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REVIEW

Drosophila, destroying angels, and deathcaps! Oh my! A review of mycotoxin tolerance in the genus *Drosophila*

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BACKGROUND: Evolutionary novelties, be they morphological or biochemical, fascinate both scientists and non-scientists alike. These types of adaptations can significantly impact the biodiversity of the organisms in which they occur. While much work has been invested in the evolution of novel morphological traits, substantially less is known about the evolution of biochemical adaptations.
METHODS: In this review, we present the results of literature searches relating to one such biochemical adaptation: α-amanitin tolerance/resistance in the genus *Drosophila*.
RESULTS: Amatoxins, including α-amanitin, are one of several toxin classes found in *Amanita* mushrooms. They act by binding to RNA polymerase II and inhibiting RNA transcription. Although these toxins are lethal to most eukaryotic organisms, 17 mushroom-feeding *Drosophila* species are tolerant of natural concentrations of amatoxins and can develop in toxic mushrooms. The use of toxic mushrooms allows these species to avoid infection by parasitic nematodes and lowers competition. Their amatoxin tolerance is not due to mutations that would inhibit α-amanitin from binding to RNA polymerase II. Furthermore, the mushroom-feeding flies are able to detoxify the other toxin classes that occur in their mushroom hosts. In addition, resistance has evolved independently in several *D. melanogaster* strains. Only one of the strains exhibits resistance due to mutations in the target of the toxin.
CONCLUSIONS: Given our current understanding of the evolutionary relationships among the mushroom-feeding flies, it appears that amatoxin tolerance evolved multiple times. Furthermore, independent lines of evidence suggest that multiple mechanisms confer α-amanitin tolerance/resistance in *Drosophila*.

Keywords *Drosophila*, mushroom-feeding, biochemical adaptations, mushroom toxins, cyclopeptides, α-amanitin

Introduction

Novel adaptations, both morphological and biochemical, have long fascinated evolutionary biologists. The evolution and diversification of such adaptations have been an active area of study, in part because novel traits can impact the biodiversity of the organisms in which they occur. Specifically, these traits can allow organisms to exploit of new niches, increase fitness and specialization, initiate adaptive radiations, and provide a means of escaping competition and other selective pressures (Simpson, 1953; Heard and Hauser, 1995; Schluter 2000; Coyne and Orr, 2004). Many studies have examined the evolution and development of novel morphological structures (e.g., Emlen, 2000; Kijimoto et al., 2012; Stansbury and Moczek, 2014; Broeckhoven et al., 2016). Far less is known about the evolution of novel biochemical and metabolic adaptations. In particular, how does a novel, biochemical adaptation of interest evolve if there are costs associated with it, and selection for the trait is inconsistent.

One such novel, biochemical adaptation that has both associated costs and experiences inconsistent selection is tolerance to the lethal mushroom cyclopeptide toxin α-amanitin. Tolerance is found in mushroom-feeding *Drosophila* species in the *immigrans-tripunctata* radiation. Within this radiation, many species are generalist feeders on a wide range of fleshy Basidiomycota mushrooms, including toxic *Amanita* species that contain mixtures of mycotoxins. These flies are among a limited number of eukaryotes known to tolerate the cyclopeptide toxins (including the notorious α-amanitin) found in *Amanita* mushrooms, despite these

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mushrooms being an unpredictable portion of their diet. As these mushrooms contain multiple distinct toxins with unique chemical properties, the flies are tolerant to more than a single toxin. Resistance to the amatoxin α-amanitin has also evolved independently in multiple strains of the frugivorous *Drosophila melanogaster*. In this review, we examine the current knowledge relating to the novel biochemical adaptation, α-amanitin tolerance/resistance.

Mycotoxins

Mushrooms are a pervasive component of the environment, and edible species provide a valuable source of nutrients (Chang and Miles, 2004). Wild edible mushrooms contain high levels of proteins, carbohydrates, and insoluble fiber and low-fat content (Kalač, 2009, 2013; Obodai et al., 2014; Toledo et al., 2016). In addition, the potassium and phosphorous content of these mushrooms is higher than that of most vegetables (Kalač 2009). Of the 14,000 named species, 100 North American mushrooms are poisonous (Broussard et al., 2001; Berger and Guss, 2005a) and can resemble harmless species (Wieland 1986; Diaz 2005). Thus, an inherent risk of collecting mushrooms is mistaking a toxic species for one that is edible (Wieland 1986; Kaul 2002).

The compounds that cause mushroom poisonings are divided into eight primary toxin classes (cyclopeptides, muscarines, monomethylhydrazines, orellanines, coprines, hallucinogenic indoles, isoxazoles, and GI irritants) based on chemical structure (see Berger and Guss, 2005a, 2005b for a detailed review of the toxin classes). These toxins produce a range of symptoms that can include gastrointestinal distress, hallucinations, and organ failure. The deadliest class of mycotoxins is the cyclopeptides. These toxins are responsible for 90%-95% of all mushroom fatalities (Wieland, 1986; Bresinsky and Besl, 1990). Furthermore, our examination of the annual National Poison Data System reports from 1995 to 2014 (Litovitz et al., 1995; Litovitz et al., 1996; Litovitz et al., 1997; Litovitz et al., 1998; Litovitz et al., 1999; Litovitz et al., 2000; Litovitz et al., 2001; Litovitz et al., 2002; Watson et al., 2003; Watson et al., 2004; Watson et al., 2005; Lai et al., 2006; Bronstein et al., 2007; Bronstein et al., 2008; Bronstein et al., 2009, 2010; Bronstein et al., 2011; Bronstein et al., 2012; Mowry et al., 2013; Mowry et al., 2014) revealed that cyclopeptide toxins poisonings resulted in more fatalities over those 20 years than bites from the four most venomous North American snakes (i.e., copperheads, rattlesnakes, coral snakes, and cottonmouths).

