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14. I thank G. Vergara, who performed all the electron microscopic work with great enthusiasm and dedication. Unfailing encouragement from J. Admiraal is also deeply appreciated.

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α-Amanitin Tolerance in Mycophagous *Drosophila*

Abstract. *Six species of* Drosophila *were tested for tolerance to the mushroom toxin α-amanitin, a potent inhibitor of RNA polymerase II. Three nonmycophagous species—D. melanogaster, D. immigrans, and D. pseudoobscura—showed very low survival and long development times in the presence of amanitin. Three mycophagous species—D. putrida, D. recens, and D. tripunctata—showed little or no sensitivity. Analysis in vitro indicated that this tolerance is not based on alteration of the molecular structure of RNA polymerase II.*

Some mushrooms of the genus *Amanita* contain substantial quantities of α-amanitin, a bicyclic octapeptide that is a potent inhibitor of eukaryotic RNA polymerase II, the enzyme which transcribes genes that encode messenger RNA's. Amanitin, therefore, is potentially very toxic to virtually all eukaryotes (*1, 2*). Larvae of wild-type *Drosophila melanogaster*, for example, cannot survive amanitin concentrations greater than about 5 μg/ml in the culture medium. However, amanitin-resistant strains of this species have been selected in the laboratory, and in at least one case (strain C4), the mechanism of resistance resides in an amanitin-insensitive RNA polymerase II (*3*). Although amanitin is considered to be a general toxin, a number of species of *Drosophila* breed, apparently with no ill effect, in various *Amanita* species that may contain up to 5000 μg of amanitin per gram (dry weight) of mushroom tissue, which is about 500 times the median lethal dose (LD_{50}) for wild-type *D. melanogaster* (*4*). The following questions arise: (i) Are mushroom-feeding species of *Drosophila* tolerant of α-amanitin, or do they avoid poisoning by not ingesting amanitin-containing tissues—for example, by selectively feeding on the yeasts and bacteria that grow on deliquescent mushrooms? (ii) If they are tolerant, is this tolerance a consequence of their possessing an amanitin-insensitive RNA polymerase II?

Six species of *Drosophila*, each from a different species group within the genus, were reared in the laboratory on artificial medium containing various concentrations of α-amanitin (*5*). These species included three that rarely, if ever, breed in mushrooms—*D. melanogaster*, *D. immigrans*, and *D. pseudoobscura*—and three—*D. putrida*, *D. recens*, and *D. tripunctata*—whose sole or principal breeding sites are mushrooms, including those that contain amanitin (*6*). For each species we determined, as a function of amanitin concentration in the larval medium, several components of fitness, including egg-to-adult survival, development time, and adult body size (*7*). In addition, RNA polymerase II activity was determined in vitro as a function of amanitin concentration (*8*).

None of the three nonmycophagous species survived amanitin concentrations of 50 μg/ml, with the exception of *D. melanogaster* strain C4, the resistant strain mentioned above that has an al-

