to α-amanitin than females. 5) The highest tolerable toxin concentration of each stock always resulted in thorax lengths lower than those at the 0-toxin concentration. 6) The higher the LC₅₀ of a stock, the more α-amanitin was necessary to bring the thorax length values below that of the 0-toxin concentration. 7) The lower the LC₅₀ of a stock, the further the thorax lengths declined below the values of the 0-toxin concentration. In summary, D. melanogaster's body size is affected by α-amanitin in a gradual, concentration-dependent manner, which stands in contrast to the sudden response in mycophagous flies at only the highest tolerable toxin concentrations. Furthermore, none of the three Asian D. melanogaster stocks showed signs of reduced or missing eyes on any α-amanitin concentration. The resistance of the three Asian D. melanogaster stocks is, although impressive compared to other susceptible stocks of this species, still two to three orders of magnitude weaker than the resistance of mycophagous flies.
Figure 1.4. Adult thorax lengths of the three original Asian stocks. A) Ama-KTT, B) Ama-MI, and C) Ama-KLM. Male and female thorax lengths (y-axis) from flies that hatched from different α-amanitin concentrations (x-axis) were measured. The data resulting from three experimental replicates were pooled. The error bars represent the s.e.m.

1.3.4 α-Amanitin in the larval food increases egg-lay performance in adult females

For the remaining tests, adults from the previous analyses that hatched within the three days of peak hatching were kept alive on non-toxic molasses agar and fresh yeast in
egg-lay chambers until they died. The flies were supplied with fresh food on a daily basis. We next asked the questions if and how different α-amanitin concentrations fed to the larvae affect female fecundity of the hatched flies. We grouped all females that hatched on the same day (day 0) from each toxin concentration into one egg-lay chamber and monitored their fecundity daily. Because females have a shorter lifespan when males are present\textsuperscript{58}, all females were accompanied by an equal number of males to balance the sex ratio across all experiments. When available, we added males of the same stock that hatched on the same day from the same toxin concentration. As an alternative, we accompanied our experimental females with young white-eyed males of the \textit{w}\textsuperscript{1118} stock because they could be easily distinguished from the toxin-resistant flies and thus excluded from the longevity experiments, as described in the next section. We performed three experimental replicates, and the data underwent ANOVA analysis. Considering the negative effects that α-amanitin exerts on the development of the three Asian \textit{D. melanogaster} stocks, we expected that higher toxin concentrations would result in lower eggs-per-female production rates and delayed egg-lay peak times. All three Asian stocks responded in a similar manner to increasing α-amanitin concentrations (Figure 1.5, Table 1.2). In contrast to our expectation, at the two to three lowest toxin concentrations, the egg-lay peak performance was shifted to one day \textit{earlier} than that of the 0-toxin concentration flies. Often, the flies on these toxin concentrations also laid more eggs than on the 0-toxin concentration. The higher α-amanitin concentrations then caused the expected concentration-dependent delay in egg-lay activity peaks by up to four days. Perhaps the most surprising result was that each stock produced about twice the amount of eggs per female at the second highest tolerable α-amanitin concentration, as compared
to the 0-toxin concentration (Table 1.2). Our results indicate that α-amanitin increases the reproductive fitness of all three Asian fly stocks.

Figure 1.5. Female fecundity of the three original Asian stocks. A) Ama-KTT, B) Ama-MI, and C) Ama-KLM. Day 0 is the day of adult female hatching. The data resulting from three experimental replicates were pooled. The error bars represent the s.e.m.
Table 1.2. Average total egg productivity of the three original Asian stocks in response to α-amanitin in the larval food. The average total eggs-per-female numbers for the three original Asian stocks in response to different α-amanitin concentrations in [µg of α-amanitin per g of larval food] are shown. All values are averages of three experimental replicates.

<table>
<thead>
<tr>
<th>Stock</th>
<th>α-Amanitin concentration</th>
<th>Average total eggs/female (± s.e.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>72.17 (± 0.89)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>96.28 (± 1.29)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>93.46 (± 1.41)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>87.95 (± 1.67)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>50.37 (± 1.69)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>109.73 (± 2.17)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>53.00 (± 1.48)</td>
</tr>
<tr>
<td>Ama-KTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>58.88 (± 1.38)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>75.60 (± 1.63)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>54.19 (± 1.77)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>37.94 (± 1.26)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>49.67 (± 2.55)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>130.89 (± 3.13)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>70.83 (± 4.64)</td>
</tr>
<tr>
<td>Ama-MI</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>33.17 (± 1.99)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>79.28 (± 1.71)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>59.38 (± 2.70)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>55.93 (± 2.19)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>86.75 (± 3.71)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>57.17 (± 2.66)</td>
</tr>
<tr>
<td>Ama-KLM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.3.5 α-Amanitin in larval food exerts a negative long-term effect on adult lifespan

We further tested if α-amanitin in the larval food affects the longevity of our flies in the egg-lay chambers. We performed three replicates for each experiment, and the data underwent ANOVA analysis. As expected, all three Asian stocks responded with reduced adult lifespans in response to larval food containing increasing amounts of α-amanitin (Table 1.3). We also noted that males of all three stocks survived longer than females in
both the presence and absence of toxin. Interestingly, at the two to three highest tolerable α-amanitin concentrations for each stock, the females died almost immediately after they hatched, while males at these concentrations lived for about a week. This observation was the second indication that males of all three stocks may be more resistant than females. Furthermore, our most resistant stock Ama-KTT also showed the highest overall lifespan, while the two less resistant stocks Ama-MI and Ama-KLM had about 30% shorter lifespans. This observation held true for both sexes with and without the toxin. For example, Ama-KTT males that were raised as larvae on non-toxic food lived 32.33 (±2.03) days, while the less resistant Ama-MI and Ama-KLM males only lived for 24.33 (±1.20) and 22.33 (±1.45) days, respectively (Table 1.3). We note that although the most resistant Asian stock has the longest life expectancy, many factors can determine lifespan, such as different genetic backgrounds. Therefore, we cannot conclude that high resistance correlates with high life expectancy.

Table 1.3. Longevity of the three original Asian stocks. The average lifespans of males (M) and females (F) for the three original Asian stocks in response to different α-amanitin concentrations in [μg of α-amanitin per g of larval food] are shown. All adult lifespan values are given in days and are averages of three experimental replicates.
<table>
<thead>
<tr>
<th>α-Amanitin concentration</th>
<th>Sex</th>
<th>Ama-KTT lifespan (± s.e.m.)</th>
<th>Ama-MI lifespan (± s.e.m.)</th>
<th>Ama-KLM lifespan (± s.e.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>M</td>
<td>32.33 (± 2.03)</td>
<td>24.33 (± 1.20)</td>
<td>22.33 (± 1.45)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>29.00 (± 1.53)</td>
<td>17.33 (± 1.45)</td>
<td>15.67 (± 0.33)</td>
</tr>
<tr>
<td>1</td>
<td>M</td>
<td>27.33 (± 0.33)</td>
<td>24.67 (± 1.20)</td>
<td>21.33 (± 1.20)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>25.00 (± 1.15)</td>
<td>15.67 (± 1.45)</td>
<td>14.67 (± 0.88)</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>24.67 (± 0.88)</td>
<td>19.33 (± 0.88)</td>
<td>16.00 (± 0.58)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>24.00 (± 1.15)</td>
<td>13.67 (± 0.88)</td>
<td>14.00 (± 0.58)</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>18.67 (± 0.88)</td>
<td>16.67 (± 0.88)</td>
<td>14.67 (± 0.88)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>15.33 (± 1.20)</td>
<td>12.33 (± 0.67)</td>
<td>12.67 (± 0.33)</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>19.33 (± 1.45)</td>
<td>12.67 (± 0.88)</td>
<td>13.33 (± 0.88)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>18.33 (± 2.03)</td>
<td>13.33 (± 0.88)</td>
<td>12.00 (± 1.15)</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>15.67 (± 1.20)</td>
<td>11.67 (± 1.45)</td>
<td>11.33 (± 0.67)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>12.67 (± 0.88)</td>
<td>9.00 (± 1.15)</td>
<td>9.67 (± 1.76)</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>13.33 (± 1.86)</td>
<td>10.67 (± 0.88)</td>
<td>9.00 (± 0.58)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>8.67 (± 1.45)</td>
<td>6.33 (± 0.88)</td>
<td>Instant death</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>11.33 (± 0.88)</td>
<td>8.67 (± 0.67)</td>
<td>6.00 (± 0.58)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>Instant death</td>
<td>Instant death</td>
<td>Instant death</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>8.67 (± 0.88)</td>
<td>6.67 (± 0.33)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>Instant death</td>
<td>Instant death</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>-</td>
<td>5.67 (± 0.33)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>-</td>
<td>Instant death</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1.4 Discussion

1.4.1 α-Amanitin resistance has no apparent fitness cost

One of the most intriguing aspects of *D. melanogaster*’s α-amanitin resistance is that the toxin is exclusively found in mushrooms, whereas the flies are not attracted to mushrooms and should not encounter α-amanitin in nature. Therefore, the resistance appears to be a cross-resistance to other toxic compounds that the flies encountered in their Asian habitats at least 50 years ago. We show that five decades after their isolation, the three Asian *D. melanogaster* stocks Ama-KTT, Ama-MI, and Ama-KLM are still more resistant to α-amanitin than the susceptible wild type stocks Oregon-R and Canton-S. Comparing the combined LC50 data of all analyzed stocks in this study, our data
strongly suggest that α-amanin resistance is conferred by many genes with smaller effects, as opposed to only two dominant alleles on the third chromosome alone, as it was suggested by Phillips et al. 14. This conclusion is further supported by our previous microarray study 52, in which we showed that several candidate genes and molecular mechanisms may be collectively contributing to the α-amanin resistance phenotype of the isochromosomal line Ama-KTT/M/2. Notably, three Cyp genes were among the resistance-conferring candidate genes of Ama-KTT/M/2, which have been associated with pesticide resistance and stress responses. It is therefore very likely that α-amanin resistance in the three Asian D. melanogaster stocks is a cross-resistance to agricultural pesticides that the flies encountered in the wild at least 50 years ago. Due to the fact that the resistance phenotype has persisted over such a long time, α-amanin resistance seems to have no major fitness costs. In a similar example, Cyp6g1-mediated DDT resistance in D. melanogaster also has no fitness cost, which caused the resistance-conferring DDT-R allele to reach global fixation even after the use of DDT was banned 61,62.

1.4.2 α-Amanitin causes developmental retardation phenotypes that resemble stress responses

When we fed increasing concentrations of α-amanin to resistant larvae, we observed the following four detrimental developmental effects in all the three Asian stocks: 1) higher pre-adult mortality, 2) prolonged larva-to-adult developmental time, 3) decreased adult body size, and 4) reduced adult longevity. The severity of the retardation symptoms was inversely correlated with the LC50 values to the toxin; i.e., the more resistant a stock, the less affected it was by α-amanin.
Our data show that pre-adult mortality and larva-to-adult developmental time increased in an α-amanitin concentration-dependent manner. In a similar study, *D. melanogaster* larvae that were fed with the mushroom toxin ibotenic acid also showed reduced pre-adult survivorship and prolonged developmental time \(^{63}\). The observed developmental retardation of larvae that feed on toxic food suggests that the detoxification processes take essential resources away from development, thereby slowing growth.

Adult body size was also affected by α-amanitin in a concentration-dependent manner. However, we noted a paradoxical increase in adult thorax lengths at the lowest toxin concentrations in all three Asian stocks, which may be explained by the fact that adult body size is affected by larval crowding in many insect species \(^{64}\). Thus, the unexpected increase in body size on low α-amanitin concentrations could be attributed to the reduced larval crowding conditions as some larvae die from the toxin. Several other studies show that thorax lengths also decrease in response to other toxins, stress, and parasitism in *D. melanogaster*, e.g. the mushroom toxin ibotenic acid \(^{63}\), temperature stress \(^{57}\), and hymenopteran parasitoid attack \(^{54}\).

