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# No evidence for behavioural adaptations to nematode parasitism by the fly *Drosophila putrida*

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## Abstract

Behavioural adaptations of hosts to their parasites form an important component of the evolutionary dynamics of host–parasite interactions. As mushroom-feeding *Drosophila* can tolerate deadly mycotoxins, but their *Howardula* nematode parasites cannot, we asked how consuming the potent mycotoxin α-amanitin has affected this host–parasite interaction. We used the fly *D. putrida* and its parasite *H. aoronymphium*, which is both highly virulent and at high prevalence in some populations, and investigated whether adult flies utilize food with toxin to prevent infection in the next generation or consume the toxin to reduce the virulence of an already established infection. First, we found that uninfected females did not prefer to eat or lay their eggs on toxic food, indicating that selection has not acted on the flies to alter their behaviour towards α-amanitin to prevent their offspring from becoming infected by *Howardula*. However, we cannot rule out that flies use an alternate cue that is associated with toxin presence in the wild. Second, we found that infected females did not prefer to eat food with α-amanitin and that consuming α-amanitin did not cure or reduce the virulence of the parasite in adults that were already infected. In sum, our results indicate there are no direct effects of eating α-amanitin on this host–parasite interaction, and we suggest that toxin tolerance is more likely maintained by selection due to competition for resources than as a mechanism to avoid parasite infection or to reduce the virulence of infection.

## Introduction

The interactions between hosts and their parasites are a powerful driver of evolutionary change. From the side of the host, immune responses are the best-studied host adaptations to combat parasites. However, even though they are less studied, changes in host behaviour that reduce the infection rate or reduce virulence of an infection may be as important as immune responses (reviewed in Moore, 2002; Parker *et al.*, 2011). For example, pre-infection strategies such as parasite avoidance can prevent contact with parasites and can be used by an organism to protect itself or its offspring from infection (e.g. Lefèvre *et al.*, 2010, 2012; Kacsoh *et al.*, 2013). Post-infection strategies such as self-medication deal more directly with parasite control and aim to reduce parasite virulence (e.g. Huffman, 2001; Bernays & Singer, 2005; Singer *et al.*, 2009; Anagnostou *et al.*, 2010; Milan *et al.*, 2012). In this study, we investigate the occurrence of both pre- and post-infection behavioural adaptations in mushroom-feeding *Drosophila*, specifically asking whether the interaction with a nematode parasite is affected by the presence of toxic compounds in the host mushrooms.

Adults of many species of *Drosophila* are attracted to mushrooms to eat and mate. Females lay their eggs on the mushrooms and the larvae develop in the decaying fruiting body. Mushroom-feeding flies are generalists on fleshy basidiomycetes and have a unique ecological adaptation: they can consume mushrooms that contain significant amounts of mycotoxins (Jaenike *et al.*, 1983; Tuno *et al.*, 2007). One of the most potent of the mushroom toxins is α-amanitin, a small bicyclic octapeptide that inhibits RNA polymerase II and is lethal to most

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eukaryotes even in low doses. Their mechanism of tolerance is unknown, although Jaenike *et al.* (1983) showed that it is not a change in the RNA polymerase II molecule itself, and recent data from Stump *et al.* (2011) suggest that cytochrome P450s may be involved.

As toxic mushrooms comprise only a small proportion of the possible mushrooms for these flies to use, it is perhaps surprising that the flies have developed a tolerance for them. Importantly, it appears that this tolerance is costly. First, *Drosophila* species that have switched hosts from mushrooms to other foods have lost their ability to tolerate α-amanitin (Spicer & Jaenike, 1996; Stump *et al.*, 2011). Second, although developing larvae of tolerant species can handle high concentrations of the toxin compared with nontolerant species, at the upper limit of what is found in naturally occurring mushrooms, even tolerant flies fail to develop, have a longer development time or have developmental defects, including a smaller adult size or missing eyes (Jaenike *et al.*, 1983; Jaenike, 1985; Spicer & Jaenike, 1996). Even with these costs, this tolerance may allow flies to escape competition or avoid predation, protect the flies from infection by eukaryotic parasites, or utilize more of what may otherwise be an ephemeral and patchy food resource.

Many species of mushroom-feeding *Drosophila* are infected by parasitic nematodes in the genus *Howardula* (Jaenike, 1992; Jaenike & Perlman, 2002; Perlman & Jaenike, 2003a). Mated female nematodes infect fly larvae as they eat through a mushroom and establish in the fly's reproductive tract as the host fly matures. The nematodes often render an adult infected female fly completely infertile: instead of laying eggs, the infected female fly will visit mushrooms and disperse juvenile nematodes (Welch, 1959). These nematodes then mature and mate in the mushroom and infect new fly larvae to begin the life cycle again. In a previous study, flies that emerged from wild-collected toxic mushrooms were almost never parasitized by *Howardula* nematodes (infection rate 0.2%), whereas flies that emerged from nontoxic mushrooms had a 65-fold higher rate of parasitism (infection rate 13%), implying that the nematodes cannot survive on toxic mushrooms (Jaenike, 1985). For this reason, it has been suggested that toxic mushrooms may provide a safe haven against virulent eukaryotic parasites such as *Howardula* and thus contribute to the origin or maintenance of toxin tolerance by the flies (Jaenike, 1985).