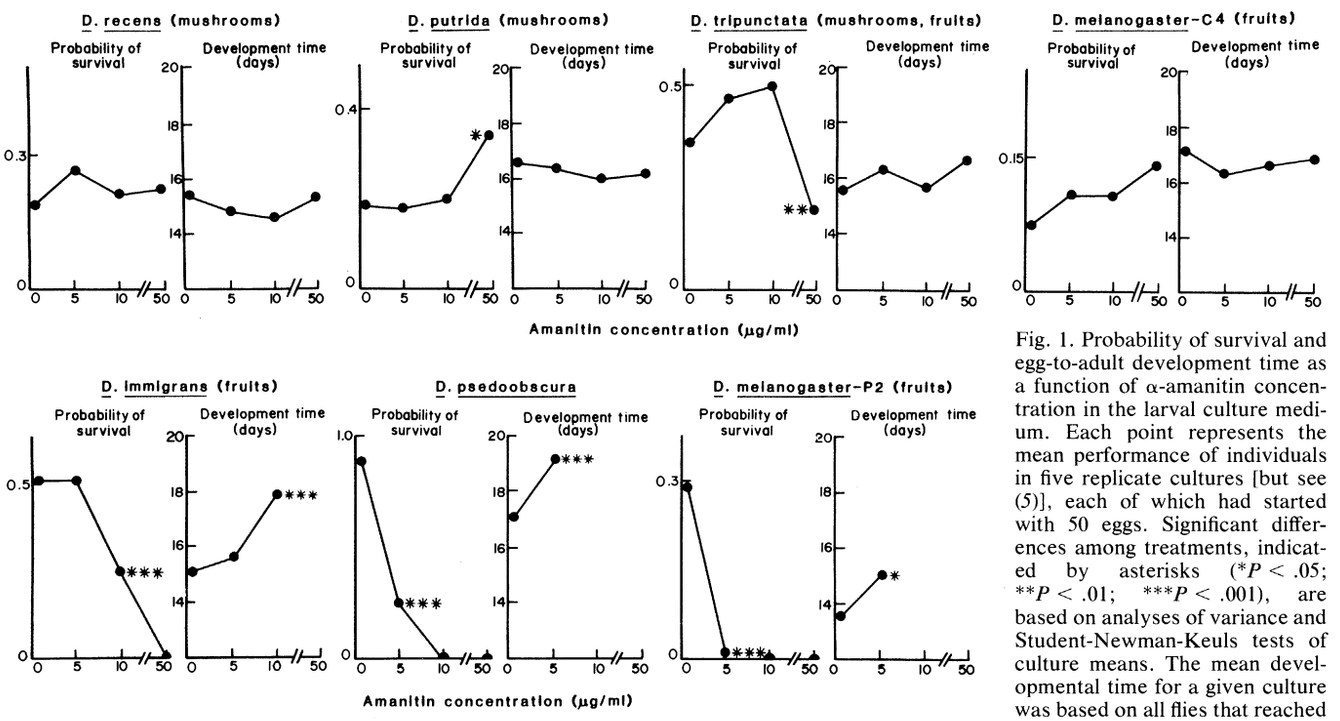

Fig. 1. Probability of survival and egg-to-adult development time as a function of α-amanitin concentration in the larval culture medium. Each point represents the mean performance of individuals in five replicate cultures [but see (*5*)], each of which had started with 50 eggs. Significant differences among treatments, indicated by asterisks (*$P < .05$; **$P < .01$; ***$P < .001$), are based on analyses of variance and Student-Newman-Keuls tests of culture means. The mean developmental time for a given culture was based on all flies that reached adulthood. Points designated by asterisks differ significantly at the level indicated from those representing lower concentrations of amanitin. The principal breeding sites of the flies are indicated in parentheses after the species name; the breeding site of *D. pseudoobscura* is unknown.

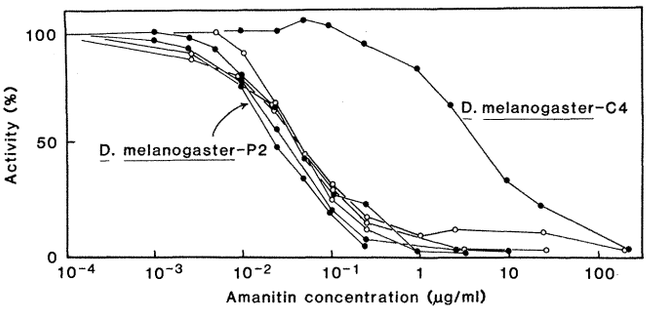

Fig. 2. Activity of RNA polymerase II in vitro as a function of α-amanitin concentration. Activities are expressed as the percent of activity with amanitin at 0 μg/ml. (●) Nonmycophagous species (*D. melanogaster*, *D. immigrans*, and *D. pseudoobscura*); (○) mycophagous species (*D. putrida*, *D. recens*, and *D. tripunctata*).

tered RNA polymerase II (Fig. 1). The mortality is due to some toxic effect of amanitin, since first-instar larvae fed actively but then generally died during molting. Of these three species, *D. immigrans* appeared to be the least sensitive, since some individuals did survive treatment with amanitin at 10 μg/ml; wild-type *D. melanogaster* (strain P2) was the most sensitive. Besides reducing larval survival, α-amanitin also significantly increased the egg-to-adult development time and decreased adult body size in these species, with the exception again of *D. melanogaster* strain C4 (*9*). Females of *D. pseudoobscura* were much more sensitive to amanitin than were males; when no amanitin was added to the medium, 56 percent of the adults produced were female, but when amanitin was added at 5 μg/ml, only 22 percent were female (*10*). Finally, *D. melanogaster* strain C4, although insensitive to the amanitin concentrations we used, was substantially less fit than the wild-type, amanitin-sensitive strain P2 when amanitin was not present in the medium. Under these conditions, the survival of C4 individuals was about one-third that of P2, and their egg-to-adult development time was about 30 percent longer. The alteration of RNA polymerase II that renders it insensitive to amanitin apparently incurs a substantial developmental cost.