When the larvae were reared on α-amanitin-containing food, adult longevity showed a negative correlation to increasing α-amanitin concentrations in the larval food, i.e., the longevity decreased in all stocks in a toxin concentration-dependent manner. These results suggest that some α-amanitin might remain in the hatched flies and affect adult longevity. This observation may be due to one of our previously suggested detoxification mechanisms, which is that the larvae sequester parts of the ingested α-amanitin in the body \(^{52}\).
In all three Asian fly stocks, adult longevity was higher in males than in females under all conditions. We further observed that at the two to three highest toxin concentrations, all females died almost instantly after they hatched, while the males lived for about one week. This observation could either mean that males are more toxin-resistant than females, or that females generally have shorter lifespans. Norry et al. showed that heat-stressed males of *D. melanogaster* live longer than heat-stressed females. Furthermore, different stress factors have been shown to reduce longevity in both sexes of *Drosophila*, e.g. stress caused by microsporidian infection and higher temperature.

### 1.4.3 α-Amanitin increases female fecundity

Exposure to low α-amanitin concentrations caused an earlier onset of female fecundity and an increase in the eggs-per-female rate in all three Asian stocks. The most dramatic increase in fecundity was observed at the second highest tolerable toxin concentration for each stock. Although the peak egg-lay time was delayed by several days at this concentration (Figure 1.5), the average total eggs-per-female productivity roughly doubled (Table 1.2). A possible explanation for the dramatic fecundity increase is that α-amanitin is sequestered in the hatched adults, causing stress responses that alter female fecundity and egg-lay behavior. Similar examples are known, where life expectancy-lowering stress factors increase female fecundity. For example, physical injury can cause female moths to lay their eggs faster and on less suitable substrates than non-injured control moths. Furthermore, stress caused by pathogens and parasitoids can also increase female fecundity in insects, e.g. in crickets.
Another surprising outcome of our study is that α-amanitin resistance seems to have no obvious fitness costs, which is in contrast to several other studies addressing insect resistance to various factors, such as the resistance of *Drosophila* to microsporidian pathogens 66 and hymenopteran parasitoids 54, that of brown planthoppers to a pesticide 70, of mosquitoes to malaria parasites 71, and the resistance of snails to schistosome parasites 72. Fitness costs can be determined by the resistance-conferring genes and/or the environment. For example, in mosquitoes, the cost of resistance to organophosphates can range from no cost to very high costs, depending on the resistance-conferring genes 73,74. In moths, resistance to *Bacillus thuringiensis* toxins has fitness costs especially when the animals are stressed or parasitized 75,76. However, when conferred by the *DDT-R* locus, the resistance of *D. melanogaster* to the pesticide DDT has no apparent fitness costs but instead benefits 61,62. Interestingly, similar to the Asian α-amanitin-resistant fly stocks, the *DDT-R* allele-carrying flies show an increased viability and female fecundity.

1.4.4 Implications

The implications of our study, which is the most detailed phenotypic analysis of naturally occurring α-amanitin-resistant *D. melanogaster* stocks to date, are two-fold: 1) *D. melanogaster* displays several stress-like responses to the complete range of sub-lethal α-amanitin concentrations, while mycophagous species remain unaffected by most sub-lethal concentrations 5,7. The observed physiological differences between mycophagous and non-mycophagous *Drosophila* species suggest that different molecular-genetic mechanisms underlie α-amanitin-resistance in ecologically distinct species. 2) The
increased fecundity of resistant *D. melanogaster* females in response to α-amanitin may have important implications on the interactions of this species with its environment: if resistant females would change their egg-lay preferences to include, for example, mushrooms, *D. melanogaster* may be well-prepared for invading the toxic mushroom niche and begin to feed on specimens with low toxicity, perhaps evolving higher resistance over time. *D. melanogaster* is already capable of completing its life cycle solely on non-toxic fungi, e.g. Baker's yeast, in the laboratory. This scenario of a non-mycophagous species entering the toxic mushroom niche is not entirely hypothetical, as ecologically intermediate species do exist. For example, *Drosophila tripunctata* feeds on both fermenting fruit and mushrooms. While *D. tripunctata* is much more resistant to α-amanitin than the three Asian *D. melanogaster* stocks are, it is also far less resistant than strictly mycophagous *Drosophila* species, which puts *D. tripunctata* in an intermediate position on the way to strict mycophagy. Taken together, if pesticides really did cause α-amanitin resistance in *D. melanogaster*, the use of them may go far beyond their intended effects and may change ecosystems in the long term.

1.4.5 Limitations

When we created the isochromosomal lines, we did not balance the X chromosome because we were working under the published assumption that α-amanitin resistance *D. melanogaster* is conferred by two dominantly acting alleles on the third chromosome. It is therefore possible that alleles derived from the X chromosome of the multi-balancer stock exert epistatic effects on the second and third chromosomes of
the isochromosomal lines, which could explain why Ama-KTT/M/5 is more resistant than Ama-KTT, even if the multi-balancer stock itself is highly susceptible to α-amanitin.

During the fecundity studies, we harvested more eggs than could be counted each day. Therefore, we stored the egg-lay vials at 4°C immediately after they were collected, which killed the eggs. It was thus not possible to assess egg fertility and offspring vigor in response to the toxin. Future studies should test if the higher amounts of eggs that result from higher α-amanitin concentrations also give rise to a larger number of viable offspring, or if the eggs show a higher mortality in response to increasing toxin concentrations.

Although courtship can lead to reduced longevity in males 79, we did not accompany excessive experimental males with white-eyed w[1118] females because doing so would have interfered with our fecundity studies.

1.4.6 Future research

In this study, we learned that all three Asian stocks display the same qualitative, but different quantitative responses to α-amanitin. Thus, the present research lays the foundation for molecular studies that can reveal the underlying causes for the observed quantitative variations in α-amanitin resistance in the three D. melanogaster stocks. One way to link the quantitative resistance phenotypes to the resistance-conferring genes would be to perform a microarray study, which includes larvae of all six isochromosomal lines. We already know that four candidate mechanisms are responsible for the resistance phenotype of Ama-KTT/M/2 larvae 52. Thus, our isochromosomal lines could be a valuable resource to verify the most important candidate genes, which can then be tested
by the transgenic rescue approach and/or mutagenesis. A similar microarray could also be performed in adults to test if stress response pathways are activated by the possibly remnant α-amanitin, which may be the cause for the increased fecundity and decreased longevity. Thus, future studies should aim for a better understanding of the molecular mechanisms that cause α-amanitin resistance and how it could persist over decades in the absence of selective pressure.

It would also be very interesting to test what factors caused the cross-resistance to α-amanitin in the first place. Cyp6a2 is one of the best candidate genes for conferring resistance in Ama-KTT/M/2 larvae. The CYP6A2 enzyme has been shown to metabolize organophosphorous and organochlorine insecticides. Thus, dose-response studies using such substances could shed light on the chemicals that caused the cross-resistance to α-amanitin in the three Asian stocks in their natural habitats more than five decades ago.

1.5 Conclusions

Our observations collectively suggest that α-amanitin resistance in the three Asian D. melanogaster stocks Ama-KTT, Ama-MI, and Ama-KLM has evolved as cross-resistance that has no apparent fitness costs. Our data further confirm the conclusion of our recent microarray study that α-amanitin resistance is a quantitative trait, rather than conferred by two dominantly acting loci on chromosome 3. The α-amanitin resistance phenotype is both interesting and important because it is likely a cross-resistance to agricultural pesticides, which suggests that pesticides may have unintentional effects on non-pest species and thus on entire ecosystems. In contrast to super-resistant
mycophagous *Drosophila* species, low α-amanitin concentrations negatively influence *D. melanogaster*’s larva-to-adult developmental time, pre-adult viability, adult body size, and adult longevity, while the toxin increases female fecundity. Although *D. melanogaster* is not a pest, the long-term persistence of the resistance phenotype and the positive effects of α-amanitin on female fecundity are somewhat alarming.

### 1.6 Materials and Methods

#### 1.6.1 Fly stocks

All *Drosophila melanogaster* (Meigen) stocks were maintained at room temperature on standard food containing cornmeal, granulated sugar, Brewer’s yeast, agar, and methylparaben as antifungal agent. The wild type stocks Canton-S and Oregon-R, the *white* mutant *w[1118]*, and the multi-balancer stock *w[1118]/Dp(1;Y)y[+]*; *CyO/nub[1] b[1] sna[Sco] lt[1] stw[3]*; MKRS/TM6B, *Tb[1]* were obtained from the Bloomington Stock Center, Bloomington, IN, USA (stocks #1, #5, #3605, and #3703, respectively). The stocks Ama-KTT (#14021-0231.07), Ama-MI (#14021-0231.06), and Ama-KLM (#14021-0231.04) were shown to be resistant to α-amanitin in 1982 and obtained from the *Drosophila* Species Stock Center at the University of California, San Diego, CA, USA. Ama-KTT and Ama-MI were originally collected in 1968 in Kenting (Taiwan) and in Mysore (India), respectively. Ama-KLM is the oldest of the three α-amanitin-resistant stocks and was collected in 1962 in Kuala Lumpur (Malaysia).
1.6.2 Generation of the isochromosomal lines

Because Ama-KTT, Ama-MI, and Ama-KLM were maintained the absence of selective pressure to toxins in the stock center over the past five decades, the stocks could have lost or become heterozygous for some of the \( \alpha \)-amanitin resistance-causing alleles. To create flies homozygous for the resistance-conferring alleles that remained in these stocks, we created isochromosomal lines that are isogenic for the second and third chromosomes (Figure 1.2). In order to guarantee that we collect most or all alleles, we started with one highly \( \alpha \)-amanitin-resistant female of each stock that survived the following concentrations: Ama-KTT: 5 \( \mu \)g \( \alpha \)-amanitin per g of food, Ama-MI: 7 \( \mu \)g \( \alpha \)-amanitin per g of food, and Ama-KLM: 4 \( \mu \)g \( \alpha \)-amanitin per g of food. We chose two resulting isochromosomal lines from each original \( \alpha \)-amanitin-resistant stock to further investigate the resistance-causing alleles. The Ama-MI/T/6 isochromosomal line differs from the other stocks by its third chromosome being balanced over the TM6B, \( Tb \) chromosome in the F1 generation, while the other five isochromosomal lines Ama-KTT/M/2, Ama-KTT/M/5, Ama-MI/M/2, Ama-KLM/M/5, and Ama-KLM/M/7 were balanced over MKRS. The isochromosomal lines were selected for three subsequent generations against the white eye color that was introduced by the X-chromosome of the multi-balancer stock, until all isochromosomal lines were purely red-eyed.

1.6.3 Dose-response studies of the fly stocks to \( \alpha \)-amanitin

In order to quantify and compare the levels of \( \alpha \)-amanitin resistance of the \( D. melanogaster \) stocks, dose-response experiments were performed, which measured the survival from freshly-hatched first-instar larvae to adulthood. Flies able to completely
hatch from their pupae were counted as survivors. The α-amanitin-resistant stocks Ama-KTT, Ama-MI, Ama-KLM, and their isochromosomal derivates were tested on a total of 11 α-amanitin concentrations, using 0 to 10 µg of α-amanitin per g of food in 1 µg increments. The α-amanitin-sensitive wild type stocks Canton-S and Oregon-R, and the multi-balancer stock w[1118]/Dp(1;Y)y[+]; CyO/nub[1] b[1] snaf Sco lt[1] stw[3]; MKRS/TM6B, Tb[1] were initially tested on five concentrations ranging from 0 to 4 µg of α-amanitin per g of food in 1µg increments. However, because they survived only the 0-toxin concentration, these stocks were further tested on 0, 0.025, 0.05, 0.075, 0.1, 0.25, and 0.375 µg of α-amanitin per g of food. α-Amanitin was purchased from Sigma-Aldrich, St. Louis, MO, USA.