Cyclopeptides contain a sulfur-linked tryptophan and unusual hydroxylated amino acids (Berger and Guss, 2005a; Li and Oberlies, 2005), and these toxins are divided into three subclasses: amatoxins (octapeptides), phallotoxins (heptapeptides), and virotoxins (heptapeptides) (Li and Oberlies, 2005). Phallotoxins and virotoxins are not readily absorbed in the digestive tract; thus, these two subclasses have little to no toxicity in humans (Karlson-Stiber and Persson, 2003; Diaz, 2005). Neither of these subclasses have been tested for toxicity in organisms other than humans. Amatoxins act at a slow rate (asymptomatic for 6-12 h and death as late as 6-8 days after ingestion; see Table 1 for details of the amatoxin toxidrome) (Wieland, 1986). They are thermostable, readily absorbed through the intestines, and are 10-20 times more toxic than phallotoxins and virotoxins (Li and Oberlies, 2005). Thus, amatoxins are primarily responsible for fatalities attributed to cyclopeptides. The long latent phase of the amatoxin toxidrome is useful in distinguishing cyclopeptide poisoning from other toxin classes that cause more immediate gastrointestinal distress after consumption (Mas, 2005). If a patient enters the hepatic phase, a liver transplantation may be needed to prevent death (Broussard et al., 2001; Diaz 2005; Escudié et al., 2007).

Table 1 Four phases of cyclopeptide poisoning (Berger and Guss, 2005a; Diaz, 2005; Mas, 2005)

Phase	Onset	Symptoms
Latent	0-12 h	Asymptomatic
Gastrointestinal Distress	6-24 h	Abdominal pain, vomiting, diarrhea
Apparent Convalescence	24-72 h	Asymptomatically rising liver enzymes
Hepatic	3-9 days	Hepatic and renal failure, coma, death

All of the reported cyclopeptide fatalities occurred after individuals unintentionally consumed a poisonous mushroom species (particularly toxic *Amanita*) (Bosman et al., 1965; Leathem et al., 1997; Broussard et al., 2001). *Amanita* is a cosmopolitan genus of ~500 species that includes edible (e.g., *A. caesarea*, Caesar's Mushroom), hallucinogenic (e.g., *A. muscaria*, the Fly Agaric), and toxic (e.g., *A. phalloides*, the Death Cap) mushrooms. Lethal *Amanita* species contain each of the three cyclopeptide subclasses. In particular, the amatoxin α-amanitin is primarily responsible for the fatalities associated with *Amanita* (Berger and Guss, 2005a). In humans, a dose as low as ~0.1 mg/kg can be fatal (Faulstich 1980; Wieland, 1986; Karlson-Stiber and Persson, 2003; Erden et al., 2013), and a single *Amanita* mushroom can contain ~10-12 mg of α-amanitin (Mas, 2005; Walton et al., 2010). Studies of amatoxin toxicity in white mice calculated an LD_{50} of ~0.3-0.7 mg/kg (Wieland, 1983). α-Amanitin acts by binding to RNA polymerase II (RNAP II), thereby inhibiting RNA transcription, which leads to cell death (Lindell et al., 1970). The exact location to which α-amanitin binds on RNAP II remains an area of active research. This toxin appears to bind to an active site below the "Bridge-Helix" of RNAP II (Bushnell et al., 2002). Specifically, α-amanitin binds to the His1085 residue on the trigger loop of RNAP II, which results in a loss of speed and accuracy of the enzyme (Kaplan et al., 2008). Due to this mode of action, α-amanitin has shown promise for the treatment of tumors that undergo rapid cell division (Duensing et al., 2007; Moldenhauer et al., 2012; Moshnikova et al., 2013; Liu et al., 2015; Kume et al., 2016). In addition to the aforementioned cytotoxicity, a recent study (Marciniak et al., 2017) demonstrated that α-amanitin also exhibits genotoxicity, the ability to cause damage to DNA and chromosomes. It would be interesting to test if α-amanitin causes an elevated mutation rate in *Drosophila* species that can tolerate this toxin.