In contrast to the nonmycophagous species, those that do regularly breed in mushrooms were little, if at all, affected by any of the amanitin concentrations we tested. In none of the three species was development time, body size, or sex ratio influenced by amanitin. With respect to egg-to-adult survival, *D. recens* and *D. putrida* were not affected by amanitin concentrations of 50 μg/ml or less (*11*), whereas the survival of *D. tripunctata* was significantly reduced at 50 μg/ml (*12*). The greater sensitivity of *D. tripunctata* to amanitin might be related to the fact that this fly also breeds in fruits to a large extent, whereas *D. putrida* and *D. recens* are more strictly mycophagous (*13*). These mycophagous drosophilids are, as far as we know, the first animals found to be normally tolerant of such high concentrations of α-amanitin. Since these flies belong to different species groups, it appears that amanitin tolerance evolved several times within the genus *Drosophila*. This tolerance is associated, perhaps causally, with an ecologically successful way of life, since these are among the most abundant drosophilids in the forests of eastern North America.

We do not know the physiological means by which these flies are rendered insensitive to amanitin. Unlike the amanitin-resistant strain C4 of *D. melanogaster*, which has an altered RNA polymerase II, the three mycophagous *Drosophila* species we studied have RNA polymerases that are almost as sensitive to amanitin as that of the wild-type, amanitin-sensitive strain P2 of *D. melanogaster* (Fig. 2). The same is true of the nonmycophagous species *D. immigrans* and *D. pseudoobscura*. Hence, we can probably rule out alterations in RNA polymerase II as the basis for amanitin tolerance in these flies. It may be that mutational tampering with this polymerase incurs such deleterious pleiotropic consequences that flies with an amanitin-resistant enzyme cannot survive in natural populations. That is, under natural conditions a C4-like strain of *D. melanogaster* might be much weaker and less fit than flies with a normal polymerase. In fact, in a recent survey of 32 wild-type strains of *D. melanogaster*, three were fairly resistant to amanitin, though not as resistant as the mycophagous species of the present study (*14*). And like the mycophagous species, these *D. melanogaster* strains also had amanitin-sensitive RNA polymerases II.

A puzzling question remains. These mycophagous species of *Drosophila* breed in a great variety of mushrooms, of which only a small fraction contain substantial, potentially poisonous levels of α-amanitin (*15*). Why, then, have these flies evolved resistance to a toxic compound that probably is encountered only rarely?

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5. Medium was prepared by mixing equal weights of brewer's yeast and instant *Drosophila* medium (Carolina Biological Supply); to 1.0 g of this mixture was added 4.0 ml of either water or an aqueous solution of α-amanitin (Sigma). This medium was used because it is the only homogeneous, uncooked medium we know of on which all six species can develop, although it is not an optimal medium for any of them. This accounts for the relatively low survival of most of these species even when no amanitin was added to the medium. Overall, this medium appeared to be about equally suitable for the mycophagous and the nonmycophagous species (see Fig. 1). Amanitin concentrations used were 5, 10, and 50 μg per milliliter of solution. These concentrations were determined by spectrophotometric absorbance at 310 nm (*2*). For each species, five replicate cultures were set up at each amanitin concentration (*7*); each replicate was started with 50 eggs. For *D. immigrans* and *D. pseudoobscura*, four and three replicates, respectively, were set up at each amanitin concentration. Cultures were maintained at 21°C and about 75 percent relative humidity.
6. The sources of the flies used are as follows: *D. melanogaster* strains C4 and P2, both derived from an Oregon-R line (*2*); *D. tripunctata*, stock number 1910.4, from the National *Drosophila* Species Resource Center, Austin, Texas; *D. pseudoobscura*, stock number 3389.28, from the National *Drosophila* Species Resource Center; *D. putrida*, descended from a female collected in Vestal, New York in 1981; *D. recens*, descended from a female collected in Chenango Valley State Park, New York, in 1981; *D. immigrans*, descended from a female collected in Chenango Valley State Park in 1981. All strains had been maintained in the laboratory in amanitin-free conditions for at least five generations before the experiment.
7. Thorax length was measured as an indicator of overall body size; this is highly correlated with ovariole number in these species (D. A. Grimaldi, unpublished data).
8. RNA polymerase II was purified from 1.5 to 2.0 g of adult flies with the procedures of A. L. Greenleaf, J. R. Weeks, R. A. Voelker, S. Ohnishi, and B. Dickson [*Cell* **21**, 785 (1980)]. The enzymes were further purified by use of a heparin-Sepharose column (D. E. Coulter and A. L. Greenleaf, *J. Biol. Chem.* **257**, 1945 (1982). Assays for the amanitin sensitivity curves were performed as described by Greeleaf *et al.*, except that the reactions were started by a transfer to 25°C (rather than 26°C).
9. Mean thorax lengths (± standard error) in millimeters for *D. pseudoobscura* at 0 μg/ml were: female, 1.167 ± 0.007; male, 1.041 ± 0.005 (N = 15 for both sexes). At 5 μg/ml the values were: female, 1.111 ± 0.033 (N = 6); male, 0.999 ± 0.016 (N = 15). For *D. immigrans*, the