Flies of mixed sexes were allowed to lay eggs on molasses agar caps that contained a streak of fresh Baker’s yeast paste at 25ºC, 70% humidity, and a 12:12 hour day/night cycle in a Drosophila chamber (Model GSDR-36VL) from Geneva Scientific, Fontana, WI, USA. The yeast was removed prior to larval hatching. Freshly hatched first-instar larvae were placed in groups of ten into 2 mL plastic test tubes (USA Scientific, Orlando, FL, USA), each containing 500 mg of non-toxic or poisoned food and two small air holes in the lid. The food consisted of 125 mg dry, instant Drosophila medium (Carolina Biological, Burlington, NC, USA) and 375 µL sterile Milli-Q water with or without dissolved α-amanitin. Ten tubes were prepared for each toxin concentration and experimental replicate, resulting in 100 larvae for each concentration and a total of 1,100 larvae per experiment. Three high-quality dose-response experiments, in which the 0-toxin concentration survival rate was at least 80%, were used to calculate the LC50 of each fly stock. The standard error of the mean (s.e.m.) was calculated for each
concentration by sampling the data points of all 30 vials of every concentration. The LC$_{50}$ was calculated using scatter plots and the logarithmic trend line function in Microsoft Excel.

1.6.4 *Thorax measurements, fecundity, and longevity measurements*

Surviving flies of the dose-response experiments were collected daily within 24 hours of hatching. To measure thorax lengths as an indicator of developmental retardation caused by the different α-amanitin concentrations, the flies were anesthetized using CO$_2$. Thorax lengths were measured from the tip of the scutellum to the base of the neck while the flies were lying on one side $^{81}$, using an Olympus SZX16 dissection microscope, an Olympus DP72 camera, and cellSens Standard 1.3 software (Olympus, Center Valley, PA, USA).

For the fecundity and longevity tests, the flies were kept in the absence of α-amanitin in 25 x 95 mm *Drosophila* plastic vials (VWR International, Radnor, PA, USA) filled with 5 mL of molasses agar and a streak of Baker’s yeast paste. The flies were housed in small groups consisting of an equal number of males and females that hatched on the same day from the same toxin concentration. Because females without male partners live longer than females in the presence of males $^{58,82}$, white-eyed $^{w[1118]}$ males were added to the experimental females who were lacking male partners to balance the male-to-female ratio across all experiments. Because of their different eye color, the $^{w[1118]}$ males could be easily excluded from the survival counts. Every day throughout their lifespan, all survivors were transferred to new molasses vials with fresh yeast paste. The eggs in the vacated vials were first stored at 4°C and then counted to assess the daily
fecundity of the females in response to different α-amanitin concentrations. In order to test if α-amanitin eaten during their larval life shortens the lifespan of the adults, the amount of the dead flies and their sexes were recorded daily.

1.6.5 Statistical analyses
Microsoft Excel was used to create the graphs and perform the one-way ANOVA analyses. A logarithmic trend line was used to calculate the LC50 values.

1.7 Acknowledgements
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1.8 Author Contributions
Conceived and designed the experiments: TW CLM. Performed the experiments: CLM RDY ZJJ SED’A KRV. Analyzed the data: CLM TW. Contributed reagents/materials/analysis tools: TW. Wrote the paper: TW CLM RDY ZJJ SED’A KRV.
Chapter 2

The Mechanisms Underlying α-Amanitin Resistance in *Drosophila melanogaster*: A Microarray Analysis

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2.1 Abstract

The rapid evolution of toxin resistance in animals has important consequences for the ecology of species and our economy. Pesticide resistance in insects has been a subject of intensive study; however, very little is known about how *Drosophila* species became resistant to natural toxins with ecological relevance, such as α-amanitin that is produced in deadly poisonous mushrooms. Here we performed a microarray study to elucidate the genes, chromosomal loci, molecular functions, biological processes, and cellular components that contribute to the α-amanitin resistance phenotype in *Drosophila melanogaster*. We suggest that toxin entry blockage through the cuticle, phase I and II detoxification, sequestration in lipid particles, and proteolytic cleavage of α-amanitin contribute in concert to this quantitative trait. We speculate that the resistance to mushroom toxins in *D. melanogaster* and perhaps in mycophagous *Drosophila* species has evolved as cross-resistance to pesticides, other xenobiotic substances, or environmental stress factors.
2.2 Introduction

How species respond to changes in their environment is a central question in biology. Insects and mammals deploy similar genes and detoxification mechanisms to defend against poisons that are present in their prey or in the environment. These include the avoidance of toxic parts of their diet, the excretion, sequestration, metabolic breakdown of the toxins, and mutations in the target proteins to avoid toxin binding. Some of the most striking natural examples of toxin resistance are snake species that feed on poisonous amphibians, caterpillars that sequester plant alkaloids in their bodies to deter predators, and toxin-resistant soft-shell clams that store algal toxins in their bodies, causing paralytic shellfish poisoning in people who eat the clams. Apart from these natural examples, the use of pesticides against insects has caused very rapidly evolving toxin resistance responses in many pest species, costing the US billions of dollars per year in crop damage and pesticide production.

Out of the vast number of eukaryotic organisms that live on our planet, a few dozen of mycophagous Drosophila species are able to breed in a variety of very toxic mushrooms, including the deadly poisonous species Amanita phalloides (Death Cap) and Amanita virosa (Destroying Angel). Among other toxins, these mushrooms contain α-amanitin as their principal toxin, which inhibits the function of RNA-polymerase II and thus brings all mRNA transcription to a halt. These resistant Drosophila species can develop on α-amanitin-containing laboratory food, showing that the resistance mechanism is not due to the avoidance of toxic parts of the mushrooms. Furthermore, the RNA-polymerase II of all tested mushroom-feeding Drosophila species is as sensitive to α-amanitin as it is in sensitive Drosophila species, showing that target mutations in the
RNA polymerase II complex are not likely to confer resistance to mushroom toxins in mycophagous *Drosophila* species.

The model organism *D. melanogaster* is a non-mycophagous species; i.e., it does not use mushrooms as a natural diet. Thus, *D. melanogaster* should not encounter toxins in nature that are solely produced by mushrooms, such as α-amanitin. However, three Asian *D. melanogaster* strains that were collected in the 1960s in Taiwan (Ama-KTT), India (Ama-MI), and Malaysia (Ama-KLM) were shown to be one order of magnitude more resistant to α-amanitin than the sensitive wild-type strain Oregon-R. In these three Asian strains, the resistance to α-amanitin was mapped to two dominantly acting loci: one situated on the left and one on the right arm of chromosome 3. Eighteen years later, a very similar phenomenon was described in a *D. melanogaster* stock collected in California. This stock showed an increased resistance to α-amanitin and surprisingly, the resistance was mapped to the seemingly same two loci on chromosome 3, as in the three Asian stocks. Even in the Californian stock, both loci acted in a dominant fashion. The Californian study concluded with the identification of two candidate genes that might confer the resistance phenotype: *Multidrug resistance 65* (*Mdr65*) on the left arm and *Protein kinase C98E* (*Pkc98E*) on the right arm of chromosome 3. Because PKC98E can phosphorylate MDR proteins and MDR proteins could potentially lead to the excretion of α-amanitin from cells, the question of how *D. melanogaster* evolved α-amanitin resistance appeared to be answered. Although the proposed scenario is simple and elegant, no conclusive evidence has been brought forward yet that demonstrates that any gene is required or necessary to confer resistance to α-amanitin. Thus, the genes that
confer mushroom toxin resistance in *D. melanogaster* (and all mycophagous *Drosophila* species) remain elusive.

In this study, we conducted a whole-genome microarray analysis, using an isochromosome stock for chromosomes 2 and 3 of the original α-amanitin-resistant *D. melanogaster* stock Ama-KTT from Taiwan. We hypothesized that genes involved in the excretion, metabolic inactivation, and/or sequestration of α-amanitin will be identified in our microarray, which can pinpoint to the mechanisms responsible for the α-amanitin resistance phenotype. To our surprise, neither *Mdr* genes nor *Pkc98E* were among the up-regulated candidate genes. Instead, we identified genes of the phase I detoxification gene family *Cyp* (Cytochrome P450), and the phase II *Gst* (Glutathione-S-transferase) and *Ugt* (UDP glucuronosyl transferase) gene families, some of which (*Cyp6a2, Cyp12d1-d*, and *Cyp12d1-p*) were several hundred-fold constitutively up-regulated in the α-amanitin-resistant fly stock. In addition, we found evidence for the possible involvement of peptidases, lipid particles, cuticular proteins, the Mayor Royal Jelly Protein homolog Yellow, and Salivary Gland Secretion (Sgs) proteins, which could provide additional protection by cleaving or immobilizing α-amanitin, or by blocking its access to cells. Because *D. melanogaster* does not feed on mushrooms in nature and α-amanitin is solely found in mushrooms, we speculate that the resistance to α-amanitin has evolved as cross-resistance to pesticides or other environmental factors that the flies encountered before they were collected in Asia 45 years ago.
2.3 Results

2.3.1 Experimental Design

In two independent studies, a total of four *D. melanogaster* stocks from Asia and North America were shown to be resistant to the mushroom toxin α-amanitin \(^{14,15}\). For each of these stocks, QTL mapping data suggested that the resistance was conferred by two dominantly acting loci on chromosome 3. Begun and Whitley identified the genes *Mdr65* and *Pkc98E* as possible candidates, with the notion that the resistance phenotype could be caused by a *cis*-regulatory change in the *Mdr65* gene \(^{15}\). In order to identify gene-regulatory changes on a whole-transcriptome scale in α-amanitin-resistant *D. melanogaster* larvae, we performed a microarray study. As starting material, we used the most resistant of the four described α-amanitin-resistant stocks, Ama-KTT \(^{14}\). Because the stock could have become heterozygous for the resistance-conferring loci during the past 45 years after being collected in the wild, we created the isochromosome stock Ama-KTT/M/2, which is isogenic for the Ama-KTT chromosomes 2 and 3. Our dose-response data show that the isochromosome stock Ama-KTT/M/2 (LC\(_{50} = 2.16 \mu g/g\) of food) is at least as resistant to α-amanitin as the original Ama-KTT stock (LC\(_{50} = 1.84 \mu g/g\) of food) (Figure 2.1), indicating that at least the majority of the α-amanitin resistance-conferring genes is located on the major autosomes. The multi-balancer stock that we used for the crosses to create the Ama-KTT/M/2 stock was very sensitive to α-amanitin (LC\(_{50} = 0.042 \mu g/g\) of food, data not shown).

We performed a whole-transcriptome gene expression microarray analysis to test what genes are differentially expressed in 1) a constitutive manner and 2) in response to
α-amanitin. The complete set of differentially expressed genes (DEGs) can be found in Table A.1. The isochromosome stock Ama-KTT/M/2 (LC$_{50}$ = 2.16 µg/g of food) was used as the experimental stock and has a 77.1 times higher LC$_{50}$ to α-amanitin than our sensitive control stock Canton-S (LC$_{50}$ = 0.028 µg/g of food, data not shown). We compared three groups with each other: 1) Canton-S larvae on non-toxic food, 2) Ama-KTT/M/2 larvae on non-toxic food, and 3) Ama-KTT/M/2 larvae that were continuously raised from the first to the third instar on α-amanitin-containing food (at 1.5 µg/g of food, a concentration that is slightly below the LC$_{50}$ of Ama-KTT/M/2). Groups 1 and 2 were prepared in 5, and group 3 in six biological replicates, each replicate consisting of ten larvae (Figure 2.2). We compared the gene expression profiles of fully-grown third-instar larvae that have not started wandering yet. For the data analysis, we focused on well-annotated genes that showed expression changes of at least 2-fold with a corrected p-value of less than 0.05. With the exception of the genome enrichment analysis and the gene $CG10226$, which is a putative $Mdr$ gene, we generally excluded genes from our analysis that solely have a CG or CR gene annotation number.
Figure 2.1. Ama-KTT/M/2 is not less resistant to α-amanitin than Ama-KTT. Ten first-instar larvae were placed on each α-amanitin concentration. The dose response curve shows the percentage of hatching flies. Error bars indicate the s.e.m. of three replicates.