Despite the well documented impacts of poisoning from cyclopeptides and particularly amatoxins, no antidote exists for α-amanitin poisoning (Garcia et al., 2014), and clinical efficacy has not been demonstrated for pharmacological treatments (e.g., benzyl penicillin G) (Wieland, 1986; Enjalbert et al., 2002; Kaul, 2002; Berger and Guss, 2005a). Instead, treatment of α-amanitin poisoning is supportive, focusing on preventing further absorption of the toxin, and attempting to eliminate it from the body. Although α-amanitin is toxic to most eukaryotic organisms, 17 mushroom-feeding *Drosophila* are tolerant of α-amanitin and other toxins found in *Amanita* mushrooms (Jaenike et al., 1983; Lacy, 1984; Jaenike, 1985b; Spicer and Jaenike, 1996; Kaul, 2002; Stump et al., 2011; Tuno et al., 2007) and a surprising number of *D. melanogaster* strains are resistant to it (Phillips et al., 1982; Begun and Whitley, 2000; Mitchell et al., 2014; Mitchell et al., 2015; Mitchell et al., 2017). Furthermore, the tolerant mushroom-feeding species can and do use toxic *Amanita* mushrooms as developmental hosts.

Amanitin tolerance in mushroom-feeding *Drosophila*

While cyclopeptide mycotoxins are toxic to most multicellular organisms, there are 17 known species of mushroom-feeding *Drosophila* that are tolerant of these toxins and use mushrooms that contain cyclopeptides and other mushroom toxins as developmental hosts. Mushrooms are considered to be a highly ephemeral host. The ability to develop in toxic mushroom species provides several benefits for the tolerant fly species: 1) flies experience less competition for an ephemeral food resource (Buxton, 1960; Grimaldi and Jaenike, 1984; Lacy, 1984) and 2) these mushrooms are lethal to nematode parasites that can lead to infertility in the flies (Jaenike 1985b; Jaenike and Perlman, 2002). Thus, while toxic mushrooms comprise only a small portion of the hosts of mushroom-feeding flies, the ability to make use of them is highly beneficial for these species. Below, we detail the natural and evolutionary history of these flies and the state of knowledge regarding amatoxin tolerance within these species.

Natural history of mushroom-feeding Drosophila

All of the mushroom-feeding species of *Drosophila* that are known to be tolerant of cyclopeptide toxins are found within the *immigrans-tripunctata* radiation of the *Drosophila* (*Drosophila*) subgenus. Within this radiation, the species that utilize toxic mushroom hosts occur in five species groups (*bizonata*, *cardini*, *quinaria*, *testacea*, and *tripunctata*). The *immigrans-tripunctata* radiation includes additional mushroom-feeding species that have not yet been assayed for toxin tolerance, but they share a similar natural history with the tolerant species. All of the mushroom-feeding species in the *immigrans-tripunctata* radiation exhibit a natural history that is simple and well-characterized (e.g., Grimaldi, 1985; Jaenike 1978a,1978b; Jaenike and James, 1991; Lacy, 1984). Adult flies are attracted to a mushroom where they mate, females lay their eggs on the mushroom, and the larvae then develop in this mushroom. As their natal mushroom disintegrates, emerging adults must disperse to find a fresh mushroom host. All of the mushroom-feeding species within the *immigrans-tripunctata* radiation are generalists across a diverse range of fleshy Basidiomycota mushrooms (Jaenike, 1978a; 1978b; Hackman and Meinander, 1979; Shorrocks and Charlesworth, 1980; Lacy, 1984; Kimura and Toda, 1989). They will utilize any suitable host species that is available, and these can include both non-toxic and toxic mushroom species. Mushroom-feeding *Drosophila* species that are not part of this radiation (*i.e.*, the subgenera *Hirtodrosophila*, *Mycodrosophila*) exhibit a more specialized strategy and feed primarily on bracket fungi (family Polyporaceae) (Lacy, 1984). Although the mushroom-feeding species in the *immigrans-tripunctata* radiation are all described as polyphagous, the state of mushroom that is most attractive to these flies differs among the species groups. For example, fresher mushrooms attract the *quinaria* group species (Werner and Jaenike, 2017), while species in the *testacea* group are attracted to mushrooms in a later stage of putrefaction (Kimura, 1980; Grimaldi, 1985). Furthermore, some of these species also utilize other non-mycophagous food sources (e.g., *D. tripunctata* feeds/develops on both mushrooms and fruits, and *D. nigromaculata* uses mushrooms rotting vegetation, and fermenting fruits).

Given the polyphagous use of fleshy Basidiomycota by the mushroom-feeding species in the *immigrans-tripunctata* radiation and the use of non-mushroom hosts, oviposition preference in these flies has been an area of active research. Parasitic infections of nematodes from the genera *Howardula* and *Parasitylenchus* are common in these species (Jaenike, 1985b,1992; Jaenike and Perlman, 2002; Perlman and Jaenike, 2003; Debban and Dyer, 2013), and these worms lead to partial to complete female infertility in flies that are infected with them. The nematodes are common on the mushroom hosts that do not contain mycotoxins, but they cannot survive on toxic *Amanita* (Jaenike, 1985b; Jaenike and Perlman, 2002). Thus, the toxic mushrooms represent a refuge for mushroom-feeding flies from these parasites, and it could be expected that the flies would actively seek out the toxic mushrooms as hosts. As the North American mushrooms that contain α-amanitin are large and white, it is possible that fly species that breed in these mushrooms prefer the large, white host mushroom phenotype. However, Debban and Dyer (2013) found that uninfected *D. putrida* did not preferentially oviposit on food containing the cyclopeptide α-amanitin. While the presence of nematodes does not appear to influence oviposition preference in *D. putrida*, significant genetic variation is present for host preference and settling behavior in *D. tripunctata* (Jaenike and Grimaldi, 1983; Jaenike 1985a,1986,1987). *Drosophila tripunctata* feeds and develops on both rotting mushrooms and fruits. Jaenike and Grimaldi (1983) allowed individuals from populations in different geographic locations and within populations to choose between tomatoes or mushrooms as locations for oviposition. They found that genetic variation for oviposition preference occurred in *D. tripunctata* both between and within populations. Following up on this work, Jaenike (1985a) determined that both the genotype and previous experience of the fly influenced oviposition preference. In addition to exhibiting genetic variation for foods and egg laying locations, populations of *D. tripunctata* also exhibit genetic variation in cyclopeptide toxin tolerance (Jaenike, 1989), which may be due, in part, to the differences in oviposition preference. While toxic mushrooms may represent only a small portion of the hosts for mushroom-feeding species and are likely sites of lower competition and parasitism, the mushroom-feeding flies do not appear to prefer these hosts over other non-chemically defended species.