Here, we focus on the mushroom-feeding fly *D. putrida* and its nematode parasite *H. aoronymphium*. *D. putrida* is a member of the testacea species group and is common in the forests of Eastern and Southern North America. It has been well studied for both its α-amanitin tolerance and interactions with the generalist nematode parasite *Howardula aoronymphium* (Allantonematidae: Tylenchida) (Jaenike *et al.*, 1983; Perlman & Jaenike, 2003b). *Howardula aoronymphium* occurs in northern North America, where it parasitizes about 10–30% of wild *D. putrida*, and female flies are nearly always rendered sterile when infected (Jaenike *et al.*, 1983; Montague & Jaenike, 1985; Jaenike, 1992; Perlman & Jaenike, 2003b; Perlman *et al.*, 2003). *Howardula aoronymphium* does not occur in the southern part of North America, presumably because the parasite is not adapted to the warmer summer temperatures (Jaenike, 1995). In the north where this parasite is both common and virulent, one might expect strong selection for pre- and/or post-infection strategies to reduce these costs. In *D. neotestacea*, another member of the testacea group found in North America that is also heavily parasitized by *H. aoronymphium*, a vertically transmitted *Spiroplasma* bacteria that decreases nematode virulence has recently spread through populations (Jaenike *et al.*, 2010). *Drosophila putrida* is not infected with *Spiroplasma*, and it is also not infected with *Wolbachia* (Jaenike *et al.*, 2010; *Drosophila* Endosymbiont Database http://flyendo.arl.arizona.edu/; K. Dyer, unpublished).

In this article, we explore whether mushroom toxins affect the host–parasite interaction between *D. putrida* and *H. aoronymphium*. First, we investigate whether uninfected flies prefer to utilize food that contains α-amanitin and whether consuming α-amanitin harms uninfected adult flies. We also compare *D. putrida* from Rochester, NY, within the geographical range of *Howardula*, with *D. putrida* from Athens, GA, outside the range of *Howardula* (Jaenike, 1995), to ask whether there is any evidence for local adaptation to using food that contains α-amanitin. Toxic mushrooms occur in both locations. Second, we ask whether *D. putrida* exhibit any post-infection behavioural adaptations that utilize the toxin. We investigate whether adult *D. putrida* prefer α-amanitin more when they are infected by *H. aoronymphium*, and whether consuming α-amanitin reduces the virulence of *H. aoronymphium* in adult infected flies. Through this study, we hope to better understand the evolution of α-amanitin tolerance in *D. putrida* and other mushroom-feeding *Drosophila* and how interactions with eukaryotic parasites have shaped this unique ecological adaptation.

## Materials and methods

### Fly and nematode strains

We used three stocks of *Drosophila putrida* in these experiments, including two from Rochester, NY, and one from Athens, GA. First, we created a genetically diverse stock from each location. We mixed together five isofemale lines collected in Rochester, NY, in 2007, and five isofemale lines collected in Athens, GA, in 2008, and allowed these stocks to interbreed for three generations before being used in any experiments. We will refer to these as the Rochester Mixed and Athens Mixed stocks. We also used a third stock of *D. putrida*,

which was also collected in 2007 in Rochester, NY, at the same time as the lines used in the Rochester Mixed stock, and was obtained from John Jaenike. This was the strain infected with *H. aoronymphium* nematodes when we received them, and we refer to this line as the Rochester Infected stock. The *H. aoronymphium* nematodes used in this study were collected in 2007 from Rochester, NY, and maintained by John Jaenike in this stock until we received them in 2011. Fly cultures were maintained on instant *Drosophila* medium (Carolina Biological, Burlington, NC, USA) supplemented with commercial mushroom (*Agaricus bisporus*), at 20 °C on a 14:10 light cycle and with 60% relative humidity.

To infect flies to use in experiments, we ground 7–9-days-old adult flies from the Rochester Infected stock with a mortar and pestle in Ringer's solution (Sullivan *et al.*, 2000). We then placed approximately 250 suspended juvenile nematodes onto a 1 $cm^2$ piece of *Agaricus bisporus* mushroom. After 24 h, we transferred the mushroom to a vial with instant *Drosophila* medium and added 20–40 adult flies from the line to be infected. The offspring from these vials—some of which we expect to be infected with nematodes—were used in the experiments below. Infected flies do not have a visible phenotype that differentiates them from uninfected flies, so to determine whether a fly was infected with nematodes, it was dissected in Ringer's solution.