Figure 2.2. Graphical representation of the groups of larvae used for the microarray and qPCR analysis. Groups 1 and 2 (Canton-S and Ama-KTT/M/2) were not treated with α-amanitin, as symbolized by the yellow color. The larvae of group 3 (Ama-
KTT/M/2) were treated with α-amanitin throughout their development, as indicated in red. Groups 1 and 2 were collected in five, and group 3 in six biological replicates (ten larvae in each replicate), as illustrated by the number of tubes and microarray chips.

2.3.2 Genes Encoding Cytochrome P450s, GSTs, and UGTs are Differentially Expressed in Ama-KTT/M/2

Assuming that gene-regulatory changes underlie α-amanitin resistance in the Ama-KTT/M/2 isochromosome stock, we expected to identify constitutive gene-expression changes in Ama-KTT/M/2 on non-toxic food, as compared to the sensitive control stock Canton-S on non-toxic food (group 2 versus group 1). We used the Plier normalization/summarization and the DEG methods to analyze our single gene microarray data. As a result, we identified 234 genes that were at least 2-fold significantly constitutively up-regulated in Ama-KTT/M/2 (Table A.2). Out of these 234 genes, 20 (8.5%) are Cyp, Gst, and Ugt genes, which are all situated on chromosomes 2 and 3 (Table 2.1). The three most highly up-regulated genes of this group were Cyp6a2, Cyp12d1-d, and Cyp12d1-p, which were between more than 300- to 197.3-fold constitutively up-regulated in the resistant stock. These three genes are expressed in the larval midgut and Malpighian tubules, which are potential detoxification organs

Interestingly, Cyp6a2 expression profiles are correlated with insecticide resistance

29,31,80,87-90, while CYP6A2 metabolizes insecticides in enzyme assays 26,80. Cyp12d1 is also associated with insecticide resistance 28,88,89,91-95 and stress response 93-95. Overexpression of Cyp12d1 increases insecticide resistance 27, and CYP12D1 from the house fly metabolizes insecticides 96. The remaining 17 detoxification genes identified in
our microarray study were 38.9 - 2.1-fold up-regulated and are presented next in the order from the highest to lowest constitutive up-regulation in Ama-KTT/M/2: Ugt36Bb, Ugt86Dd, GstD5, GstE1, GstE6, GstE5, Ugt36Bc, Cyp6a20, Ugt37c1, Ugt36Ba, Cyp4c3, Ugt37b1, Cyp6w1, Cyp305a1, Cyp49a1, GstD8, and GstE9. Some of these genes are associated with various phenotypes: Ugt86Dd and Cyp6w1 (inducibility by the xenobiotic phenobarbital)\textsuperscript{89}, GstD5 and GstE1 (stress responses)\textsuperscript{97,98}, GstE5 (insecticide resistance)\textsuperscript{33}, Cyp6a20 (aggressive behavior)\textsuperscript{99-101}, and Cyp305a1 (ecdysteroid synthesis and lipid storage regulation)\textsuperscript{102}.

We were curious to see if the constitutive up-regulation of detoxification genes is a specific characteristic for the α-amanitin-resistant stock Ama-KTT/M/2 or if there are other detoxification genes that show higher expression levels in Canton-S, as compared to Ama-KTT/M/2. Surprisingly, 15 Cyp and Ugt genes were between 2.1 and 186.8-fold lower expressed in the resistant stock Ama-KTT/M/2 than in Canton-S. From the lowest to highest expression difference, these genes are: Cyp12a4, Cyp304a1, Cyp313a2, Cyp12e1, Cyp6t1, Cyp4ac2, Cyp4s3, Ugt86Dj, Cyp4d2, Cyp6a23, Cyp4ac3, Cyp4p2, Cyp28d1, Cyp4d8, and Cyp6a17 (Table 2.1). Correlative or functional data exists for Cyp12a4 and Cyp4p2 (insecticide resistance)\textsuperscript{29,103}, Cyp304a1 and Cyp4d2 (methanol resistance)\textsuperscript{104}, and Cyp6a17 (thermosensory behavior)\textsuperscript{105}.

**Table 2.1.** Single gene analysis for Ama-KTT/M/2 versus Canton-S on no toxin (group 2 versus 1) Type I and II detoxification, Mdr, and transcription factor genes with possible functions in detoxification processes are shown. The at least 2-fold differentially
expressed genes are sorted by positive and negative fold-changes, followed by the genes that are not significantly differentially expressed. All p-values are corrected. The chromosomes, FlyBaseID, and probe ID numbers are presented.

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2.3.3 Genes Encoding Cytochrome P450s and GSTs are Inducible by α-Amanitin

Our next question was what genes are inducible by α-amanitin in the resistant Ama-KTT/M/2 stock as compared to Ama-KTT/M/2 on non-toxic food (group 3 versus group 2). We found that 143 genes were significantly inducible by α-amanitin (Table A.3), eleven of which (7.7%) belong to the Cyp and Gst gene families (Table 2.2). Cyp316a1 was the strongest inducible Cyp gene (11.8-fold) in the resistant stock Ama-KTT/M/2. However, when we compared resistant Ama-KTT/M/2 on toxic food to sensitive Canton S without toxin (group 3 versus group 1), Cyp316a1 was only 1.9-fold (p=0.0941, Table A.1) more expressed in Ama-KTT/M/2 on toxic food, making the 11.8-fold induction within the Ama-KTT/M/2 stock less convincing. The remaining ten Cyp and Gst that were up-regulated by α-amanitin in the resistant stock were induced between 7.2- and 2.0-fold and are listed in the order from highest to lowest induction: Cyp6d2, Cyp4d8, Cyp28d1, Cyp6t1, GstD3, GstD6, Cyp4d2, GstD9, GstD10, and Cyp4d14. Four of these genes, Cyp4d8, Cyp28d1, Cyp4d2, and Cyp4d14, are expressed in the larval midgut and/or Malpighian tubules, suggesting that they could play a role in the detoxification of xenobiotic compounds. Some genes are associated with various phenotypes: Cyp6d2 (camptothecin resistance)\textsuperscript{106}, GstD6 (oxidative stress response)\textsuperscript{107}, and Cyp4d2 (methanol resistance)\textsuperscript{104}. Notably, both Cyp316a1 and Cyp4d8 are situated at cytological position 66A2, which is relatively close to region 65A10 to which α-amanitin resistance was QTL-mapped in four independent D. melanogaster stocks in the past\textsuperscript{14,15}. We next asked what Cyp, Gst, and Ugt genes were down-regulated in response to α-amanitin in the resistant stock. As a result, nine genes were 2.1- to 3.8-fold down-regulated in response to α-amanitin, which are presented in the order from lowest to
highest down-regulation: Ugt37b1, Cyp4c3, Cyp28d2, Ugt86Dd, Cyp6a23, Cyp9b2, Ugt37c1, and Cyp28a5 (Table 2.2). Out of these, Ugt86Dd is inducible by the xenobiotic phenobarbital \(^{89}\) and Cyp28a5 by methanol \(^{104}\). Some of the most strongly \(\alpha\)-amanitin-inducible genes (>300-fold) were the salivary gland secretion genes Sgs1, Sgs3, Sgs5, Sgs7, and Sgs8 (Table A.3). We will speculate about their role later.

Table 2.2: Single gene analysis for Ama-KTT/M/2 on \(\alpha\)-amanitin versus Ama-KTT/M/2 on no toxin (group 3 versus 2). Type I and II detoxification, Mdr, and transcription factor genes with possible functions in detoxification processes are shown, sorted by positive and negative fold-changes. The at least 2-fold differentially expressed genes are sorted by positive and negative fold-changes, followed by the genes that are not significantly differentially expressed. All p-values are corrected. The chromosomes, FlyBaseID, and probe ID numbers are presented.
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2.3.4 Mdr Genes are Neither Constitutively Up-Regulated nor Inducible in Ama-KTT/M/2

In 1982, QTL mapping data suggested that two loci on chromosome 3 of the Asian Ama-KTT, Ama-MI and Ama-KLM stocks confer resistance to α-amanitin in a dominant fashion 14. Eighteen years later, a Californian D. melanogaster stock showed α-amanitin resistance that was QTL-mapped to virtually the same two loci on chromosome 3 15. It was concluded that Mdr65 and Pkc98E were possible candidate genes for causing the resistance. Furthermore, sequence comparisons between the most and the least resistant Californian stocks pointed out differences in the non-coding regions, but not in the coding regions of Mdr65. Thus, if Mdr65 would confer resistance, the prediction was that a cis-regulatory change in the Mdr65 gene is responsible for the resistance α-amanitin. We thus asked the question if Pkc98E, Mdr65 or any other Mdr genes
(CG10226, Mdr49, and Mdr50) were either constitutively up-regulated or inducible by α-amanitin in the Ama-KTT/M/2 stock. Comparing group 2 with group 1, Mdr65 showed a statistically significant but very low (1.2-fold) constitutive up-regulation in Ama-KTT/M/2 (Table 2.1), while Mdr65 was 1.2-fold down-regulated in response to α-amanitin when group 3 was compared to group 2 (Table 2.2). CG10226, a predicted Mdr gene that directly flanks the Mdr65 gene on the left arm of chromosome 3, showed a statistically significant 1.4-fold constitutive up-regulation in the resistant Ama-KTT/M/2 stock as compared to Canton-S (Table 2.1), while this gene was 1.2-fold down-regulated in response to α-amanitin (Table 2.2). The remaining two Mdr genes of D. melanogaster, Mdr49 and Mdr50, are both situated on the right arm of chromosome 2. Mdr49 showed a mere 1.1-fold constitutive up-regulation in Ama-KTT/M/2 (Table 2.1), and it is 1.1-fold inducible by α-amanitin (the latter value is statistically insignificant) (Table 2.2). The observed 1.6-fold constitutive induction of the Mdr50 gene was statistically significant (Table 2.1), and the same gene was significantly 3.3-fold down-regulated in response to α-amanitin (Table 2.2). Furthermore, Pkc98 is 1.1 times lower expressed in Ama-KTT/M/2 as compared to Canton-S on no toxin (Table 2.1), while this gene is 1.2-fold inducible by α-amanitin (both values statistically insignificant) (Table 2.2). In summary, our data show that Mdr genes and Pkc98E were far less than 2-fold (if at all) up-regulated, neither constitutively nor in response to α-amanitin. Mdr genes are thus not likely to confer the α-amanitin resistance, at least not by increasing Mdr gene expression.

We also specifically analyzed the regulation of two transcription factor genes that are known to play a role in regulating responses to xenobiotic factors. Hr96 encodes a nuclear receptor that is involved in xenobiotic responses in D. melanogaster. Our data
in Table 2.1 show that Hr96 is 1.6-fold constitutively higher expressed in Canton-S (group 1) than in Ama-KTT/M/2 on no toxin (group 2). In response to α-amanitin, the Hr96 gene was 1.2-fold (statistically insignificant) down-regulated in Ama-KTT/M/2 (group 3 versus group 2, Table 2.2). The other gene of interest was the leucine zipper transcription factor cnc, which is known to activate oxidative stress and detoxification responses in D. melanogaster. In our microarray, the cnc gene is 1.2-fold constitutively higher expressed in Canton-S (group 1) than Ama-KTT/M/2 (group 2, Table 2.1), while α-amanitin treatment caused a 1.2-fold induction in the Ama-KTT/M/2 stock (group 3 versus group 2, Table 2.2), thus bringing cnc gene expression to the same level that was observed in Canton-S without toxin. These results, at least at the transcriptional level, do not suggest the involvement of both transcription factors in the resistance to α-amanitin.