Amatoxin tolerance in the **immigrans-tripunctata** *radiation*

Even though toxic mushrooms are a small part of the potential diet, every mushroom-feeding species in the *immigrans-tripunctata* radiation that has been assayed can tolerate high concentrations of cyclopeptide toxins, despite these toxins being lethal to nearly every other eukaryote. The toxic mushrooms, in which these species feed and develop, can contain up to 1600 µg of α-amanitin per gram of dried mushroom tissue (Wieland, 1968, 1986), and the flies are deleteriously affected by α-amanitin in the range of 750 to 1000 µg/g (Jaenike, 1985b). For comparison, the human LC_{50} is 0.1 µg/g bodyweight, which can make the consumption of a single mushroom lethal (Wieland et al., 1978).

Early reports (Buxton, 1960; Shorrocks and Wood, 1973; Jaenike, 1978a,1978b; Jaenike and Selander, 1979) noted that adults of the mushroom-feeding species from the *quinaria* and *testacea* groups emerged from *Amanita* mushrooms containing cyclopeptide poisons. The first quantification of toxin tolerance was completed by Jaenike et al. (1983), who demonstrated that mushroom-feeding flies from the *quinaria*, *testacea*, and *tripunctata* groups could tolerate high doses of α-amanitin. They reared larvae of three mushroom-feeding species, three frugivorous species, and a *D. melanogaster* strain (C4) known to be resistant to amatoxins on diets containing a range of α-amanitin concentrations. At the highest toxin concentration (50 µg/mL α-amanitin), none of the frugivorous species survived. At lower concentrations, survival dropped significantly, and development time increased significantly. The three mushroom-feeding species survived at this concentration, but *D. tripunctata*, a species that feeds on both mushrooms and fruits, exhibited a significant reduction in survival at this concentration. The high concentration of α-amanitin did not impact the development time of any of these three species. Furthermore, Jaenike et al. (1983) noted that the RNAP II of the three mushroom-feeding species was as susceptible to α-amanitin binding as that of the three frugivorous species. Thus, the mechanism of tolerance in these species was not due to a mutation that would inhibit the toxin from binding.

Following the initial identification of toxin tolerance in the three mushroom-feeding species by Jaenike et al. (1983), Stump et al. (2011) conducted a broader survey of α-amanitin tolerance in the *immigrans-tripunctata* radiation. They assessed 16 species that represented the *quinaria*, *cardini*, *tripunctata*, *immigrans*, *calloptera*, and *funebris* species groups. Four of the species sampled feed on only rotting vegetation or fermenting fruits. The remaining 12 species feed only on mushrooms or exhibit mushroom-feeding in addition to utilizing fruits or vegetation (see Table 2 regarding which species are included in the different analyses). In all of the species that do not use mushrooms as a host, survival on a diet containing 50 µg/mL of α-amanitin was zero. In addition, *D. funebris*, a species that feeds on both polypore mushrooms and vegetation, did not survive on a diet containing the toxin. The other mushroom-feeding species that are polyphagous for Basidiomycota mushrooms (Table 2) did survive on this diet. Stump et al. (2011) also surveyed the RNAP II gene of the amanitin tolerant, mushroom-feeding species for non-silent mutations that might confer tolerance. They identified two, non-synonymous mutations in the RNAP II of these species. However, the two mutations do not occur in the funnel loop of RNAP II (α-amanitin binding site). These two mutations are also present in *Drosophila* species that are susceptible to α-amanitin. Furthermore, Stump et al. (2011) assessed the impact of type I and II detoxification enzymes on tolerance in these species. Using chemical inhibitors, they inhibited either Cytochrome P450s (P450s; type I detoxification enzymes) or Glutathione-S-Transferases (GSTs; type II detoxification enzymes) and measured survival on a diet containing the toxin. While inhibition of GSTs did not cause a loss of tolerance, inhibiting P450s resulted in a loss of tolerance in four of the eight species surveyed. Thus, their findings suggest that P450s play an important role in detoxification in some but not all mushroom-feeding species, implying that there are multiple mechanisms for tolerance.