### Feeding assays

To visualize whether a fly consumed food and to decipher which food it chose, we used an agar–sugar medium that contained a red or blue food dye, the colour of which shows through the abdomen of the fly. The final concentration of the blue dye was 21.5 $\mu g\ mL^{-1}$ erioglaucine (Sigma 861146), and the red dye was 45 $\mu g\ mL^{-1}$ sulforhodamine-$\beta$ (Sigma S9012). In most experiments, we used a final concentration of 100 $\mu g\ mL^{-1}$ of α-amanitin (Sigma A2263). This concentration amounts to 1.44 mg $g^{-1}$ dry matter of α-amanitin, within the range of the average content of *Amanita phalloides, A. bisporigera* and *A. virosa* mushrooms, which have means of 1.1–2.6 mg $g^{-1}$ dry weight (Jaenike, 1985).

Some experiments utilized feeding behaviour choice assays, which used plates that contained both red- and blue-dyed agar food. Each choice plate consisted of a 2 well by 2 well square cut from a 96-well PCR plate, with each well filled halfway with hot glue. The remainder of each well was filled with 100 $\mu L$ of the dyed agar food described above. Two wells of each plate contained red agar food and were placed diagonally from each other, and the other two wells contained blue agar food. Some plates had α-amanitin in one colour of the dyed agar food, at a final concentration of 100 $\mu g\ mL^{-1}$. Each choice plate was placed in an empty standard *Drosophila* vial, where the preference assay took place. Before being placed in a preference assay, flies were starved overnight in empty vials that contained moistened filter paper. Preference assays were conducted for 3 h, after which the flies were immediately frozen. Later, the abdominal colour of each fly was scored (red, blue or purple), and when relevant, the fly was dissected to assay for nematode infection.

### Infection avoidance behaviours

We tested for differences in the feeding and oviposition behaviour of flies from two populations, Rochester and Athens, that differ in the presence of *H. aoronymphium*. We used the choice assay described above to assay feeding preference for toxin. We starved 5–9-day-old uninfected nonvirgin male and female flies from the Rochester Mixed and Athens Mixed stocks overnight and then placed approximately half of the flies on choice plates with toxin in red food and the other half on choice plates without toxin in either colour. After 3 h, the flies were frozen, and the colour of the abdomen of each fly was recorded. Each replicate included one assay vial per treatment (sex/population/toxin, for total of eight vials per replicate), and we replicated this design seven times. Each assay vial contained an average of 27 flies (SE = 2.8), and each treatment included an average of 188 flies (SE = 14) summed over the seven replicates, for a total of 1500 flies in the experiment. A fly was scored as 1, 0 or 0.5 if it ate red, blue or both colours, respectively, and the overall preference index (PI) was calculated as the sum of these values/total number of flies that ate. A PI of one indicates that all flies ate only red food, a PI of 0 indicates that none ate red food and a PI of 0.5 indicates that the flies did not discriminate between food types. We then asked whether the preference differed between the assays with and without toxin in the red food. We analysed the food preference of females and males separately using an ordinal logistic regression by general linear model (GLM), with the population and toxin exposure nested within population as fixed effect variables, and with the replicate as a random effect variable. We used PROC GLIMMIX in SAS v9.3 (SAS Institutes, Cary, NC, USA) with a multinomial error distribution and cumulative logit link function. Data were also analysed excluding the flies that ate both colours, and results were consistent.

We next compared the oviposition preference for toxin of females from Rochester and Athens. We made a mushroom–agar food by boiling a blended mix of 100 mL water, 50 g fresh store bought *Agaricus bisporus* mushrooms, 5 g agar, 2.5 g sucrose, 2.5 g Brewer's yeast and 6 mL of a 10% tegosept solution. We combined 4 mL of this with 4 mL of 200 $\mu g\ mL^{-1}$ α-amanitin (for toxin food) or 4 mL water (for nontoxin food) and allowed it to cool in 1-dram glass vials. This

resulted in a final concentration of 1.29 mg $g^{-1}$ dry weight of α-amanitin, which is within the range found in toxic *Amanita* mushrooms (Jaenike, 1985). We put four slices of each type of food in alternating order in a petri dish with at least 40 nonvirgin female flies from either the Rochester or Athens Mixed stocks. After 48–72 h, we removed the flies and counted how many eggs had been laid on each type of food. We used the proportion of eggs laid on food with toxin as a measure of female preference. In this way, we conducted four replicate experiments, including three for 48 h and one for 72 h; the latter we let go longer because not as many eggs were laid on either toxic or nontoxic food in this trial as in the others. Each replicate was conducted on a different day with a different batch of flies and used freshly prepared medium. We tested for a difference between the oviposition preference of Athens vs. Rochester females and for a preference for toxin of each population (i.e. vs. 50:50) using Cochran–Mantel–Haenszel tests. Because the number of eggs laid varied across experiments, to equally weight the different experiments, we multiplied each proportion by the number smallest number of eggs from any experiment ($n = 84$).