2.3.5 Genome Enrichment Analysis Confirms 30-Year-Old QTL Mapping Data

In order to identify the regulatory pathway components that lead to the α-amanitin resistance phenotype, we performed a genome enrichment analysis to look for clusters of significantly differentially expressed genes along the four chromosomes. In accordance with the two previous studies that mapped α-amanitin resistance to the polytene bands 95 and 98 on chromosome 3, we found signatures for both constitutive (group 2 versus group 1) and α-amanitin-inducible (group 3 versus group 2) clusters of differentially expressed genes. The only constitutively differentially expressed gene cluster is situated at cytological band 38B on the left arm of chromosome 2, which contains the genes CG10659, Taf13, CG17570, phr6-4, dia, and CG31674 at the peak of differential expression (Figure 2.3 and Table A.4). However, their predicted and experimentally
proven functions do not explain how α-amanitin resistance is genetically controlled. The remaining four clusters of differentially expressed genes responded to α-amanitin in the larval food. The most interesting induced gene cluster is situated at cytological band 66A, which is close to Mdr65-containing region 65A10 on the left arm of chromosome 3, to which α-amanitin resistance was previously mapped \(^{14,15}\). The genes at the peak of differential expression are \(mp, Hsc70-4, pst, CG8562, Cyp316a1, Cyp4d8, CG33276\), and \(RNaseX25\) (Figure 2.3 and Table A.4). Interestingly, two predicted Cytochrome P450 genes with unknown functions, \(Cyp316a1\) and \(Cyp4d8\), were 11.8- and 7.1-fold inducible by α-amanitin (see also Table 2.2). We further identified differentially expressed gene clusters at cytological bands 68A (left arm of chromosome 3), 92A, and 96D (right arm of chromosome 3). Most of these genes are poorly annotated and none of the genes were linked to any known toxin response (Figure 2.3 and Table A.4). It is worth noting that the transcription factor gene \(Hr96\) is close to the previously identified \(Pkc98\) locus, to which α-amanitin resistance was mapped \(^{14,15}\). Although our single gene analysis did not show significant up-regulation of the \(Hr96\) gene, it is nevertheless possible that \(Hr96\) contributes to the resistance on a post-transcriptional level.
Figure 2.3: Genome enrichment analysis for genomic correlates. Genomic correlates are likely disrupted in Ama-KTT/M/2 versus Canton S (red) and Ama-KTT/M/2 on α-amanitin versus Ama-KTT on non-toxic food (blue). Colored lines above the gray line indicate significant enrichment of a genomic correlate. Of the five genomic correlates rising above the cutoff value, two genomic correlates are similar to those found in previous linkage studies on the Ama-KTT stock.

2.3.6 Gene Ontology Enrichment Analysis Suggests Additional α-Amanitin Resistance Mechanisms

In order to explore if multiple mechanisms confer the resistance phenotype to α-amanitin in the Ama-KTT/M/2 stock, we performed a gene ontology enrichment analysis. First, we compared the constitutive gene expression differences between Ama-KTT/M/2
and Canton-S on non-toxic food (group 2 versus group 1). As a result, we identified three molecular functions that could be relevant for the α-amanitin resistance phenotype in Ama-KTT/M/2 (Figure 2.4): 1) 'Oxidoreductase activity' genes (GO 0016491) were on average 4.6-fold higher expressed (p=1.06E-18) in Ama-KTT/M/2. This result confirms the single gene analysis results (Table 2.1), which indicated that the three highest constitutively expressed Cyp genes (Cyp6a2, Cyp12d1-d, and Cyp12d1-p might be important for the resistance to α-amanitin. 2) "Transferase activity' genes (GO 0016740) were on average 4.6-fold higher expressed in Ama-KTT/M/2 (p=7.61E-11), confirming our single gene analysis for the Gst and Ugt genes (Table 2.1). 3) 'Structural constituents of chitin-based cuticle' genes (GO 0005214) were on average 10.5-fold (p=1.87E-18) higher expressed in Ama-KTT/M/2, including 45 insect cuticle genes of the Cpr, Lcp, and Ccp gene families, which belong to the top 190 constitutively up-regulated genes in Ama-KTT/M/2 (Table A.2). It is possible that cuticular proteins provide a protective layer against α-amanitin in organs that are covered by a cuticle, such as the epidermis and the gut. For example in honey bees, 'structural constituents of chitin-based cuticle' genes have been suggested to protect venom gland cells from toxins that are stored in the gland 111. It is interesting to note that like α-amanitin, the bee venom ingredient Mast Cell Degranulating (MCD) Peptide is a bicyclic peptide. Structural constituents of the chitin-based cuticle could perhaps bind to bicyclic peptides and prevent them from entering cells. Furthermore, we identified two significant biological processes in this comparison (group 2 versus group 1) (Figure 2.4). 1) 'Oxidation-reduction process' genes (GO 0055114) were on average 5.6-fold higher expressed in Ama-KTT/M/2 (p=5.25E-18), confirming the possible role of Cyp genes in α-amanitin detoxification. 2) The 'cellular
amino acid metabolic process' genes (GO 0006520) showed a 1.2-fold higher expression average in Ama-KTT/M/2 (p=2.55E-13) and was divided into two sub-processes. 2a) The 'cellular modified amino acid process' (GO 0006575) contained 16 Gst genes, which were on average 1.8-fold higher expressed in Ama-KTT/M/2 (p=4.08E-03), suggesting that GST enzymes might help detoxifying α-amanitin via the phase II detoxification process.

2b) 'Alpha-amino acid metabolic process' genes (GO 1901605), such as glutathione metabolism genes, were on average 2.3-fold constitutively up-regulated in Ama-KTT/M/2 (p=6.49E-03). Some of these genes might provide the substrate glutathione for the GST enzymes. Interestingly, yellow (y), a well-known pigmentation gene in Drosophila, was among the genes of this gene ontology term (14.7-fold up-regulated, p=0.0448, Table A.2). yellow is closely related to Major Royal Jelly Protein (MRJP) genes in honey bees, which were previously suggested to protect the venom gland cells from the bee venom. It is thus possible that yellow plays a role in keeping α-amanitin outside of tissues or perhaps even modifying it so that it becomes less toxic.
Next, we aimed to identify the gene ontologies that respond to α-amanitin in the resistant stock Ama-KTT/M/2. We thus compared Ama-KTT/M/2 on α-amanitin-containing food to Ama-KTT/M/2 on non-toxic food (group 3 versus group 2). As a result, we identified genes with two molecular functions that are significantly induced by α-amanitin (Figure 2.5). 1) The 'oxidoreductase activity' genes (GO 0016491) are on average 4.7-fold induced (p=2.36E-10) by the toxin, again suggesting that a phase I detoxification process mediated by Cytochrome P450s is involved in conferring α-amanitin resistance. Among the 37 Cyp genes of this gene ontology term, we found seven genes that we already identified in our single gene analysis (Table 2.2): Cyp316a1, Cyp6d2, Cyp4d8, Cyp28d1, Cyp6t1, Cyp4d2, and Cyp4d14. 2) 'Peptidase activity, acting on L-amino acid peptides' genes (GO 0070011) were on average 15.4-fold induced (p=3.95E-05). Because α-amanitin is a peptide, peptidases are good candidates to cleave it. To date, however, no specific enzyme is known that can inactivate α-amanitin by cleaving this bicyclic octapeptide. Besides molecular functions, we further identified two biological processes that were of interest. 1) The 'oxidation-reduction process' genes (GO 0055114) were on average 5.0-fold induced (p=3.40E-13), again confirming that Cyp
genes could play a role in detoxifying α-amanitin. 2) We identified the 'cellular amino acid metabolic process' (GO 0006520) with an average up-regulation of 1.2-fold (p=4.09E-11) in response to α-amanitin. The most interesting genes in this gene ontology group are 11 Gst genes and the yellow gene, again showing that the phase II detoxification process is inducible by α-amanitin and that yellow could play a protective role. Our gene ontology enrichment analysis further identified cellular components that respond to α-amanitin exposure (Figure 2.5). 1) 'Cytoplasm' genes (GO 0005737) were on average 681.2-fold induced (p=5.26E-13), some of which are yellow, eight Cyp genes, and 13 Gst genes. The eight Cyp genes belong to the gene ontology term 'cytoplasmic part' (GO 0044444), which is on average 859.6-fold induced (p=9.57E-10). Unexpectedly, the most highly induced gene ontology term for the cellular component was the 'lipid particle' with an average gene induction of 5,271.5-fold (p=8.62E-10). Lipid particles are subcellular structures that play roles in detoxification processes and the innate immune system. In insects, lipid particles form coagulation products, thereby protecting cells from pathogens and toxic products of the phenol oxidase cascade. In yeast cells, lipid particles detoxify excessive amounts of lipophilic substances. Even in humans, liposomes are used for detoxifying patients with overdoses of drugs, such as heroin, opioids, and cocaine. The fact that the Ama-KTT/M/2 stock responds to α-amanitin with a several thousand-fold induction of lipid particle genes suggests that cytoplasmic lipid particles contribute to the resistance to α-amanitin in the Ama-KTT/M/2 stock.
2.3.7 The Domain Enrichment Analysis Verifies the Gene Ontology Enrichment Analysis

Because many proteins have more than one functional domain and the gene ontology enrichment analysis cannot reveal what domain of a protein is important for the resistance to α-amanitin, we further performed a domain enrichment analysis with our microarray data. As shown in Table 2.3, when comparing Ama-KTT/M/2 with Canton-S
(group 2 versus group 1) on non-toxic food, the following protein domains were identified as significantly enriched: Cytochrome P450 (p=4.72E-11), UDP-glucuronosyl/UDP-glucosyltransferase (p=1.26E-10), Cytochrome P450, conserved site (p=5.93E-10), insect cuticle protein (p=1.55E-09), Cytochrome P450, E-class, group I (p=6.05E-09), Glutathione S-transferase, C-terminal (p=6.39E-06), Glutathione S-transferase, C-terminal-like (p=1.02E-05), Glutathione S-transferase/chloride channel, C-terminal (p=1.28E-05), and Glutathione S-transferase, N-terminal (p=4.77E-05). Thus, the domain enrichment analysis confirms the possible importance of phase I and II detoxification reactions in conferring α-amanitin resistance. When comparing Ama-KTT/M/2 on α-amanitin-containing food to Ama-KTT/M/2 on no toxin (group 3 versus group 2, Table 2.4), we identified the following significantly enriched protein domains: major royal jelly (p=0), pupal cuticle protein C1 (p=0), Cytochrome P450 (p=1.20E-12), Cytochrome P450, conserved site (p=2.90E-12), insect cuticle protein (p=1.91E-11), chitin binding domain (p=3.38E-11), Cytochrome P450, E-class, group I (p=1.77E-10), peptidase M17, leucyl aminopeptidase, N-terminal (p=4.38E-06), UDP-glucuronosyl/UDP-glucosyltransferase (p=4.93E-06), leucine aminopeptidase/peptidase B (p=8.48E-06), and peptidase M17, leucyl aminopeptidase, C-terminal (p=8.49E-06).

These results confirm the results from the gene ontology enrichment analysis, suggesting that Cytochrome P450s and transferases can detoxify α-amanitin via the phase I and II detoxification pathways. Furthermore, peptidases might cleave α-amanitin, and Royal Jelly Protein domain-containing proteins might protect tissues from α-amanitin, similar to the situation in the honey bee venom gland 111.
Table 2.3: Domain enrichment analysis for Ama-KTT/M/2 versus Canton-S on no toxin (group 2 versus 1). This table shows the selected and significantly enriched domains without toxin treatment. “DEGs w/ domain” are the differentially expressed genes that have a particular domain. “DEGs” is the number of all differentially expressed genes in this comparison. “Genes w/ domain” is the total number of genes with a particular domain in the genome. “Genes” is the total number of genes in the genome. All p-values are corrected.