While the works of Jaenike et al. (1983) and Stump et al. (2011) demonstrated that the mushroom-feeding species are tolerant of the lethal toxin α-amanitin at concentrations far above those observed in non-mushroom-feeding species, the level of tolerance found in these flies is not absolute. The average concentration of α-amanitin in *Amanita bisporigera*,

Table 2 Summary of survival and toxin tolerance in mushroom-feeding *Drosophila* under different dietary conditions.

Group	Species	Host sites	α-amanitin concentrations (µg/mL) [a]					Ibotenic acid concentrations (µg/mL)[b]			
			5	10	50	50[c]	50[d]	62.5	125	250	500
quinaria	*brachynephros*	M	–	–	N	–	–	N	N	N	N
	phalerata	M	–	–	N	L	SD	–	–	–	–
	falleni	M	–	–	SD	N	–	–	–	–	–
	angularis	M	–	–	N	N	N	N	N	N	N
	deflecta	V	–	–	D	–	–	–	–	–	–
	recens	M	N	N	N	L	–	–	–	–	–
	quinaria	V	–	–	D	–	–	–	–	–	–
	subquinaria	M	–	–	N	–	–	–	–	–	–
	nigromaculata	M, V, F	–	–	N	N	N	–	–	–	–
	guttifera	M	–	–	SD	N	N	–	–	–	–
tripunctata	*tripunctata*	M, F	N	N	SD,N[e]	–	–	–	–	–	–
cardini	*cardini*	M, F	–	–	N	SD	SI	–	–	–	–
	acutilabella	M, F	–	–	N	L	N	–	–	–	–
testacea	*putrida*	M	N	N	SI	–	–	–	–	–	–
bizonata	*bizonata*	M	–	–	–	–	–	–	–	–	N
immigrans	*immigrans*	F, M	N	SD	D	–	–	–	SD	SD	D
	albomicans	F	–	–	D	–	–	–	–	–	–
calloptera	*ornatipennis*	F	–	–	D	–	–	–	–	–	–
funebris	*funebris*	F, M	–	–	D	–	–	–	–	–	–
pseudoobscura	*pseudoobscura*	F	SD	D	D	–	–	–	–	–	–

M = Mushrooms; V = Vegetation; *F* = Rotting fruits. *N* = No significant change in survival; D = Died; SI = Significant increase in survival; SD = Significant decrease in survival; L = Loss of tolerance.

[a] Concentrations based on the works of Jaenike et al. (1983) and Stump et al. (2011)

[b] Concentrations based on the work of Tuno et al. (2007)

[c] Inhibitors of Phase I detoxification genes included in diet

[d] Inhibitors of Phase II detoxification genes included in diet

[e] Species included in multiple studies, where findings differed between studies. Results reported as Jaenike et al. (1983), Stump et al. (2011).

A. phalloides, and *A. virosa* is 250 µg/g, but can range as high as 1 mg/g (Tyler et al., 1966; Faulstich and Cochet-Meilhac, 1976; Yocum and Simons 1977; Beutler and Der Marderosian, 1981). When Jaenike (1985b) reared three mushroom-feeding species from the *quinaria* species group (*D. falleni*, *D. recens*, and *D. phalerata*) on diets containing a range of α-amanitin concentrations from 0 µg/mL to 1 mg/mL, he found that at a concentration of 750 µg/mL and higher, adults developed for only two of the species (*D. recens* and *D. phalerata*). In *D. falleni*, the larvae did not develop beyond the second instar. While adults of two of the mushroom-feeding species developed at the extreme concentrations, negative physiological impacts were observed in both species. *D. phalerata* experienced a significant decrease in survival, and the adults that did emerge were smaller than those that developed on a diet with a lower α-amanitin concentration. In *D. recens*, survival was not significantly impacted, but the development time increased (~3 days on average), and some adults emerged with malformed or absent eyes. Furthermore, non-mushroom-feeding *quinaria* group species that are closely related to amatoxin-tolerant species have rapidly lost tolerance to α-amanitin (Spicer and Jaenike, 1996). These findings along with the earlier work of Jaenike et al. (1983) suggest that toxin tolerance is a costly adaptation.

Evolution of toxin tolerance

To understand the number of evolutionary events that gave rise to cyclopeptide tolerance, the occurrence of this physiological adaptation must be examined within the phylogenetic context of the *immigrans-tripunctata* radiation. Several studies (Hatadani et al., 2009; Morales-Hojas and Vieira, 2012; Izumitani et al., 2016) examined the evolutionary relationships of the nine species groups within in the *immigrans-tripunctata* radiation, using data sets based on different combinations of mitochondrial and/or nuclear markers. While each of these analyses recovered the monophyly of the *immigrans-tripunctata* radiation, the relationships that they found both among and within the species groups were incongruent. For example, the phylogenies of Hatadani et al. (2009) and Izumitani et al. (2016) each contained clades composed of species from the *tripunctata*, *cardini*, and *guarani* groups, but in the phylogeny of Morales-Hojas and Vieira (2012), the species representing the *cardini* and *guarani* groups occurred in a clade that also included species from the *testacea* group, which was more closely related to the *quinaria* group. In each of these analyses, the mushroom-feeding species did not form a monophyletic cluster. Also, non-mushroom-feeding species separated the species that are tolerant of mushroom toxins. Thus, it is likely that amatoxin tolerance evolved multiple times within the *immigrans-tripunctata* radiation.