Third, we asked whether eating toxin reduces the fitness of flies that are not infected, implying there would be a cost for uninfected adults to consume the toxin. As a proxy for fitness, we tested for an effect of α-amanitin on survival of adult uninfected females. We used uninfected virgin females from both the *D. putrida* Rochester Mixed and Athens Mixed stocks and placed four flies per 1.5-mL centrifuge tube that contained 100 μL blue agar food with 100 μg $mL^{-1}$ toxin, made as described above. We limited our assay to virgin flies to avoid the confounding effects that mating has on fly survival (e.g. Fowler & Partridge, 1989). In this way, we assayed 232 flies per population, with half on food with toxin and half on food without toxin. Fly survival was recorded every 1–3 days until every fly died. To analyse the survival data, we used a Cox proportional hazard model with PROC PHREG in SAS, with population and toxin nested within population as fixed effect variables and the vial as a random effect variable. Three flies escaped during the experiment and were included as censored data.

### Post-infection behavioural adaptations

First, we asked whether *D. putrida* already infected with *H. aoronymphium* prefer to eat food that contains α-amanitin. We conducted preference assays using choice plates (as described above) with nonvirgin females from the Rochester Mixed and Rochester Infected stocks that had been reared on mushrooms containing infective juvenile *H. aoronymphium*. The infection status of each fly was unknown until after the assay when it was dissected, thus both infected and uninfected flies were assayed together in the same vials. We conducted a total of 16 assays, half of which had toxin in red, and the other half had toxin in blue. There was an average of 15 females per assay vial, for a total of 247 females assayed, of which 66 were infected. Each fly was scored as 1, 0 or 0.5 if it ate food with toxin, food without toxin or both colours, respectively, and the overall preference index (PI) within each treatment was calculated as the sum of these values/total number of flies that ate. A PI of 1 indicates that all flies ate only toxic food, a PI of 0 indicates that none ate toxic food and a PI of 0.5 indicates that the flies did not discriminate between food types. We used an ordinal logistic regression by GLM to analyse female toxin preference, with the food colour that contained toxin and infection status nested within toxic colour as fixed effect variables and with the assay vial nested within toxic colour as a random effect. We used PROC GLIMMIX in SAS with a multinomial error distribution and cumulative logit link function. We combined data from the two Rochester stocks, as there was no difference in the results. We also analysed the data excluding the flies that ate both colours, and the results were consistent.

Second, we asked whether eating α-amanitin provides any curative effect for flies infected with *H. aoronymphium*. Flies were placed three to four per vial, and each vial was a 1.5-mL centrifuge tube that contained 100 μL of blue agar food with a 22-gauge hole in the top. Half the flies were placed on food that contained α-amanitin at a final concentration of 100 μg $mL^{-1}$. After 1 week, each fly was dissected and scored for the presence and number of nematode motherworms, and if the female was infected, we noted whether she had any mature eggs in her ovaries. We included four assays in this experiment using flies that had been reared on mushrooms containing infective juveniles: (i) virgin 5–7-day-old females from the Rochester Infected stock ($n = 19$ vials and 55 flies), (ii) virgin 5–7-day-old females from the Rochester Mixed stock ($n = 31$ vials and 91 flies), (iii) nonvirgin females from the Rochester Mixed stock ($n = 20$ vials and 67 flies) and d) nonvirgin males from the Rochester Mixed stock ($n = 20$ vials and 63 flies). These different assays also allow us to detect any differences based on mating status and sex. To determine whether the infection prevalence decreased after exposure to the toxin, we used a logistic regression by GLM with assay (a–d) and toxin exposure within assay as fixed effects, and the vial nested within assay as a random effect. This was carried out in SAS using PROC GLIMMIX, with binomial error distribution and logit link function.

To test for an effect of host toxin consumption on motherworm size and thus parasite fecundity, we also took a photograph of each motherworm from the first two assays (for a total of 103 motherworms), using the same magnification for all images and calculated the

area (# pixels) of each motherworm using NIHimageJ (NIH, www.rsbweb.nih.gov/ij/). To ask whether motherworm size decreased if the host ate the toxin, we used an analysis of variance that considered the number of motherworms within a host (1, 2, $\geq 3$), toxin exposure and the interaction between the two variables. Statistical analyses of motherworm size were completed on square root transformed data that conformed to normality, using JMP version 10 (SAS Institutes).

## Results

### Infection avoidance behaviours

First, we tested whether females from Athens, where the nematode does not occur, differ in eating preference for food containing α-amanitin compared with flies from Rochester, where the nematode is found at high prevalence. For females, there is no difference in toxin preference between the two populations ($F_{1,850} = 0.88$, $P = 0.34$), but there is an effect of toxin within population ($F_{2,850} = 4.1$, $P = 0.017$). This is due to avoidance of the toxin by the Athens females ($t_{850} = -2.65$, $P = 0.008$), whereas females from Rochester did not display a difference in feeding behaviour in the presence of toxin ($t_{850} = -1.06$, $P = 0.29$) (Fig. 1). This avoidance by Athens but not Rochester females is consistent with a cost to larvae of developing on toxic substrate without a benefit of lower parasitism rates. Males did not differ in feeding behaviour when there was toxin present in the food (Fig. 1; Population: $F_{1,628} = 2.4$, $P = 0.12$; Toxin within population: $F_{2,628} = 0.33$, $P = 0.72$).