<table>
<thead>
<tr>
<th>Domain</th>
<th>DEGs w/ Domain</th>
<th>DEGs</th>
<th>Genes w/ Domain</th>
<th>Genes</th>
<th>p-Value</th>
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<tr>
<td>Cytochrome P450</td>
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<td>2609</td>
<td>35</td>
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<tr>
<td>Insect cuticle protein</td>
<td>51</td>
<td>2609</td>
<td>107</td>
<td>11890</td>
<td>1.55E-09</td>
</tr>
<tr>
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<td>81</td>
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</tr>
<tr>
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<td>2609</td>
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</tr>
<tr>
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<td>51</td>
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<td>1.02E-05</td>
</tr>
<tr>
<td>Glutathione S-transferase/chloride channel, C-term.</td>
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<td>2609</td>
<td>40</td>
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</tbody>
</table>

Table 2.4: Domain enrichment analysis for Ama-KTT/M/2 on α-amanitin versus Ama-KTT/M2 on no toxin (group 3 versus 2). This table shows the selected and significantly enriched domains in response to toxin treatment. “DEGs w/ domain” are the differentially expressed genes that have a particular domain. “DEGs” is the number of all differentially expressed genes in this comparison. “Genes w/ domain” is the total number of genes with a particular domain in the genome. “Genes” is the total number of genes in the genome. All p-values are corrected.
### 2.3.8 The RT-qPCR Results Confirm the Microarray Data

We used real-time quantitative reverse transcription PCR (RT-qPCR) to confirm the fold-changes of ten genes, which we selected because of their high fold-changes and predicted importance for the resistance phenotype (Figure 2.6 and Table A.5). When comparing Ama-KTT/M/2 to Canton-S (group 2 versus group 1), the genes Cyp6a2, 12d1-d, Ugt86Dd, GstD5, and GstE1 were between 1366.9 and 10.7-fold up-regulated (p<0.001 for all values, randomization test, B=2000). When we compared Ama-KTT/M/2 treated with α-amanitin to Ama-KTT/M/2 (group 3 versus group 2), Cyp316a1, 6d2, 4d8, 28d1, and 6t1 were up-regulated between 14.1 and 8.4-fold (p=0.002 for Cyp316a1 and p<0.001 for the other genes, randomization test, B=2000). In summary, the microarray analysis fold-induction changes perfectly correlate with our RT-qPCR results, such that the microarray results slightly underestimate the fold-changes that resulted from the RT-qPCR analysis.

<table>
<thead>
<tr>
<th>Domain</th>
<th>DEGs w/ Domain</th>
<th>DEGs</th>
<th>Genes w/ Domain</th>
<th>Genes</th>
<th>p-Value</th>
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<td>2642</td>
<td>9</td>
<td>11890</td>
<td>4.38E-06</td>
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<td>20</td>
<td>2642</td>
<td>35</td>
<td>11890</td>
<td>4.93E-06</td>
</tr>
<tr>
<td>Leucine aminopeptidase/peptidase B</td>
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<td>11</td>
<td>11890</td>
<td>8.48E-06</td>
</tr>
<tr>
<td>Peptidase M17, leucyl aminopeptidase, C-term.</td>
<td>9</td>
<td>2642</td>
<td>11</td>
<td>11890</td>
<td>8.49E-06</td>
</tr>
</tbody>
</table>
Figure 2.6: The qPCR results confirm the microarray data. A) Relative expression distribution (Y-axis) of ten selected genes is shown as a ratio comparing Ama-KTT/M/2 and Canton-S (group 2 versus group 1). Each measurement contains 15 replicates (3 replicates for each of the five biological controls of groups 1 and 2). B) Gene expression differences between Ama-KTT/M/2 treated with α-amanitin and Ama-KTT/M/2 (group 3 versus group 2) are compared. Group 3 contributes to 18 data points (three replicates for each of the six biological controls), while group 2 contributes to 15 data points, as previously mentioned. All comparisons were normalized with two reference genes, Sucb.
and alpha-Tub84B. Ratios above one indicate that a gene is up-regulated in the comparison.

2.4 Discussion

2.4.1 Several Mechanisms Seem to Confer α-Amanitin Resistance

α-Amanitin is the principal toxin in some of the most deadly poisonous mushrooms, which inhibits the function of RNA-polymerase II by binding to it. Our results presented here comprise the first whole-transcriptome scale investigation to identify the molecular and cellular mechanisms that underlie the resistance to this very potent toxin in any organism. Using larvae of the resistant stock Ama-KTT/M/2 and the sensitive stock Canton-S, we identified both constitutive and α-amanitin-inducible mechanisms that can explain the resistance to α-amanitin in the Ama-KTT/M/2 stock. Based on an array of bioinformatics analyses of our microarray data and RT-qPCR validation, we found that four main mechanisms are likely to contribute in concert to the resistance: 1) constitutive and α-amanitin-inducible toxin entry blockage, mediated by cuticular proteins, the MRJP domain of the Yellow protein family, and Sgs proteins, 2) constitutive and α-amanitin-inducible phase I and II detoxification, mediated by the Cytochrome P450, GST, and UGT enzyme families (likely followed by excretion), 3) α-amanitin-inducible lipid particle gene induction, possibly leading to the sequestration of α-amanitin in cytoplasmic lipid particles, and 4) α-amanitin-inducible peptidase genes, perhaps leading to the digestion of α-amanitin either inside or outside (e.g. gut lumen) of cells (Figure 2.7).
Figure 2.7: A model of the four mechanisms that contribute to the resistance to α-amanitin in concert. The bicyclic octapeptide α-amanitin is shown as a red 8. Cuticular proteins block some of the α-amanitin from entering the cells (blockage). α-Amanitin that entered the cytoplasm is either sequestered in lipid particles, cleaved by peptidases, or detoxified by phase I and II detoxification enzymes, possibly followed by excretion.

In honey bee venom glands, the Major Royal Jelly Protein 8 (MRJP8) was shown to be a part of the cuticular layer that forms the inner lining of the gland. It was suggested that MRJP8 protects the venom gland cells from the stored toxins. The closest relatives to the MRJP genes in Drosophila are the proteins of the Yellow family.
yellow gene itself was together with numerous cuticular protein genes identified as significant in our single gene, gene ontology, and domain enrichment analyses. It is thus possible that Yellow, together with other cuticular proteins, block the entry of α-amanitin into cells protected by a cuticular layer, such as the larval epidermis and gut epithelium (Figure 2.7). In a similar manner, the products of the five strongly α-amanitin-inducible salivary gland secretion genes Sgs1, Sgs3, Sgs5, Sgs7, and Sgs8 (each >300-fold induced) could perhaps bind to α-amanitin and reduce its uptake in the midgut. Another possibility is that α-amanitin is simply a stress factor that induces these and other genes. After all, α-amanitin blocks messenger RNA transcription in poisoned cells, which is certainly stressful for the organism.

Besides being involved in environmental stress responses, hormone metabolism, and other metabolic functions, some Cytochrome P450, GST, and UGT proteins catalyze detoxification reactions, which transform a broad variety of xenobiotic substances into less toxic molecules that can be more easily excreted from the body. Cytochrome P450 proteins, which are encoded by Cyp genes, are known for their broad range of substrates that they chemically modify. Several Cyp genes have been associated with single or multiple toxin resistance in diverse insect species, such as Cyp6g1, Cyp6g2, Cyp6a2, Cyp12a4, and Cyp12d1. Our single gene and gene ontology enrichment analyses identified three of these detoxification-implicated Cyp genes, which are more than about 200-fold constitutively up-regulated in Ama-KTT/M/2: Cyp6a2, Cyp12d1-d, and Cyp12d1-p (Table 2.1). It is thus possible that one or all three of these genes contribute to the resistance to α-amanitin. There is also evidence that Cyp12d1 is inducible by environmental stress factors, such as heat, oxidative stress,
and air pollutants. Because Cyp6a2, Cyp12d1-d, and Cyp12d1-p are constitutively up-regulated in our double-controlled study, stress is not a likely cause for the up-regulation of these three genes.

Some GST and UGT proteins perform phase II detoxification reactions that make toxic molecules bulkier and more hydrophobic, preparing the toxins for their excretion from the body. Several of these genes have been linked to insecticide resistance, while others are involved in several types of stress responses. Our single gene analysis showed that several Gst and Ugt genes are constitutively up-regulated in Amak-TT-M/2 and that both gene families are significantly enriched in our gene ontology enrichment analysis, while their specific domains were identified as significant in the protein domain enrichment analysis. It is thus likely that some of them help detoxifying α-amanitin by making it both bulkier to prevent it from binding to RNA-Polymerase II and more water-soluble to augment its secretion via the Malpighian tubules (Figure 2.7). It is, however, possible that the α-amanitin-induced genes simply respond to stress caused by the effects of the toxin.

In our gene ontology enrichment analysis, we identified two other interesting mechanisms, which are inducible in response to α-amanitin in the larval food: the possible sequestration of α-amanitin in lipid particles and the cleavage of α-amanitin by peptidases. A group of genes involved in the cellular component 'lipid particle' were on average more than 5200-times induced by α-amanitin in the larval food. Natural and artificial lipid particles have been shown to be involved in various detoxification processes in very diverse organisms such as yeast, insects, and humans. We
therefore speculate that cytoplasmic lipid particles aggregate around $\alpha$-amanitin molecules and trap them, thereby preventing the toxin from entering the nucleus, where RNA-Polymerase II performs its function. Furthermore, a variety of peptidase genes were identified in our various data analyses, suggesting that $\alpha$-amanitin is cleaved either in the gut lumen, in the cells, or perhaps even in the food, if the larvae secrete peptidases from their mouths (Figure 2.7).

2.4.2 Implications

Our data does not support the previously held view that an MDR mechanism confers $\alpha$-amanitin resistance in *D. melanogaster*. In 1982 and 2000, two studies based on QTL mapping suggested that $\alpha$-amanitin-resistance in four wild-caught *D. melanogaster* stocks is conferred by two major loci on chromosome 3 $^{14,15}$, the more recent of which pointed out *Mdr65* and *Pkc98E* as possible candidates. However, our single gene and genome enrichment analyses identified two $\alpha$-amanitin-inducible *Cyp* genes, *Cyp316a1* and *Cyp4d8*, which are situated close to the *Mdr65* locus and *Hr96* close to the *Pkc98E* locus. Because Begun and Whitley used QTL mapping, not deletion mapping, the two *Cyp* and the *Hr96* genes could instead be the resistance-conferring genes. Taking all the observations from our study together, we conclude that $\alpha$-amanitin resistance has evolved as a quantitative complex trait that is based on entry blockage, phase I and II detoxification followed by secretion, peptidase cleavage, and sequestration.

Cross-resistance to a broad variety of toxins could explain how some *Drosophila* species evolved into mushroom-feeding specialists that can use mushroom toxins to their
own advantage. For example, various mycophagous *Drosophila* species are frequently infected with parasitic nematodes that render about 20% of the adult flies sterile. Feeding on poisonous mushrooms not only kills the nematode parasites, it also provides a unique food source that is not accessible to many animals. *D. melanogaster* is a non-mycophagous species and should thus not be exposed to α-amanitin in nature. However, as discussed earlier, Cytochrome P450 enzymes can provide cross-resistance to multiple toxins, such as manufactured pesticides and natural xenobiotic products. We speculate that α-amanitin resistance in *D. melanogaster* has evolved in response to agricultural pesticides or other environmental factors, to which the flies were exposed before they were collected in the 1960s. Thus, if unrelated toxins can induce α-amanitin resistance, such a cross-resistance could prime a species to a radical host switch. If *D. melanogaster* females were to change their egg-laying behavior and oviposit on less toxic mushrooms, a niche change could result, followed by selection to feed on more toxic mushrooms. Being a species with such high fecundity, *D. melanogaster* could then even drive rare mycophagous *Drosophila* species out of their niche.