values at 0 μg/ml were: female, 1.340 ± 0.008; male, 1.231 ± 0.024. At 5 μg/ml the values were: female, 1.359 ± 0.010; male, 1.234 ± 0.046. At 10 μg/ml, they were: female, 1.301 ± 0.015; male, 1.135 ± 0.019 (N = 15 for each sex and concentration). The single individual of *D. melanogaster* P2 that emerged at 5 μg/ml was not measured. For each species and sex, a one-way analysis of variance was used to assess the significance of size variation of flies reared at different amanitin concentrations. One-way analysis of variance was also used to detect significant variation in development time as a function of amanitin concentration.

10. The difference between treatments in the sex ratios was highly significant (χ^2 = 13.8; d.f. = 1; $P < .001$). Based on 133 flies raised at 0 μg per milliliter of amanitin and 37 flies at 5 μg/ml.
11. The survival of *D. putrida* was actually greater when amanitin was added at 50 μg/ml than when the lower concentrations were used. But since this difference is significant at only the $P = .05$ level (Student-Newman-Keuls test), we refrain from speculating on the possible causes of this finding.
12. $P < .01$ by a Student-Newman-Keuls test.
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14. J. P. Phillips, J. Willms, A. Pitt, *Can. J. Genet. Cytol.* **24**, 151 (1982). The LD_{50}'s for these strains were 10, 30, and 35 μg/ml of α-amanitin in the larval medium.
15. α-Amanitin has been detected in other, nonpoisonous species of mushrooms but at extremely low, presumably innocuous, levels [H. Faulstich and M. Cochet-Meilhac, *FEBS Lett.* **64**, 73 (1976)].
16. We thank A. Ben-Yishay for help with the rearing experiments and P. Feeny, F. J. Kull, R. C. Lewontin, R. K. Selander, and D. M. Simons for helpful comments on the manuscript. Supported by NSF grant DEB80-08574 (to J.J.) and NIH grant GM 28078 (to A.L.G.).

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Combined Lipase Deficiency (*cld*): A Lethal Mutation on Chromosome 17 of the Mouse

Abstract. *Two triglyceride lipases, lipoprotein lipase and hepatic triglyceride lipase, participate in the metabolism of plasma lipoproteins. A single recessive mutation,* cld, *on mouse chromosome 17 causes an apparent deficiency of both lipoprotein lipase and hepatic triglyceride lipase activities. Mice homozygous for this defect develop lethal hyperchylomicronemia within 2 days postpartum as a consequence of nursing. Plasma triglyceride values in affected mice often reach 20,000 milligrams per deciliter (100 times higher than that in normal littermates), and total lipase activity in plasma or tissues is 5 to 20 percent of that in controls.*

The clearance of chylomicrons and very low density lipoproteins (VLDL) is dependent on an initial interaction with the enzyme lipoprotein lipase (LPL). This triglyceride hydrolase is present at the capillary endothelium of most extrahepatic tissues (for example, heart, muscle, adipose tissue) (*1*). A second lipase, hepatic triglyceride lipase (HTGL), is probably confined to the capillary endothelium of the liver, and recent studies suggest its involvement in the final conversion of VLDL to low-density lipoproteins (LDL) (*2*). The metabolism of high-density lipoproteins (HDL) may also be affected by this enzyme (*3*). Both LPL and HTGL are released into plasma by the intravenous injection of heparin (postheparin plasma).