Another analysis examined the evolutionary relationships within the *quinaria* group and also formulated a hypothesis of the evolution of toxin tolerance in the group (Spicer and Jaenike, 1996). They recovered a strongly supported topology for the *quinaria* group based on three mitochondrial genes, which suggested that toxin tolerance evolved once and was lost multiple times. However, mitochondrial genes can be misleading due to indirect selection, hybridization, and endosymbiotic bacteria (Hurst and Jiggins, 2005; Galtier et al., 2009). Within the *quinaria* group, incomplete lineage sorting is likely due to high rates of speciation (Spicer and Jaenike, 1996; Perlman et al., 2003) and large, effective population sizes (Dyer and Jaenike, 2005; Dyer et al., 2007; Dyer et al., 2011; Dyer et al., 2013). Furthermore, many species within the group can hybridize (Jaenike et al., 2006; Dyer et al., 2011; Bray et al., 2014; Humphreys et al., 2016) and are also infected with *Wolbachia* (a maternally-inherited endosymbiont that produces cytoplasmic incompatibility) (Werren and Jaenike, 1995; Shoemaker et al., 1999; Jaenike et al., 2006; Dyer et al., 2011). Thus, it is possible that evolutionary relationships within the *quinaria* group and our understanding of toxin tolerance within it would likely be distinctly different in a phylogeny constructed using different markers.

Amanitin resistance in *Drosophila melanogaster*

Beyond the mushroom-feeding *Drosophila* species, the first report of α-amanitin resistance in a eukaryotic organism was identified by Greenleaf et al. (1979) in a laboratory-generated ethyl methanesulfonate mutant of *Drosophila melanogaster* (*i.e.*, Ama-C4 mutant). The Ama-C4 mutant had an altered RNAP II, which rendered the mutant ~250 times less susceptible to the lethal effects of α-amanitin (Greenleaf et al., 1979). In the early 1980s, Canadian researchers tested wild-caught *D. melanogaster* stocks to identify naturally occurring α-amanitin-resistant strains. Out of 32 different stocks tested, three showed unusually high resistance to the toxin. These resistant fly stocks were originally collected in the 1960s in Taiwan (Ama-KTT), India (Ama-MI), and Malaysia (Ama-KLM). Because amatoxins are solely produced by mushrooms (Hallen et al., 2002; Hallen et al., 2007; Walton et al., 2010), it is quite astonishing that any strains of this mushroom-avoiding species, which is frugivorous and does not exhibit any mushroom-feeding behavior (Werner, 2017), have evolved resistance to the potent amatoxin, α-amanitin. Their α-amanitin resistance was approximately two orders of magnitude higher than that of the susceptible reference strain Oregon-R (*i.e.*, the resistant strains tolerated up to ~10 µg of α-amanitin per gram of food, as compared to ~0.1 µg/g in susceptible strains). The resistance phenotype was further mapped to two dominantly acting loci on the left and the right arms of chromosome 3, respectively (Phillips et al., 1982). Two decades later, a Californian *D. melanogaster* stock (III-25) was isolated, which also showed increased resistance to α-amanitin (Begun and Whitley, 2000). Subsequent mapping identified the seemingly same two loci on chromosome 3 that were observed in the three Asian strains analyzed in 1982 (Begun and Whitley, 2000). Interestingly, both loci also acted in a dominant fashion. The Californian study went one step further though and suggested two responsible candidate genes conferring the peculiar α-amanitin resistance phenotype: *Multidrug resistance 65* (*Mdr65*) (left arm) and *Protein kinase C98E* (*Pkc98E*) (right arm of chromosome 3). This model seemed appropriate because PKC98E can phosphorylate MDR proteins (Chambers et al., 1990), and MDR proteins might lead to the excretion of α-amanitin from the cells.

Half a century after the resistant Asian strains Ama-KTT, Ama-KLM, and Ama-MI were collected, Mitchell et al. (2015) re-characterized the three strains to test if they were still resistant to α-amanitin despite being maintained on non-toxic food in the stock center for nearly 50 years. This study showed that the three strains largely maintained their resistance over ~1200 generations, except in one line (Ama-MI), where the resistance seemed to have dropped to half of the original value, possibly due to genetic drift. However, the establishment of isochromosome lines for the second and third chromosome re-established the resistance in the Ama-MI/M/2 line close to the original values that were measured in the early 1980s by Phillips et al. (1982). The re-characterization study of Mitchell et al. (2015) looked further into the physiological parameters of the larvae and adult flies, which were grown on a variety of α-amanitin concentrations in the food. All three Asian strains displayed several stress-like responses to all sub-lethal α-amanitin concentrations in a nearly linear manner: the larva-to-adult development time increased, while pre-adult viability, adult body size, and adult longevity decreased with increasing toxin concentrations. Unexpectedly, females hatching from the second highest tolerable toxin concentration displayed a ~2-fold increase in fecundity, which was true for all three Asian strains.