### 2.4.3 Limitations

The most obvious limitation of every microarray is that the observations and conclusions are entirely based on mRNA transcription differences. It is thus possible that some important mechanisms escaped detection. Furthermore, many *D. melanogaster* genes are still poorly annotated and their true functions are elusive. We thus excluded the most poorly annotated genes from our analysis. However, in doing so, we might have inadvertently lost some important genes that could contribute to the resistance to α-
amanitin. Furthermore, because we used whole larvae in our study, we cannot determine the relative importance that the different tissues play in the resistance to α-amanitin.

Our microarray data analysis did not reveal any gene-regulatory pathways that lead to the resistance to α-amanitin. *Hr96* and *cnc* have been shown to be upstream of detoxification genes\(^{108,109,120}\). *Hr96* is situated on the right arm of the third chromosome, where the genome enrichment analysis shows a peak in response to α-amanitin. However, the expression levels of both *Hr96* and *cnc* revealed nothing that would lead us to conclude their role in α-amanitin resistance. One reason for this could be that these genes encode transcription factors, which are already present in the cytoplasm to await activation, and we might not expect dramatic differences in their RNA regulation.

Another reason could be that our larvae were feeding on α-amanitin from the first instar until they were collected at the late third instar. Thus, we might have missed the critical time period during which the upstream components of the pathway were up-regulated.

We also noticed a lack of dramatic *Cyp*, *Gst*, and *Ugt* gene inducibility in response to α-amanitin. In the resistant stock Ama-KTT/M/2, many *Cyp*, *Gst*, and *Ugt* genes were constitutively expressed at higher levels than in Canton-S, while in larvae that were fed on toxic food, a completely different set of *Cyp* and *Gst* genes showed a much weaker induction than we initially expected. This weak gene induction is perhaps not surprising because in a previous microarray study using six different toxins, the detoxification gene families were not much inducible either\(^{121}\). It is thus possible that at least for the *Cyp*, *Gst*, and *Ugt* genes, the resistance to α-amanitin is mostly a constitutive trait.
Based on the mapping data from the two previous studies, we expected to find the α-amanitin resistance-conferring genes on chromosome 3\textsuperscript{14,15}. Because the original Ama-KTT stock is 45 years old, we wanted to make sure that the genes on both major autosomes are homozygous before performing the microarray. One limitation to our approach is that we did not balance the X chromosome when we created the isochromosome stock Ama-KTT/M/2. However, we showed that the Ama-KTT/M/2 stock is not less resistant than original Ama-KTT stock (Figure 2.1), indicating that most if not all resistance-conferring alleles are present in the isochromosome stock that we used for the microarray. Most genes that we identified as significant are situated on chromosomes 2 and 3 (Tables 2.1 and 2.2). However, a few highly expressed genes, like yellow, are on the X chromosome. Thus, these X-chromosomal genes could either be the original alleles from Ama-KTT or the alleles from the multi-balancer stock. If they derived from the multi-balancer stock, the regulation of these genes could be explained by epistasis, such that the inducers of the X-chromosomal genes are situated on the two major autosomes, which are derived from the original Ama-KTT stock.

**2.4.4 Future Studies**

In order to identify the upstream components of the pathways that lead to the resistance to α-amanitin in the Ama-KTT/M/2 isochromosome stock, future microarray studies should include samples of larvae that have been exposed to α-amanitin for different periods of time. Because first instar larvae are very small, the exposure to α-amanitin should happen during the third larval instar, and samples should be collected at a series of subsequent time points thereafter. This approach should be efficient to detect
gene-regulatory differences of the upstream pathway components. Furthermore, it would be interesting to investigate the mechanisms that confer α-amanitin resistance in mycophagous Drosophila species, using the RNA sequencing approach. Mycophagous species are several orders of magnitude more resistant to α-amanitin than D. melanogaster. The higher toxin resistance of those species could produce clearer signals for the determination of the factors that make Drosophila resistant. After we gain a clearer picture about the candidate genes that might confer α-amanitin resistance in several Drosophila species, the next step would be to provide conclusive genetic evidence if the candidate genes are sufficient and necessary for the α-amanitin resistance phenotype. This could be done by transgenically overexpressing the resistance-conferring alleles in either D. melanogaster or other sensitive species that are closely related to highly resistant mycophagous species. In D. melanogaster, overexpression of candidate genes can be achieved using the Gal4-UAS system with visible effects in different organs such as the gut, fat body, and Malpighian tubules. Such tests can reveal the organs and tissues that contribute to the resistance to α-amanitin. Because toxic mushrooms contain more than one toxin, mycophagous Drosophila species must be resistant to a variety of toxins that target different biological processes. Thus, other commercially available mushroom toxins, such as β-amanitin, phalloidin, ibotenic acid, and muscimol should be used to test if cross-resistance or independent mechanisms provide protection against the variety of mushroom toxins that mycophagous larvae encounter in their food source. Another pressing question is where α-amanitin goes once it entered a larva. Is it digested in the gut? Does it enter the cytoplasm of all or just a subset of cells?
Radioactive α-amanitin could be a means to answer this question, but the analysis of the data might prove very difficult.

### 2.5 Conclusions

We suggest that the α-amanitin resistance phenotype in *D. melanogaster*, a species that does not feed on mushrooms in nature, has evolved as cross-resistance to pesticides or other factors in the environment. Entry blockage of α-amanitin into epithelial cells, phase I and II detoxification mediated by Cytochrome P450, GST, and UGT enzymes (likely to be followed by excretion from the body), sequestration of α-amanitin in cytoplasmic lipid particles, and proteolytic cleavage by peptidases are four likely mechanisms to contribute to the resistance phenotype in concert. In contrast, we did not detect any evidence for multidrug resistance efflux systems to be important for the resistance to α-amanitin. Future studies should include a time series of α-amanitin exposure, *Drosophila* species that actually feed on toxic mushrooms in nature, and more mushroom toxins. Candidate genes resulting from these experiments should then undergo sufficiency and necessity tests by transgenic rescue.

### 2.6 Materials and Methods

#### 2.6.1 Fly Stocks

All fly stocks were maintained at room temperature on food containing Brewer’s yeast, cornmeal, granulated sugar, agar, and methylparaben as antifungal agent. The wild-
type stock Canton-S and the multi-balancer stock w[1118]/Dp(1;Y)y[+]; CyO/nub[1] b[1] sna[Sco] lt[1] stw[3]; MKRS/TM6B, Tb[1] were obtained from the Bloomington Stock Center, Bloomington, Indiana (stocks #1 and #3703, respectively). The α-amanitin-resistant Ama-KTT stock (# 14021-0231.07) was originally collected in 1968 in Kenting (Taiwan) and obtained from the Drosophila Species Stock Center at the University of California, San Diego.

2.6.2 Generation of the isochromosome stock Ama-KTT/M/2

Because Ama-KTT was maintained in the absence of selective pressure to toxins in the stock center over the past five decades, the stock could have lost, or become heterozygous for, some of the α-amanitin resistance-causing alleles. In order to create flies homozygous for the resistance-conferring alleles, we crossed the Ama-KTT stock to the multi-balancer stock w[1118]/Dp(1;Y)y[+]; CyO/nub[1] b[1] sna[Sco] lt[1] stw[3]; MKRS/TM6B, Tb[1]. As a result, we created the isochromosome stock Ama-KTT/M/2, which is isogenic for the second and third chromosomes.

2.6.3 Dose-Response Studies of the Fly Stocks to α-Amanitin

In order to quantify and compare the levels of α-amanitin resistance of the D. melanogaster stocks, dose-response experiments were performed, which measured the survival from freshly-hatched first-instar larvae to adulthood. Flies able to completely hatch from their pupae were scored as survivors. The α-amanitin-resistant stocks Ama-KTT and Ama-KTT/M/2 were tested on 11 α-amanitin concentrations, using 0 to 10 µg
of $\alpha$-amanitin per g of food in 1 $\mu$g increments. The $\alpha$-amanitin-sensitive wild-type stocks Canton-S and the multi-balancer stock w[1118]/Dp(1;Y)y[+]; CyO/nub[1] b[1] snl[Sco] lt[1] stw[3]; MKRS/TM6B, Tb[1] were initially tested on five concentrations ranging from 0 to 4 $\mu$g of $\alpha$-amanitin per g of food in 1 $\mu$g increments. However, because they survived only the zero-concentration, these stocks were further tested on 0, 0.25, 0.5, 0.75, 0.1, 0.25, and 0.375 $\mu$g of $\alpha$-amanitin per g of food.

In order to obtain first-instar larvae for the dose-response experiments, flies of mixed sexes were allowed to lay eggs on molasses agar caps that contained a streak of fresh Baker’s yeast paste at 25°C, 70% humidity, and a 12:12 hour day/night cycle. The yeast was removed prior to larval hatching. Freshly hatched first-instar larvae were placed in groups of ten into 2-mL plastic test tubes (USA Scientific), each containing 500 mg of non-toxic or poisoned food and two small air holes in the lid. The food consisted of 125 mg dry, instant $Drosophila$ medium (Carolina) and 375 $\mu$L sterile Milli-Q water with or without dissolved $\alpha$-amanitin. Ten tubes were prepared for each toxin concentration and experimental replicate, resulting in 100 larvae for each concentration and experiment. Three high-quality dose-response experiments, in which the zero-concentration survival rate was at least 80%, were used to calculate the LC$_{50}$ of each fly stock. The standard deviation of the mean (s.e.m.) was calculated for each concentration by sampling the data points of all 30 vials of every concentration. The LC$_{50}$ was calculated using scatter plots and the logarithmic trendline function in Microsoft Excel.

73
2.6.4 Sample Preparation for the Microarray Analysis

In order to compare the constitutive gene-regulatory differences across the entire transcriptome between α-amanitin-sensitive and -resistant stocks, freshly-hatched first-instar larvae of the sensitive Canton-S stock (group 1) and the resistant Ama-KTT/M/2 stock (group 2) were placed in groups of ten into 2-mL plastic test tubes (USA Scientific), containing 500 mg of non-toxic food. To identify the genes that are inducible by α-amanitin, Ama-KTT/M/2 larvae were raised on 1.5 µg of α-amanitin per g of food (group 3), which is slightly lower than the LC50 concentration of this stock. All larvae were raised until they reached the late third instar at 25°C, 70% humidity, and a 12:12 hour day/night cycle. Because not all larvae survived in the tubes and the larvae on α-amanitin-containing food had a slower growth rate, initially 600 first-instar larvae (60 tubes) for each group were started over three subsequent days (20 tubes per group and day). When the majority of larvae reached the late third instar, the tubes were emptied and groups of ten late, but still feeding third-instar larvae were randomly picked from across all tubes and flash-frozen in batches of ten in liquid nitrogen, each batch providing the RNA for one microarray chip. Five biological replicates (ten larvae each) were prepared for groups 1 and 2, whereas group 3 was prepared in six biological replicates (ten larvae each). All samples were collected on the same morning. RNA extraction was performed without delay, using the RNeasy microarray tissue kit (Qiagen), according to the manufacturer’s instructions.
2.6.5 Affymetrix Array Target Preparation, Hybridization, and Scanning

Collection and analysis of data were compliant with MIAME standards. The microarray experiment was performed using the Affymetrix GeneChip Drosophila Genome 2.0 Arrays (Affymetrix, Santa Clara, CA, USA) with biotinylated targets derived from total RNA. Each array contains 18,952 probes that interrogate ~18500 transcripts of genes present in the transcriptome of *D. melanogaster*. Prior to labeling, total RNA samples were checked for purity and concentration, using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher, Waltham, MA, USA) and for integrity, using RNA 6000 Nano Chips in a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). cDNA for hybridization was synthesized and biotin-labeled from 400 ng of total RNA, using a MessageAmp Premier IVT kit (Ambion, Austin, TX, USA) according to the manufacturer's specifications. Biotinylated cDNA was fragmented, then hybridized, washed, and stained using a GeneChip Hybridization, Wash, and Stain Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's specifications. Arrays were post-processed on the AFX 450 Fluidics Station before they were scanned on an AFX GC3000 G7 Scanner (Affymetrix, Austin, TX, USA). Data were extracted from the raw images, using the Affymetrix Expression Console v.1.2 software. The RNA quality check, labeling, hybridization, and imaging procedures were performed according to Affymetrix protocols at the Center for Genomics Research and Biocomputing, University of Wisconsin.