Second, we tested whether the differences we observed in feeding behaviour between females from Athens and Rochester translate to differences in oviposition behaviour. We presented mated females with a choice of food with and without α-amanitin and scored the number of eggs on each type of food. As shown in Fig. 2, there was substantial variation across experiments. Overall, neither Athens nor Rochester females showed a significant preference or avoidance for ovipositing on toxin (Athens: $\chi^2_{1\,\mathrm{MH}} = 0.63$, $P = 0.4$; Rochester: $\chi^2_{1\,\mathrm{MH}} = 0.98$, $P = 0.3$). Comparing the two populations, females from Rochester oviposited on toxic food somewhat less than females from Athens ($\chi^2_{1\,\mathrm{MH}} = 3.7$, $P = 0.052$).

Third, to test whether α-amanitin harms uninfected adult *D. putrida*, we assayed the survival of females from Rochester and Athens that were kept on food with and without the toxin. The flies from Athens lived for an average of 30 days whether or not they were on food with α-amanitin, and the Rochester flies on toxin lived for 32.4 days compared to 28.4 without toxin (Fig. 3). Using a Cox proportional hazards model, population (Athens vs. Rochester) was not a significant effect (Wald $\chi^2_{0.9} = 0.87$, $P = 0.10$), but toxin nested within population was marginally significant (Wald $\chi^2_{1.7} = 1.72$, $P = 0.053$). The effect of the toxin differs between the populations: within Athens, there was no difference in survival of flies kept on toxin (Wald $\chi^2_1 = 0.06$, $P = 0.8$), whereas among the females from Rochester, the flies that were kept on toxin lived longer than those that were not (Wald $\chi^2_1 = 5.3$, $P = 0.022$). Thus, α-amanitin did not exert a detrimental effect on uninfected adult flies, and in the Rochester population, it extended lifespan.

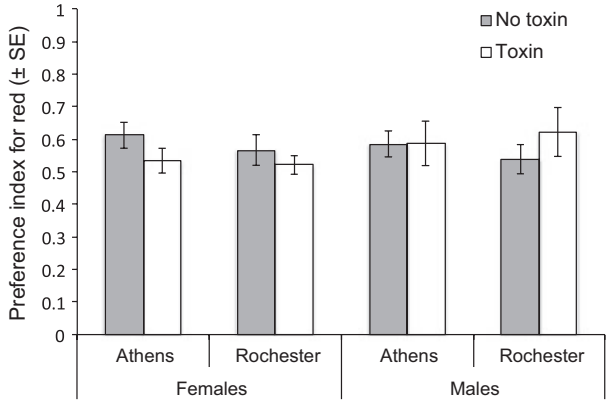


**Fig. 1** Feeding preference for α-amanitin by adult uninfected flies from Rochester, NY, and Athens, GA. White bars indicate assays where α-amanitin was added to the red food, and grey bars indicate assays where no toxin was included in either colour food. Each bar represents the mean of the seven replicate feeding preference assays, and the error bars indicate the standard error among replicates.

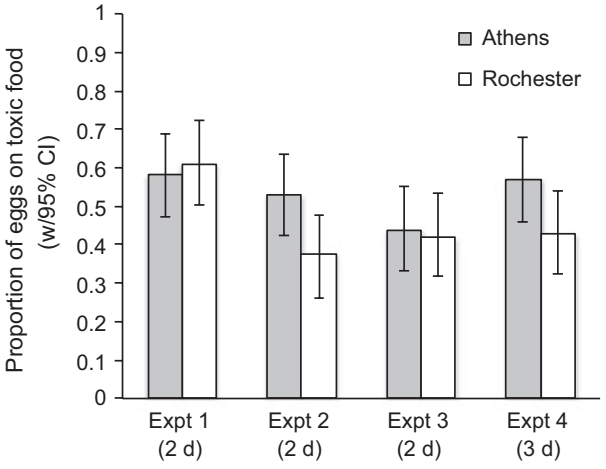


**Fig. 2** Oviposition preference of uninfected female flies towards food with α-amanitin. Results are shown for each replicate experiment. Error bars indicate the 95% confidence intervals, calculated using a binomial distribution.