Focusing on an isochromosome line derived from the Taiwanese Ama-KTT stock (*i.e.*, Ama-KTT/M/2), Mitchell et al. (2015) performed a whole-genome microarray expression analysis to identify genes involved in the α-amanitin resistance phenotype. Neither *Mdr65* nor *Pkc98E* were among the upregulated candidate genes, as was expected from previous mapping data (Begun and Whitley, 2000). Instead, they identified four possible candidate mechanisms for α-amanitin resistance: 1) blockage by cuticular proteins, 2) detoxification by phase I (P450s) and phase II detoxification enzymes (GSTs and UDP glucuronosyl transferases), 3) cytoplasmic sequestration in lipid particles, and 4) cleavage by peptidases (Fig. 1). Remarkably, three Cytochrome P450

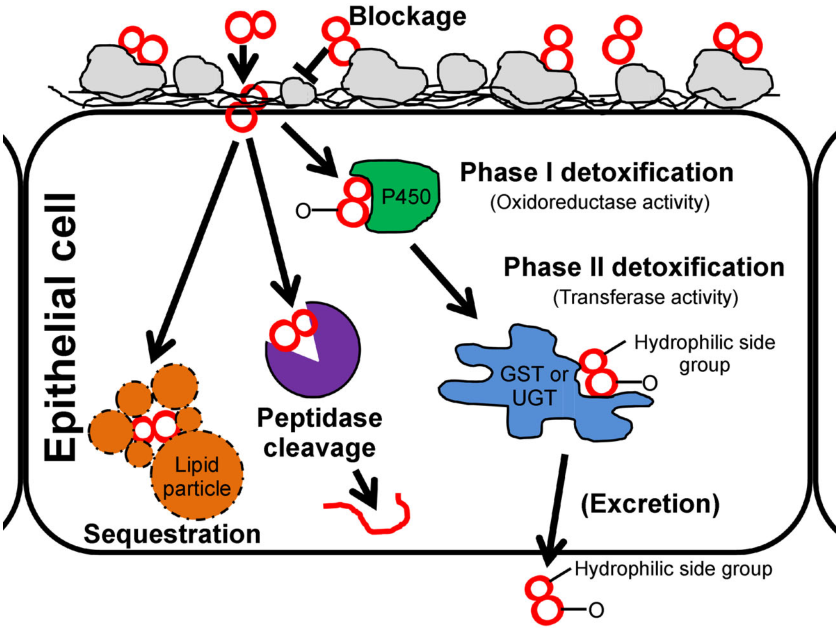

Figure 1 Four mechanisms that are hypothesized to contribute to the resistance to α-amanitin in the *D. melanogaster* Ama-KTT strain. α-Amanitin is shown as a red 8. Cuticular proteins block some of the α-amanitin from entering the cells (blockage). α-Amanitin that entered the cytoplasm is either sequestered in lipid particles, cleaved by peptidases, or detoxified by phase I and II detoxification enzymes, possibly followed by excretion. Figure adapted from Mitchell change to et al. (2014).

genes were at least 200-fold constitutively upregulated in the resistant Ama-KTT/M/2 larvae: *Cyp6a2*, *Cyp12d1-d*, and *Cyp12d1-p*. All three genes have been shown to respond to, or detoxify, various chemically unrelated substances, including the pesticides DDT, imidacloprid, dicyclanil, atrazine, and the drug phenobarbital (Brun et al., 1996; Amichot et al., 2004; Festucci-Buselli et al., 2005; Le Goff et al., 2006; Daborn et al., 2007; Kalajdzic et al., 2012). The emerging picture from this experiment suggests that α-amanitin resistance in the Asian *D. melanogaster* flies may have evolved as cross-resistance to pesticides instead of a direct response to mushroom toxin exposure.

In the most recent study investigating α-amanitin resistance in *D. melanogaster*, Mitchell et al. (2017) performed a GWAS analysis to test if cases of α-amanitin resistance were rare accidents, or if resistant strains were more common in nature than previously anticipated. They tested ~180 of the *Drosophila* Genetic Reference Panel (DGRP) strains, which originated in Raleigh, North Carolina (Mackay et al., 2012; Huang et al., 2014). The results were quite surprising. There was continuous variation of α-amanitin resistance among all tested lines, ranging from highly susceptible to as resistant as the three Asian strains were shown to be in previous studies (Phillips et al., 1982; Mitchell et al., 2014; Mitchell et al., 2015). A further surprise was that the candidate genes were not associated with the previously identified mechanisms in the microarray study (Mitchell et al., 2015) in the Taiwanese *D. melanogaster* strain Ama-KTT/M/2. Instead, the researchers identified three new candidate genes, *Megalin* (*mgl*), *Tequila* (*teq*), and *Widerborst* (*wdb*) that may interact with the Target of Rapamycin (TOR) pathway. TOR is a critical repressor of autophagy (Kim and Guan, 2015) and Megalin-mediated endocytosis (Gleixner et al., 2014). TOR inactivation may play a role in the elimination of cytoplasmic α-amanitin by de-repression of the autophagic process. As a result, the toxin would become entrapped in a cytoplasmic phagophore, and the autophagosome would then undergo lysosomal fusion, followed by degradation of α-amanitin (Fig. 2).