2.6.6 Microarray Data Normalization

The quality of microarray data sets was first checked by examining the distribution of the Studentized deleted residuals, using a previously described procedure.
and only high-quality microarray data were used for normalization. Probeset-level normalization was performed with the PLIER (Probe Logarithmic Intensity Error) algorithm with quantile normalization and mismatch intensity adjustment, using the Affymetrix Power Tools software v.1.14.4.1. Probesets were annotated using release 32 of the Affymetrix annotation for the Drosophila 2.0 array platform. The CEL files and summarized (normalized) microarray data resulting from this study have been deposited in the NCBI's Gene Expression Omnibus database at NIH (http://www.ncbi.nlm.nih.gov/geo/) with the accession number of GSE52782.

2.6.7 Genome Enrichment Analysis

To find genome regions containing more differentially expressed genes than expected by chance, we used the binomial coincidence detection algorithm with modifications specific for this dataset. Because *D. melanogaster* has a smaller genome and shorter regions of genetic linkage than mammalian genomes, we reduced the length of the overlapping bins to 500 kb spaced at 250 kb intervals. In order to reduce the total noise and find the strongest signal, we used only the top 0.01 most differentially expressed genes in the dataset. Briefly, under a null hypothesis of no significant enrichment in a genome region, the probability of finding a significantly differentially expressed gene within each bin will follow a binomial distribution with a probability of any given gene being significantly differentially expressed at no more than 0.01. The algorithm calculates a binomial probability for the empirical quantity of differentially expressed genes within each bin across the entire genome. The decimal log of the inverse of these probabilities is graphed. A decimal log of 2 corresponding to the horizontal line
through each graph indicates a probability of a cluster occurring 1 in 100 times under the null hypothesis, the cutoff used for this method. The resulting graphic shows clustering over the whole genome and spikes indicate clusters unlikely to have occurred by chance. This is statistical evidence that a genome region is likely implicated in a gene expression phenotype. The assumptions for the inferential statistics used for this analysis necessitate inclusion of low copy genes as differentially expressed, thus the inferential statistics used to generate the genome enrichment figure were performed in the limma package in Bioconductor v.2.10. Cytoband visualization is derived from annotation tables of the UCSC dm3 genome, which represents cytobands as alternating light and dark bands.

2.6.8 Identification of Differentially Expressed Genes (DEGs)

A nonparametric method, RankProd (RP), was used to identify differentially expressed genes (DEGs) between Ama-KTT treated with α-amanitin slightly below the LC$_{50}$ concentration, and untreated Ama-KTT, or Canton-S conditions. We chose RP because it had been implicated to be more accurate for ranking genes by differential expression than t-statistics or derived methods. Kadota et al. once evaluated eight DEG ranking methods and concluded that RP is one of the best performing methods. Laing et al. indicated RP is one of most efficient method when replicate numbers is less than 10. In this study, we applied multiple testing corrections to the p-values resulting from RP using Benjamini and Hochberg False Discovery Rate and all genes with corrected p-values (< 0.05) were defined as DEGs.
2.6.9 Gene Ontology Enrichment Analysis

The DEGs identified from each comparison, namely, Ama-KTT/M/2 versus Canton-S and Ama-KTT/M/2 on α-amanitin versus Ama-KTT/M/2 on no toxin, were used as the input for the gene ontology enrichment analysis. We employed an online tool, AmiGO’s Term Enrichment, to identify the enriched gene ontologies (http://amigo.geneontology.org/). This tool uses the Perl module GO:TermFinder available at CPAN (http://search.cpan.org/) to identify the enriched gene ontology terms associated with a DEG list, using the hypergeometric probability function. We applied multiple testing corrections to calculate the p-values of all GO terms and then corrected p-values using Benjamini and Hochberg False Discovery Rate (130). All gene ontology terms with a corrected p-value < 0.05 were considered to be significantly enriched.

2.6.10 Protein Domain Enrichment (PDE) Analysis

Protein domains were analyzed with InterproScan (131). We first downloaded and installed InterproScan and associated databases to our Linux server and performed the standalone analysis to identify protein domains of all target sequences provided by FlyBase (http://flybase.org/static_pages/docs/datafiles.html). The enrichment of each domain in the differentially expressed gene list was compared to the occurrence of the respective domain in the background of all genomic genes, and two parameters were introduced to show the enrichment of each domain as described in (132): (1) Enrichment factor, EF = k/(nM/N); and (2) the E_score, which is the hypergeometric probability of
identifying at least k domains from DEG list. It is calculated using the following formula:

\[ E_{\text{score}} = 1 - \sum_{r=0}^{k-1} \binom{M}{i} \binom{N-m}{n-i} \binom{N}{i} \]

N is the total number of domains associated with all genomic genes, M is total number of a specific domain for all genes in the genome, n is the number of all domains associated with the DEGs, and k is the number of a specific domain present in the DEGs list. We applied multiple testing corrections to the p-values calculated via hypergeometric probability using Benjamini and Hochberg False Discovery Rate (FDR) \(^{130}\). The significantly enriched protein domains are those that have a corrected p-value < 0.05.

2.6.11 RT-qPCR Validation of the Microarray Results
Quantitative real-time PCR (qPCR) was performed on ten genes of interest to confirm the results of the microarray analysis. Each gene of interest and biological replicate was repeated three times to ensure the statistical significance of the result. The genes included two Cyp, one Ugt, and two Gst genes that were up-regulated when comparing the resistant group Ama-KTT/M/2 to the control group Canton-S (group 2 versus group 1) and five Cyp genes that were up-regulated when comparing Ama-KTT/M/2 on α-amanitin to Ama-KTT/M/2 on no toxin (group 3 versus group 2). Two reference genes, Scub and alpha-tub84B, were used as controls to normalize the results. These genes were selected because their fold-changes were nearly zero for each comparison. The primer pairs used were a part of the Taqman Gene Expression Assays
kit (Applied Biosystems): Dm02361072_s1, Dm01831596_g1, Dm01840671_g1, Dm01830394_g1, Dm01822311_g1, Dm01804633_g1, Dm01799869_s1, Dm02147253_g1, Dm01817955_g1, Dm02152265_s1, Dm01826948_s1, and Dm02374415_g1. The reactions were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems). The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to reverse transcribe RNA to cDNA in an Eppendorf PCR machine for 96 reactions (Eppendorf, Model 96S). We used REST 2009 to calculate the RT-qPCR p-values.

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2.8 Author Contributions
Conceived and designed the experiments: CM TW. Performed the experiments: CM TW. Analyzed the data: CM MS LL HW TW. Contributed reagents/materials/analysis tools: MS HW TW. Wrote the paper: CM MS LL HW TW.
Conclusions

In this study, we investigated the molecular mechanisms underlying resistance to the most potent mushroom toxin, to which only a handful of animal species on Earth are resistant. \( \alpha \)-Amanitin-containing mushrooms cause 90% of the mushroom fatalities in the United States \(^{12} \). As of right now, there is no good cure for \( \alpha \)-amanitin poisoning. Because \( D. \ melanogaster \) is a model organism and widely used for human medical research, we now have a valuable tool to understand the cause of \( \alpha \)-amanitin resistance. Studying this resistance may lead to the development of a cure for \( \alpha \)-amanitin and other types of poisoning. We already know that the \( Cyp \) family of genes can detoxify a variety of xenobiotic substances \(^{26-31} \), and manipulating these genes in humans could become the key to help and treat patients that fall victim to poisoning.

We also tried to understand how \( \alpha \)-amanitin affects \( D. \ melanogaster \) physiologically. We concluded that the overuse of pesticides may have caused this curious cross-resistance to \( \alpha \)-amanitin. It is important to note that \( \alpha \)-amanitin resistance could be the beginning of \( D. \ melanogaster \)’s expanding into another ecological niche in the environment. Because only a few species of \( Drosophila \) currently occupy the mushroom-feeding niche, and these species lay relatively few eggs, \( D. \ melanogaster \) could very easily drive some of the mushroom-feeding species to extinction due to \( D. \ melanogaster \)’s high fecundity. The overuse of pesticides could thus potentially cause shifts in ecosystems, helped by a few random mutations in the resistant flies.
Future Research

Based on our research, the foundation has been laid to further determine what has caused mushroom toxin resistance in *Drosophila*. We would like to create transgenic *D. melanogaster* stocks using the candidate genes from our microarray study and test these lines by transgenic rescue to determine if the candidate genes from a resistant strain can cause α-amanitin resistance in a susceptible strain. As our microarray data suggest, we expect that multiple genes act together to confer resistance, not just one single gene. We would also like to use the CRISPR/Cas9 system to delete possibly resistance-conferring genes in resistant strains. These experiments are expected to provide conclusive genetic evidence for our candidate gene’s involvement in α-amanitin resistance.

Besides the moderately resistant *D. melanogaster* stocks that we have studied so far, *Drosophila guttifer*a is a species that eats extremely toxic mushrooms in nature. We would further like to perform an RNA deep-sequencing study to investigate α-amanitin resistance in a species that actually feeds on deadly toxic mushrooms. The RNA deep-sequencing experiment would be performed using a similar experimental design as in the microarray study. After we identify some candidate genes, we would again be incorporating transgenic rescue experiments and the CRISPR/Cas9 system to identify how much each candidate gene contributes to the α-amanitin resistance in this mushroom-feeding species. Because mushroom-avoiding flies are much less resistant than mushroom-feeding flies (three orders of magnitude), we predict that the α-amanitin resistance will be conferred by largely different genes and molecular mechanisms.
References


Appendix A

All supplemental tables can be found at the following webpage:
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0093489

Table A.1: Differentially expressed genes (DEGs) between Ama-KTT/M/2 on no toxin versus Canton-S (group 2 versus 1), Ama-KTT/M/2 on toxin versus Canton-S (group 3 versus 1), and Ama-KTT/M/2 on toxin versus Ama-KTT/M/2 on no toxin (group 3 versus 2). This table contains 4209 DEGs that are differentially expressed in at least one of the three comparisons.

Table A.2: Complete single gene analysis for Ama-KTT/M/2 versus Canton-S on no toxin (group 2 versus 1). This table contains well-annotated genes that are at least 2.0-fold constitutively up-regulated in the resistant stock, as compared to the sensitive stock, on no toxin. The p-value cutoff is p<0.05.

Table A.3: Complete single gene analysis for Ama-KTT/M/2 on toxin versus Ama-KTT/M/2 on no toxin (group 3 versus 2). This table contains well-annotated genes that are at least 2.0-fold inducible by feeding larvae of the resistant stock with α-amanitin-containing food, as compared to resistant larvae on no toxin. The p-value cutoff is p<0.05.

3 The material contained in this chapter was previously published in PLoS ONE.
Table A.4: Genome enrichment analysis for group 2 versus 1 and group 3 versus 2. This table shows the genes behind the peaks in Figure 3. The peak at band 38B is the only locus that is differentially expressed between Ama-KTT/M/2 and Canton-S on no toxin (group 2 versus group 1). The remaining peaks 66A, 69A, 92A, and 96D show differentially expressed loci in response to α-amanitin treatment (group 3 versus group 2). All p-values are corrected and fold-changes are given for the individual genes. Peaks 66A and 96D are very close to the two QTL mapping peaks identified in previous studies 14,15.

Table A.5: Comparison of qPCR and microarray fold-induction values. The first five genes were constitutively over-expressed in Ama-KTT/M/2, as Compared to Canton-S (Group 2 versus group 1). The last five genes were induced by α-amanitin in Ama-KTT/M/2, as compared to Ama-KTT/M/2 on no toxin (group 3 versus group 2). The RT-qPCR p-values are uncorrected, while the array p-values are corrected.
Appendix B

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