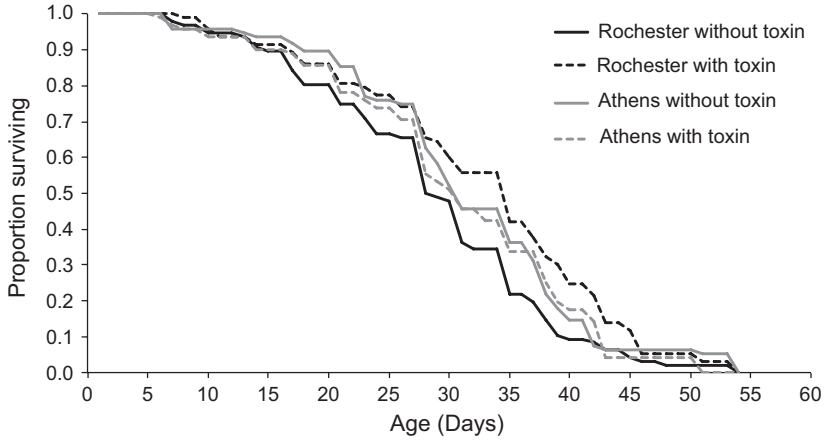


**Fig. 3** Survival of uninfected females from Athens, GA, and Rochester, NY, on food with and without α-amanitin.

### Post-infection behavioural adaptations

We used preference assays to ask whether flies that are infected with *H. aoronymphium* prefer food with α-amanitin relative to uninfected flies. As can be seen in Fig. 4, *D. putrida* generally prefers food with red colouring (toxic colour: $F_{1,14} = 18.7$, $P = 0.0007$). Across both colours, infected flies ate more food with toxin than uninfected flies, although this trend is not significant (infection nested within toxic colour: $F_{1,319} = 1.7$, $P = 0.18$). We also did not find a significant relationship between the number of motherworms per female and toxin preference ($r^2 = 0.004$, $F_{2,244} = 0.5$, $P = 0.6$).

Next, we tested whether eating toxin reduced the virulence of *H. aoronymphium* in flies that were already infected. We tested both virgin and nonvirgin females, as well as nonvirgin males, and we found that across all assays, the flies kept on food with α-amanitin were just as likely to be infected as flies kept on food without toxin (Fig. 5; assay: $F_{3,82} = 19$, $P < 0.0001$; toxin consumption within assay: $F_{4,186} = 0.3$, $P = 0.87$). Thus, we can also infer that the reproductive status and the sex of the fly did not have a significant effect on whether the toxin cured the host. The overall lower infection prevalence of the virgin Rochester Mixed flies (Fig. 5) could be due to a lower density of infective juveniles in the culturing medium or higher density of larvae in these vials. Considering only infected females, there was no difference in the number of motherworms per fly depending on whether the female was kept on toxin (No toxin: $1.79 \pm 0.15$ [mean ± SE], Toxin: $1.83 \pm 0.20$; $F_{1,89} = 0.02$, $P = 0.88$). All infected flies had live, moving juvenile nematodes in their abdomen,

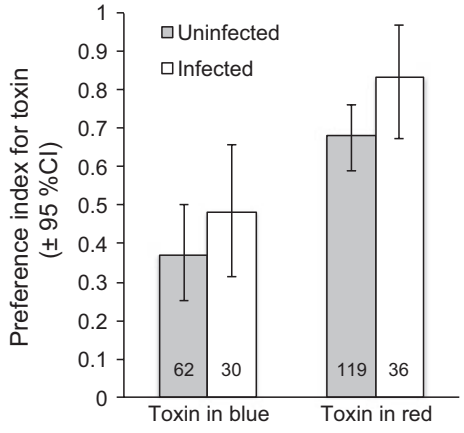


**Fig. 4** Feeding preference for α-amanitin by infected and uninfected Rochester flies, separated by the food colour the toxin was in. Data are summed across the eight assay vials, and error bars indicate the 95% confidence intervals of the preference index, calculated using a binomial distribution. The number inside each bar indicates the total number of flies in that category.

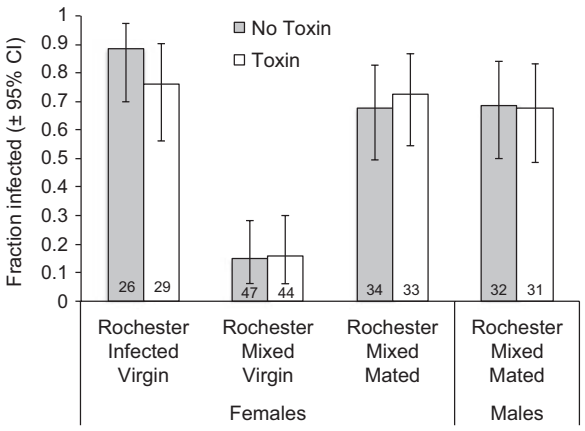


**Fig. 5** The proportion of flies infected with *Howardula* after being kept on food with or without α-amanitin for 1 week. The total number of flies assayed is shown at the base of each bar, and error bars indicate the 95% confidence intervals, calculated using a binomial distribution. Results are broken down by experimental assay; see text for details.

and no infected female had ovaries with any mature eggs.