As mentioned at the beginning of this section, *D. melanogaster* has no known history of mushroom-feeding in the wild, and their resistance to α-amanitin would not be sufficient to safely consume most mushrooms that contain this toxin. α-Amanitin resistance, perhaps a by-product of pesticide resistance, may, however, represent a pre-adaptation for a possible mushroom niche invasion in the future. The doubled fecundity of females observed at the second highest sub-lethal α-amanitin concentrations in laboratory tests could give this species a reproductive advantage at the time of toxic mushroom niche invasion (Mitchell et al., 2015).

Concluding remarks

With the exception of one laboratory-generated mutant strain of *Drosophila melanogaster* (Greenleaf et al., 1979), all tested wild-caught fruit flies of this and the mushroom-feeding *Drosophila* species have an RNAP II that is very susceptible to the effects of α-amanitin (Jaenike et al., 1983; Stump et al., 2011). Thus, *Drosophila* species that are either

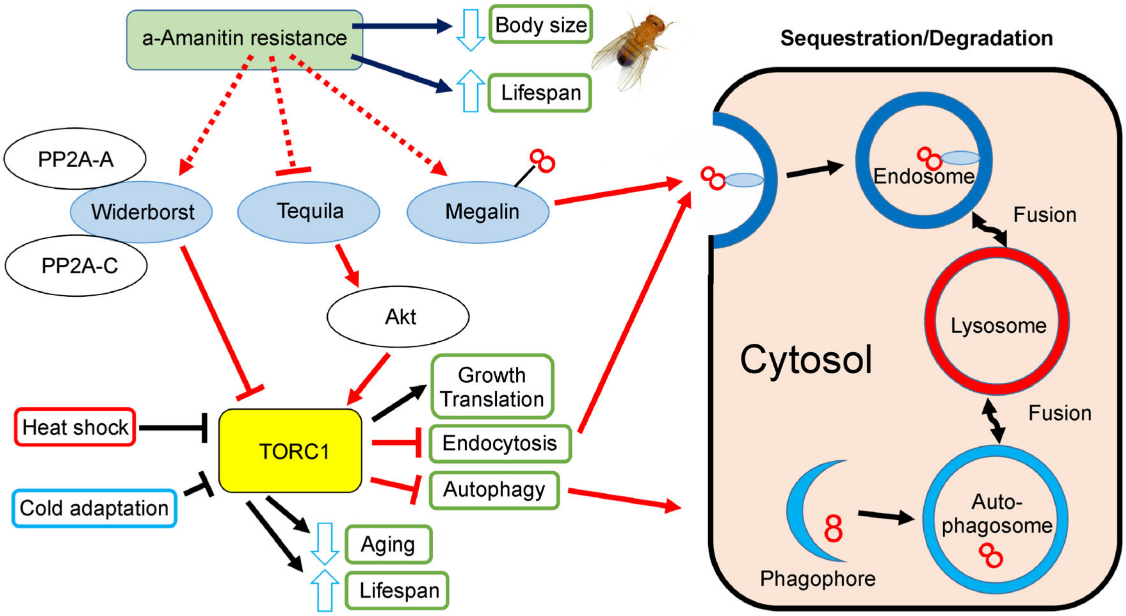

Figure 2 The TOR pathway may mediate α-amanitin resistance in the DGRP lines. α-Amanitin is shown as a red 8. The proteins Widerborst and Tequila are upstream regulators of TOR, influencing autophagy. TOR is a critical repressor of autophagy and Megalin-mediated endocytosis. Both the endocytic and autophagic catabolic processes end with the degradation and recycling of macromolecules in lysosomes. Megalin protein is hypothesized to sequester α-amanitin to the endosome, followed by the degradation of α-amanitin. Figure adapted from Mitchell change to et al. (2017).

tolerant of or resistant to mushroom toxins must employ detoxification mechanisms that prevent α-amanitin from entering the nuclei of the cells, where RNAP II is active. In some but not all species, type I detoxification enzymes appear to play a critical role in toxin tolerance. However, it is important to acknowledge that poisonous mushrooms contain mixtures of several toxins, including multiple types of amatoxins and phallotoxins (Enjalbert et al., 1993; Vetter, 1998; Hallen et al., 2007; Kaya et al., 2013; Kaya et al., 2015), muscimol, ibotenic acid, and other peptide toxins (Chilton and Ott, 1976) specifically produced by mushrooms. Because different toxins target a variety of tissues, organs, and biological processes, fruit flies must be multi-toxin resistant to all poisonous substances that are present in a mushroom. Along these lines, Tuno et al. (2007) demonstrated that three species of mushroom-feeding fly are tolerant of both muscimol and ibotenic acid toxins found in *Amanita muscaria* and other hallucinogenic mushrooms, and these same species are also tolerant of α-amanitin. Taking into consideration what is known of the evolutionary relationships of the *immigrans-tripunctata* radiation, the current state of knowledge suggests that amatoxin tolerance has evolved multiple times within the radiation, and there is more than one mechanism that can confer tolerance. Furthermore, if toxin tolerance evolved concurrently with mushroom-feeding in the ancestor of the *quinaria* group, then this tolerance might represent a novel adaptation that initiated the group's adaptive radiation.

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Compliance with ethics guidelines

Authors declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by the authors.

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