Deficiency of LPL activity is a rare familial trait in humans (*4*). This recessive genetic disorder produces a hypertriglyceridemia that is expressed primarily as a buildup of chylomicrons after consumption of a fatty meal. Although, when uncontrolled, severe hyperchylomicronemia can produce pancreatitis in humans, this complication is effectively managed by restricting dietary fat. The same clinical syndrome results from genetic deficiency of the activator of LPL, apoprotein C II (*5*). In both disorders, HTGL activity is present in plasma at normal or reduced levels after heparin injection. Deficiency of HTGL activity was described in a single family with an associated accumulation of intermediate-density lipoproteins (IDL) and lighter HDL (HDL_2) (*6*). No disorder resulting in deficiency of both enzyme activities has been described in humans. There are no reports of a primary deficiency of either LPL or HTGL activity in laboratory animals.

We now report on a mouse autosomal recessive mutation, named combined lipase deficiency (*cld*), which causes a deficiency of both LPL and HTGL activities. The *cld* mutation was extracted from a chromosome bearing mutations at the *T/t* complex of mouse chromosome 17 (*7*). The original *t* haplotype was characterized as carrying t^{w73}, a recessive embryonic lethal acting in homozygotes at 5 days of gestation (*8*). Any chromosome carrying one recessive lethal could carry undetected numerous other later-acting lethals with no further genetic detriment. Genetic dissection of *t* chromosomes by recombination analysis has separated the different component genes carried by complete *t* haplotypes (*9*). In the chromosome bearing t^{w73}, a parasitic lethal gene, *cld*, acting postnatally, was uncovered (Fig. 1). The two relevant *cld* phenotypes can be distinguished immediately by inspection of the tail phenotype because of the close linkage of the tail mutation and *cld* (94 percent co-inheritance of the two traits through the female and 98 percent through the male). Such a distinction can be made with confidence even on animals delivered by cesarean section.

Although apparently normal at birth, tailless (*cld/cld*) animals show decreasing mobility, poor weight gain, paleness, and progressive cyanosis leading to death between 36 and 48 hours postpartum. Gross examination of tissue reveals small hemorrhages in the heart, lungs, and liver, suggesting diffuse microinfarction. Plasma glucose levels taken during the first 48 hours postpartum are within normal limits (40 to 80 mg/dl) in both normal and affected mice, indicating that the mice are not diabetic. Hypertriglyceridemia is not present in the affected mice examined in utero, and cholesterol levels are comparable to those in normals (Fig. 2). During the first 2 days, a progressive rise in plasma triglycerides occurs. The serum becomes opaque, and triglyceride concentration is often more than 20,000 mg/dl by the second day postpartum. Cholesterol values also rise from approximately 60 mg/dl in utero to more than 450 mg/dl after 48 hours (Fig. 2). Sudan black staining of plasma lipoproteins separated by electrophoresis in 1 percent agarose (*10*) shows chylomicron accumulation in plasma of affected mice. A pre–β-lipoprotein (VLDL) band is less discrete, and the α-lipoprotein (HDL) band shows reduced staining in comparison with that of normal animals. β-Lipoprotein (LDL) is virtually undetectable in affected and unaffected mice. It seems highly probable that the extreme increase in chylomicrons results in coalescence of particles and obstruction of capillary fields in vital organs.

We assayed endothelial bound lipase activity released into the plasma by heparin, as well as liver and heart tissue triglyceride lipases. The lipolytic activity of postheparin plasma and tissue homogenates in the affected animal was 5 to 20 percent of control activity for both LPL and HTGL. The liver homogenate of normal neonatal mice showed an increase in activity with the addition of serum, a property normally ascribed to LPL. Total liver lipolytic activity was reduced by 1.5M sodium chloride or protamine sulfate (data not shown). This is compatible with previous reports of an activity similar to LPL in addition to HTGL activity in the neonatal rat liver (*11*). The decreased lipase activity observed in *cld/cld* mice is not the result of an inhibitor present either in the plasma or in tissue homogenates, because addition of an equal volume of heart or liver homogenate from an affected mouse did not reduce the activity of homogenates from normal mice. Some inhibition of lipolytic activity (20 to 25 percent) was seen when an equal volume of post-