The size of the nematode motherworm can indicate the fecundity of the parasite (Perlman & Jaenike, 2003a), thus we also tested whether keeping the infected adult females on toxin reduced the size of the motherworms. Because the size of the motherworm may depend on the number of motherworms per host, we categorized flies by how many motherworms each had (1, 2, $\geq 3$). We found no decrease in motherworm size if the host ate toxic food, although the average motherworm size in more heavily parasitized flies was smaller (Fig. 6; full model: $F_{5,97} = 2.9$, $P = 0.018$; motherworm category: $F_{2,100} = 4.7$, $P = 0.012$; toxin consumption: $F_{1,101} = 0.25$, $P = 0.61$; interaction: $F_{2,100} = 0.64$, $P = 0.53$). In summary, these results suggest that the toxin does not cure adult flies of the nematode infection, and it also does not decrease the virulence in infected flies.

## Discussion

Here, we test for both pre- and post-infection behavioural adaptations of *D. putrida* to the nematode *H. aoronymphium*, which is a parasite that is both virulent and common in the northern part of this fly's geographical range. Because the infection status of uninfected females' offspring will depend in large part on whether those offspring develop on a toxic mushroom, and flies that develop on toxic mushrooms are protected from *Howardula* parasitism (Jaenike, 1985), we predicted strong selection on uninfected females to prefer to lay their eggs on toxic mushrooms. However, we did not observe any significant preference by uninfected females to prefer food with α-amanitin. Flies from Athens where the parasite is not found showed some avoidance of consuming the toxin; however, this is not due to a cost to adults of eating toxin, as the lack of a reduced adult survival on toxic food suggests that adult flies are tolerant of the toxin like larvae.

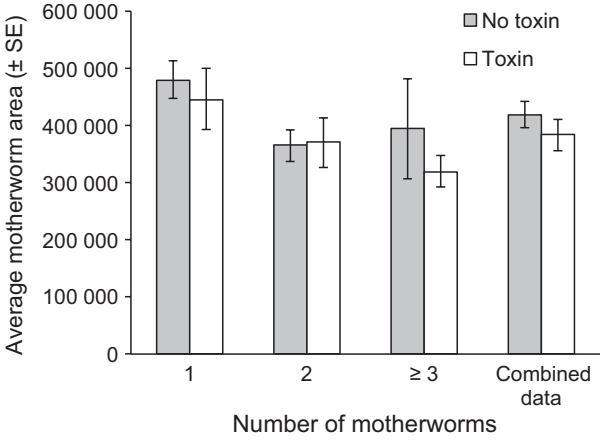


**Fig. 6** The average motherworm size after the host female was kept on food with or without α-amanitin. Because the size of the motherworm varies depending on how many parasitize a female, the data are shown for all females as well as broken down by the number of motherworms/fly. The nematode motherworm area is measured in number of pixels.

We can think of several reasons why we did not detect any preference for uninfected females to utilize toxic food. First, the flies may not use the toxin itself as a cue, but rather some other character that is correlated with toxin presence that was not included in our assays. Many different mushroom species contain α-amanitin, including species in the *Amanita*, *Galerina*, *Lepiota* and *Conocybe* genera (Arora, 1986). Poisonous mushrooms do not tend to be more colourful than edible mushrooms (Sherratt *et al.*, 2005), and the resemblance of toxic and nontoxic species is one reason that mushroom poisonings in humans occur. However, even after phylogenetic corrections, poisonous mushrooms do tend to have more distinct odours than their edible counterparts (Sherratt *et al.*, 2005). Thus, there may be olfactory or gustatory cues associated with the presence of toxins that flies cue in on; for example, *Amanita phalloides* and *A. virosa*, two mushroom species that are especially toxic, have both been noted to have a 'sickly sweet' odour (e.g. Phillips 2005). Future experiments should use fresh poisonous mushrooms to investigate this possibility.

A second possibility why we did not detect a signature of a host preference is that there may not have been enough time for it to evolve. Based on a lack of genetic variation in the nematode, Perlman & Jaenike (2003a) hypothesized that *H. aoronymphium* may have recently expanded into North America, and thus, coevolution with hosts such as *D. putrida* may be recent on an evolutionary time scale. Unfortunately, because of the absence of genetic variation, it is not possible to calculate the age of this host–parasite interaction. However, we note that *Drosophila* are parasitized by many other eukaryotic parasites in addition to *Howardula* nematodes, and one might expect that selection from other parasites that have longer associations with *D. putrida* could also cause behavioural changes towards consuming toxic mushroom hosts.

Third, host gene flow may affect the strength of selection for parasite resistance (e.g. Gandon, 2002; Morgan *et al.*, 2005; Greischar & Koskella, 2007). At least half of the range of *D. putrida* does not experience infection by *Howardula*, and there is a substantial amount of gene flow among populations of *D. putrida* in the eastern United States (Lacy, 1983; K. Dyer, unpublished). Thus, it is possible that gene flow among populations with and without the parasite may affect the strength of selection for resistance mechanisms that decrease the virulence or transmission of the nematode. When the host migrates more than the parasite, as is the expectation in this system, the theoretical

expectation is that this would favour local host adaptation, especially when the parasite is virulent (Gandon *et al.*, 1996; Lively, 1999; Gandon, 2002). However, extremely high host migration, even in the face of no gene flow among parasite populations, can homogenize populations and results in a lack of local host adaptation. Further studies that use rapidly evolving molecular markers would be useful to address the potential here for gene flow to dampen host local adaptation.

Finally, even at a high population level infection prevalence, the strength of selection to prefer toxic mushrooms may not be strong, or it may come at a significant cost. An assay of mushroom usage by genus found that 6% of collected *D. putrida* in both New York and Tennessee were off of *Amanita* mushrooms, some of which are highly toxic (Lacy, 1984). This is small but not insignificant, although it is not known what the overall relative abundance of toxic mushrooms was relative to nontoxic species. If the mechanism of tolerance is specific to mycotoxins, then the cost of tolerance when no other food sources are available may be much less than seeking out a different food source. We found no evidence for a cost of toxin tolerance in adults, and for Rochester females eating toxin even extended lifespan (Fig. 3). We note that although we do not know the source of this increased longevity, two possibilities are that the toxin may have a hormetic effect on the fly or that it may protect the adults from fungal infections, even in lab stocks such as we used here. (We are not aware of any infections in the stocks we used here other than the intended nematode infection.) However, for selection to act on adults to reduce infection in their offspring, it is the cost to larval development that is key, and this has been shown to be significant (Jaenike, 1995). Thus, the cost of reduced growth rate in toxic food may be sufficient to combat any selection pressure on the adults to prefer toxic food sources, even when infection rates are high.

The cost of infection to adult flies is significant: adult female *D. putrida* infected with *H. aoronymphium* have reduced fertility and reduced longevity, and males have reduced mating success (Perlman & Jaenike, 2003b). We find that there is no benefit for an infected adult fly to consume toxic food—the toxin does not cure or reduce the parasite virulence in infected adult flies. Regardless of whether an infected female consumes toxin, she will have few to no offspring, implying little or no selection for infected females to preferentially consume the toxin. Thus, it is not surprising that we find that infected females do not strongly prefer to eat food that contains the toxin. In addition, considering that α-amanitin does not reduce nematode virulence, our results suggest that when an infected adult fly feeds on toxic food, the toxin never reaches the nematodes in the abdomen of the fly. α-amanitin is a small molecule that would have no trouble crossing the cell membrane, and thus it is probably broken down before it can cross the digestive tract barrier into the haemolymph of the abdomen, where the nematodes reside. We attempted to expose nematodes to the toxin *in vitro*, but there was generally low survival of the nematode outside of the fly, regardless of whether the toxin was applied (results not shown).

As noted earlier, the closely related species *D. neotestacea* is infected with *Spiroplasma* that confers resistance against the effects of this same *Howardula* parasite (Jaenike *et al.*, 2010). Recent work showed that when this *Spiroplasma* is artificially transferred into *D. putrida*, it also confers resistance to *Howardula* in this novel host (Haselkorn *et al.*, 2013). It will be interesting to follow whether this endosymbiont naturally horizontally transfers between hosts. Both *D. neotestacea* and *D. putrida* are often found together at the same mushrooms, and *Spiroplasma* has been shown to be able to move between *Drosophila* hosts via mite vectors (Jaenike *et al.*, 2007). Thus, it may be a matter of time until *Spiroplasma* infects *D. neotestacea* and protects it from *Howardula*, making behavioural adaptations by the host unnecessary.

In conclusion, our results suggest that the evolution of toxin tolerance by mushroom-feeding *Drosophila* may not have been driven by parasite avoidance, at least by *Howardula* nematodes. Instead, it seems to be more likely that selection has acted on flies to be able to utilize any available mushroom food source or as a way to escape competition from other insects and larger animals. Mushrooms are not likely to be a reliable food source in all conditions, and selection to maintain the ability to utilize a variety of host mushroom species may be stronger than selection for parasite resistance. In line with this and our findings here, previous work found that even individual *D. putrida* flies were attracted to more than one species of mushroom, although none of the especially toxic mushroom species were tested (Jaenike, 1978; Jaenike & Grimaldi, 1983). Although our studies here suggest that neither pre- nor post-infection behavioural avoidance has evolved in *D. putrida* in response to parasitism by *Howardula* nematodes, tolerance to α-amanitin and other mycotoxins is likely very important to the ecology of these flies. Mycotoxins may still provide protection from other eukaryotic parasites, for example, other nematodes or fungi, and these toxic mushrooms may also provide a valuable source of food in an otherwise unpredictable environment.

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